ABSTRACT

Cancer still remains one of the major health problems worldwide and the identification of novel biomarkers that could be used for the risk assessment, early detection and prognosis, as well as for the prediction of the response to treatment and disease recurrence is of great importance for coping with this disease. Immunoglobulin G class antibodies that are produced against tumour-derived proteins are attractive biomarker candidates for the development of non-invasive serological tests since they are observed in most if not all cancer patients, detected in early to late stages of the disease, possess significant degree of specificity and are stable in serum. However, their clinical utility so far has been hampered by low frequencies against each individual antigen, heterogeneity of induced antibody responses among individual patients and tumours, and uncertain biological roles. These limitations in part could be overcome by the expanding the list of serologically active tumour antigens and the elaboration of approaches that enable high-throughput profiling of autoantibody responses in cancer patients.

The application of the conventional SEREX approach (serological identification of tumour antigens by recombinant expression cloning) to different human tumours has led to the identification of more than 1500 tumour-associated antigens (TAAs) to date, including novel relevant immunotherapy targets; however, it is not suitable for the assessment of autoantibody responses in large-scale, which has been tried to overcome by its combination with the phage surface display. The overall aim of the current study was to evaluate the potential of the conventional and phage display-based SEREX approaches for the assessment of antigen repertoires eliciting B cell responses in cancer patients and to identify a comprehensive set of antigens associated with melanoma, prostate and gastric cancer that further could be searched for novel autoantibody biomarkers as well as potential immunotherapy targets.

The conventional SEREX approach was applied to gastric adenocarcinoma, which resulted in the identification of 14 TAAs, eight from which were novel. Preliminary characterisation of the antigens revealed no mutated, overexpressed, cancer-testis or differentiation antigens, nonetheless, antibody responses against a half of them were cancer-specific thus suggesting their relevance as sero-diagnostic or predictive markers. Detailed molecular characterisation of two of the TAAs – TACC1 and NUCB2 – revealed that the expression of cancer-associated alternative splice isoforms and altered, cancer-specific posttranslational modifications, respectively, may underlay their immunogenicity in cancer patients.

Next, two different lytic phage surface display vector systems – λKM8 and T7Select 10-3b – were chosen and shown to be capable of displaying antigens from 15 known TAA families as hybrids with phage surface protein gpD N-terminus and 10B C-terminus, respectively. Immunoscreening of the antigen-encoding phage clones with 21 melanoma patients' sera demonstrated relatively good concordance between the two serum-reactive antigen repertoires. However, T7Select 10-3b system was shown to be more sensitive for autoantibody-mediated detection of the antigens that most likely was due to their better spatial accessibility when displayed on T7 phage in comparison to λ phage. Both phage systems were shown to be equally suitable for the development of phage-displayed antigen microarrays as well as for microarray-based profiling of autoantibody responses in cancer patients. Noteworthy, it was demonstrated that the microarray-based screening and plaque immunoscreening have comparable, yet not identical sensitivities for the detection of autoantibodies.

Due to the higher sensitivity, availability of commercial antibodies against the phage capsid and more favourable biological properties, the T7Select 10-3b display system was chosen for the elaboration of the phage display-based SEREX approach, which included the assessment of optimal biopanning conditions and cycle numbers – single biopanning cycle was defined as optimal and ensured 60-80-fold enrichment of a cDNA expression library with serum-reactive clones. The elaborated T7 phage display-based SEREX technique was applied for the assessment of spontaneously occurring and immunotherapy-induced autoantibody responses in 29 melanoma, 22 prostate cancer, 27 gastric cancer and 15 gastritis patients, which resulted in the identification of 529, 179, 233 and 123 individual antigens, respectively. The clone insert sequences and antigen-related information was deposited in the Tumour Antigen data base.

The clone insert sequence analysis revealed that only minority of the recombinant proteins (~9%) are expressed in-frame to T7 phage surface protein 10B while the vast majority of them most likely represent mimotopes of other, yet unknown antigens. Serum antibody responses to selected out-of-frame as well as in-frame antigens were confirmed by Western blot. Approximately 20% of the in-

frame antigens represented gene products that have been previously identified by the conventional SEREX studies while ~37% of them belong to protein families, other members of which have been identified by SEREX thus indicating that the antigen repertoire defined by T7 phage display-based SEREX partially overlaps with that of conventional SEREX as well as demonstrates that the developed approach enables the identification of novel TAAs.

Further, a prototype of antigen microarray comprising 700 antigen-displaying and 30 non-recombinant phage clones was developed and used for the profiling of autoantibody responses in 121 melanoma, 55 prostate cancer patients' and 136 healthy donors' sera. Preliminary data obtained from these analyses showed that 245 and 182 antigens are preferentially recognised by sera form patients with prostate cancer and melanoma, respectively, and not with healthy donors' sera.

Besides it was estimated that on the basis of the identified cancer-specific autoantibody signatures it would be possible to detect prostate cancer and melanoma with 100% specificity and 91% and 70% sensitivity, respectively, i.e. – with significantly higher accuracy than any other of the currently used biomarkers. Besides, the results indicated that these cancer-specific signatures could be used for the early detection of cancer.

The development of the final version of the antigen microarray comprising 1300 antigen clones identified in this study is underway, and it will be applied for the analyses of autoantibody repertoires in larger sets of sera from patients with cancer and premalignant lesions, patients undergoing immunotherapy as well as healthy donors and patients with various autoimmune conditions in order to define autoantibody biomarkers for cancer diagnosis and early detection, and prediction of the patients' response to immunotherapy. Apart form biomarker discovery, the identified in-frame antigens will be systematically analysed in order to search for novel targets that could potentially be used for immunotherapeutic intervention.

CONTENTS

LIST OF THE ORIGINAL PUBLICATIONS

The current dissertation is based on the following publications referred in the text by their Roman numerals. However, large proportion of the presented work includes hitherto unpublished data.

I Linē A, Stengrēvics A, **Slucka Z**, Li G, Jankevics E and Rees RC. Serological Identification and Expression Analysis of Gastric Cancer-associated Genes. *Br J Cancer*, 2002, 86 (11):1824-30. Original paper.

II **Kalnina Z**, Silina K, Bruvere R, Gabruseva N, Stengrevics A, Barnikol-Watanabe S, Leja M, Line A. Molecular characterisation and expression analysis of SEREX-defined antigen NUCB2 in gastric epithelium, gastritis and gastric cancer. *Eur J Histochem,* 2009, 53 (1):7-18. Original paper.

III Linē A, **Slucka Z**, Stengrēvics A, Li G and Rees RC. Altered Splicing Pattern of TACC1 mRNA in Gastric Cancer. *Cancer Genet Cytogenet*, 2002, 139 (1):78-83. Short communication.

IV **Kalniņa Z**, Siliņa K, Meistere I, Zayakin P, Rivosh A, Ābols A, Minenkova O, Schadendorf D and Linē A. Evaluation of T7 and Lambda phage display systems for survey of autoantibody profiles in cancer patients. *J Immunol Methods*, 2008, 334 (1-2):37-50. Original paper.

V **Kalnina Z**, Silina K and Line A. Autoantibody profiles as biomarkers for response to therapy and early detection of cancer. *Curr Canc Ther Rev*, 2008, 4 (2):149-156. Review.

My contribution to the articles referred in the current dissertation is as follows:

I Accomplished (i) allogeneic screening of the gastric cDNA expression library, (ii) antigen identification and (iii) comparative mRNA expression analyses of the identified antigens.

II (i) Accomplished majority of the experimental work; (ii) wrote the paper.

III (i) Participated in TACC1 mRNA transcript variant characterisation.

IV Accomplished (i) TAA mini-library construction, (ii) TAA mini-library immunoscreening and antigen identification; (iii) tested λ and T7 phage suitability for the generation of microarray; iv) participated in the preparation of the manuscript.

V (i) Literature overview on proteomic approaches for the identification of antigens and auto-antibody profiling; (ii) co-writer of the manuscript.

The results of this work have been presented in 24 international and four local conferences, from these 11 have been presented by author.

ABBREVIATIONS

ADCC	antibody-dependent cellular cytotoxicity
APC	antigen presenting cell
BCR	B cell receptor
CDC	complement-dependent cytotoxicity
CDCC	complement-dependent cellular cytotoxicity
CSC	cancer stem cell
CT	cancer-testis
CTL	cytotoxic T lymphocyte
CTLA4	cytotoxic T-lymphocyte antigen 4
DC	dendritic cell
ECM	extracellular matrix
EST	expressed sequence tag
GC	germinal centre
HLA	human leukocyte antigen
IFN	interferon
IL	interleukin
mAb	monoclonal antibody
MALT	mucosa-associated lymphoid tissue
MCFS	macrophage chemotactic factor
MHC	major histocompatibility complex
miRNA	micro-RNA
MDSC	myeloid-derived suppressor cell
MS	mass spectrometry
NAA	natural autoantibody
NK cells	natural killer cells
NKT cells	natural killer T cells
ORF	open reading frame
pfu	plaque forming unit
PSA	prostate-specific antigen
PTM	posttranslational modification
RT-PCR	reverse transcriptase polymerase chain reaction
SEREX	serological identification of antigens by recombinant expression cloning
TAA	tumour-associated antigen
TCR	T cell receptor
TD	thymus-dependent
TGF	tumour growth factor
T_{FH}	follicular B helper T cell
Th1	type 1 helper T cells
Th17	IL-17 secreting helper T cell
Th2	type 2 helper T cells
TI	thymus-independent
TIL	tumour-infiltrating lymphocytes
TNF	tumour necrosis factor
Treg	regulatory T cell
UTR	untranslated region
VEGF	vascular endothelial growth factor
WHO	World Health Organisation

INTRODUCTION

Despite enormous advances in scientific research cancer still remains one of common causes of morbidity and mortality worldwide. This is a multistep, multipath disease arising from a single cell and its progeny, which, through sequential rounds of mutation and selection, have gradually accumulated multiple genetic and epigenetic changes in critical genes that alter normal programmes of cell proliferation, differentiation and death[1]. It is not a single disease – more than 100 different cancer types are known, and subtypes of tumours can be found in particular tissues. Cancer genome, epigenome, transcriptome and proteome are being extensively studied in order to define novel therapeutic targets and to identify tumour-specific signatures that could provide definitive degree of certainty in diagnosis, staging, and progression, as well as prediction of the natural course of cancer and of response to treatment[2]. To date, despite the expanding list of individual biomolecules or their groups that have been shown to have promising biomarker qualities for defined types of cancer, in majority of cases their role as cancer biomarkers has not been yet validated in the context of a defined clinical application[3].

In general, the use of cancer biomarkers is limited in solid malignancies – tissue material, in which biomarkers can be measured, is available on diagnosis or when the tumour is resected; however, there are limitations to the use of predictive biomarkers in patients with solid tumours since tumour-derived rather than germline DNA is required for the analyses, and it is often difficult to gain access to. As an option, efforts have been made to identify blood-born biomarkers that could be detected by non-invasive tests. Circulating tumour cells as well as cell-free DNA, RNA and plasma proteome are being interrogated for novel biomarkers[3]. Currently blood-born protein biomarkers such as CA125, CA19-9 and carcinoembryonic antigen are used to monitor the response to therapy and disease recurrence in ovarian, pancreatic and colon cancer, respectively[4] while the detection of elevated PSA levels is widely used in prostate cancer screening programmes[5].

Apart from that, the patient's immune system may serve as a biosensor. Tumour-induced autoantibody production seems to be nearly ubiquitous event and it has been shown to have early-to late stage occurrence in cancer patients[6-8]. Immunoglobulin-G class antibodies are attractive biomarker candidates since they are extremely stable in serum and possess significant degree of specificity, which make them very promising targets for the development of non-invasive serological tests that could be used for diagnostic or prognostic purposes as well as for monitoring of response to therapy and recurrence of defined malignancy. Yet, no serological tests for such purposes are available in clinics at reasonable sensitivity and specificity, and this is mainly due to the low frequencies of antibody responses in cancer patients against a single antigen[9], heterogeneity of particular tumour type-induced autoantibody responses[10] and overlap of cancer-associated autoantibody repertoires with inflammation, autoimmune disorder or viral-induced humoural responses. Therefore, expanding the list of serologically active tumour antigens as well as the elaboration of approaches that enable high-throughput profiling of autoantibody responses in cancer patients may represent important step towards the generation of serological tests encompassing panels of tumour-associated antigens, which would have significant clinical utility.

Several approaches have been developed to study tumour-induced antibody responses in cancer patients; however, the major advancement in this field was the introduction of SEREX (serological identification of antigens by recombinant expression cloning) approach, which is based on tumour-derived cDNA library expression library construction and expression in bacteriophage-based vector system and immunoscreening with the patient's serum antibody repertoire[11,12]. The application of this approach has lead to the discovery of novel important cancer immunotherapy targets and significantly contributed to the definition of cancer immunome[13]. In recent years several modifications of the conventional SEREX approach have been introduced to overcome specific limitations of this approach and their application has shown promising results[14-20].

The overall aim of the current study was to evaluate the potential of the conventional and phage display-based SEREX approaches for the assessment of antigen repertoires eliciting B cell responses in cancer patients, and to identify a comprehensive set of tumour-associated antigens in melanoma, prostate and gastric cancer that would be further interrogated for novel autoantibody biomarkers as well as for potential immunotherapy targets. The present work was carried out at the Latvian Biomedical Research and Study Centre in collaboration with Latvian Oncology Center, German Cancer Research Center, Onyvax Vaccine Therapies Ltd and Radium Hospital, Norway.

1. LITERATURE REVIEW

1.1. MOLECULAR BASIS OF CARCINOGENESIS

1.1.1. THE NATURE OF CANCER EVOLVEMENT

Cancer has been known to humans since ancient times and since then scientists have made an incredible effort to unravel the origins of tumourigenesis. Cancer was initially thought to be an infectious disease. Afterwards, the aetiology of common cancers was predominantly viewed as environmental[21]. In 1890 David von Hansemann reported multipolar mitoses and aberrant chromosome segregation monitored in malignant cells[22] indicating that cancer is a disease with genetic changes. Today we know that, at least to some extent, all of this is true.

The nature of cancer evolvement is complex. It involves host-intrinsic and extrinsic factors, the interplay of which is known to be crucial in the aetiology of cancer. Many lifestyle factors such as environmental impact, diet, smoking (e.g. exposure to viral infections and physical or chemical carcinogens, many of which are mutagens) and reproductive life (hormonal modifications) significantly influence human carcinogenesis and thus can be altered to prevent the development of this disease. Apart from that, there are risk factors that are inherent in our own physiology – mutagenic metabolism by-products, unresolved inflammation, errors that occur during DNA replication and inherited genetic changes may represent a substantial step toward malignancy[23].

Cancer as a genetic disease

Cancer is known as a disease at the cellular level. Various factors different in origin and mechanism of action contribute to the initiation, promotion and progression of cancer. However, all neoplastic cells gradually acquire multiple genetic changes – alterations in genomic as well as mitochondrial DNA sequence – which lead to altered protein sequences and changes in gene expression that drive cancer development. These cancer genes broadly have been classified in three major categories – oncogenes, tumour suppressor genes and stability genes[24]. In general, an oncogene is any gene whose gain-of-function contributes to the neoplastic phenotype of a cell, and alterations in a single allele are enough to exert the oncogenic effect. Tumour suppressor gene products, on contrary, protect a cell from the onset and progression of cancer. Mutations leading to the loss-of-function or reduced activity of tumour suppressors are generally required to occur in both gene alleles to confer selective advantage to the cell[24,25]. Finally, stability genes or caretakers represent a group of genes responsible for the reparation of mistakes made by DNA replication, mitotic recombination and chromosomal segregation or those introduced by mutagen exposure. Just as for tumour suppressor genes, both alleles of stability genes must be inactivated to reach physiological effect that results in a phenomenon commonly seen in cancer – the genetic and genomic instability, which leads to increased mutation rates. Mutations implicated in the carcinogenesis may also occur in and be transferred through germ line thus significantly contributing to the individual's susceptibility to the disease.

The complexity of genetic alterations commonly evidenced in cancer cells is broad – from subtle intragenic mutations to large-scale intrachromosomal inversions and deletions, chromosomal rearrangements and chromosome number changes – aneuploidy and polyploidy. These alterations often serve as a mechanism for oncogene activation and tumour suppressor inactivation. To date, perhaps only 50% of cancer mutations have been identified[26]. A study of human breast and colorectal cancers revealed that individual tumours accumulate an average of 90 mutant genes but only a subset of them (an average of 11 per tumour) was supposed to carry "driver" mutations[27]. More recently in a Cancer Genome Project study, coding exons of 518 protein kinase genes in 210 different human cancers (>50% were primary tumours) were systematically sequenced. The results revealed more than a thousand somatic mutations in 274 Mb of the sequenced DNA, and two thirds of them were novel indicating the existence of a larger repertoire of cancer genes than it was previously anticipated[28]. The results of the study also revealed that most somatic mutations are likely to be "passenger" mutations – i.e. they seem not to contribute to oncogenesis. With regard to the large-scale genetic aberrations in cancer, gains and losses of whole chromosomes are likely to occur in most human malignancies, and looses of heterozygosity are widespread and often are accompanied by a gain of the opposite allele; it has been estimated that on average 25-30% of the alleles, which are present in normal cells may be lost in neoplastic cells as a result of chromosomal instability[29]. The determination of genetic

alterations that are necessary and sufficient for the development of malignancies has already lead to the novel anti-cancer therapies and identified new biomarkers for risk stratification and early detection of cancer.

Epigenetic changes in cancer

In cancer cells, apart from genetic changes – inherited or acquired during a lifetime – gene expression and protein functions are deregulated also through other means, which include aberrations of transcription factors, splicing machinery, miRNAs and different translational or posttranslational mechanisms[30]. In addition to above listed, there are two main hallmark processes of epigenetic gene regulation, which have been recognised as important contributors to carcinogenesis – DNA methylation and modifications of histone structure[31]. By definition, epigenetic changes refer to all heritable changes in gene expression that are not associated with alterations in the corresponding DNA sequence[30] and therefore may be reversible.

In humans, DNA methylation occurs at CpG dinucleotides, which are known to be accumulated throughout genome in regions called CpG islands. CpG islands are found in the proximal promoter regions of approximately half of the genes and usually are not methylated in normal cells[32]. Nevertheless, some regions in human genome normally are subjected to methylation to ensure genomic imprinting and the inactivation of certain tissue-specific genes, and to dampen down repetitive genomic sequences and proviral elements[33]. Hypermethylation and hypomethylation denotes relative state that represents a change from the "normal" methylation level, and both of them have been identified in all cancers examined leading to inappropriate gene silencing and activation, respectively[31,34]. These aberrations are believed to occur due to spontaneous defects in DNA methyltransferases (DNMTs)[35], although the molecular causes are not definitively understood. It has been estimated, that in general the level of hypomethylation increases and the level of hypermethylation decreases in tumour cells with the progression of neoplasm[31]. The changes in DNA methylation lead to the activation of oncogenes, inactivation of tumour suppressor genes, and contribute to the genomic instability. The latter occurs due to the reactivation of repetitive and endoparasitic DNA sequences through their demethylation, leading to gene disruption or chromosomal rearrangements[33].

Histones are the main protein components of the chromatin. There are six classes of histone proteins encompassing multiple different histone protein variants (e.g. H3A1, H1H1), and the epigenetic information is stored by posttranslational modifications (PTMs) that occur in the histone protein aminoterminal regions, which alter their interactions with DNA and nuclear proteins. These PTMs are of different nature (methylation, acetylation and phosphorylation) and have different degrees of methylation (mono-, di-, or trimethylation) and may occur at different histone protein aminoterminal residues (e.g. lysine, arginine or serine)[31] thus generating a diverse array of histone PTM variant combinations that have been termed the “histone code”, which considerably extends the information potential of the genetic code[36]. Histone acetylation and methylation have a direct effect on a variety of processes including gene transcription, DNA replication and repair, and chromosomal organisation. In cancer, enzymes that modify histones are known to be altered through various mechanisms[31] leading to the disruption of the histone code associated with a number of adverse outcomes. Moreover, modifications in histone structure may influence the process of DNA methylation – tumour suppressor gene inactivation through promoter region hypermethylation in cancer cells is associated with a particular combination of histone markers[31].

Micro-RNAs and cancer

Micro-RNAs (lately designated as miRNAs or miRs) are 19-25 nucleotides long non-coding RNAs that recently have emerged as important regulators of gene expression at the translational level. They may subject target mRNAs to degradation or prevent their translation through sequence-based pairing to their untranslated regions (UTRs)[37], and it has been established that individual miRNAs can affect the expression of hundreds of proteins[38,39]. MiRNAs are known to be involved in important biological processes such as apoptosis, development, differentiation and proliferation[40,41]. In cancer, the deregulation of miRNA expression seems to be nearly ubiquitous[37], these molecules are involved in the initiation as well as progression of tumourigenesis and may function as tumour suppressors, oncogenes, or both in different cellular contexts[42] contributing to the acquisition of the tumour key physiological features described hereinafter[1]. In general, global numbers of miRNAs tend to be reduced in tumours although selected miRNA gene clusters are upregulated in neoplastic cells[43] – for

example, a group of six co-transcribed miRNAs known as the miR-17-92 cluster is found to be amplified in lymphomas and solid tumours[44].
The differential expression of miRNA genes in cancerous compared to non-cancerous cells is thought to be a consequence of alterations in the complex miRNA processing machinery, deregulated epigenetic mechanisms, or the location of miRNA genes in cancer-associated genomic regions[37]. Moreover, germline sequence discrepancies have been identified in miRNA genes, transcripts and in the respective regions in their target mRNAs[37]. Currently there are hundreds of confirmed and more than a thousand predicted human miRNAs, and miRNA profiling, alike gene expression profiling, is extensively exploited to identify signatures associated with diagnosis, staging, progression and prognosis of cancer, as well as response to treatment[37,45]. Apart from that, miRNAs may provide additional targets for cancer treatment.

Cancer stem cells

The concept of cancer stem cells (CSCs), also referred to as cancer stem-like cells, as tumourigenic cells was first indirectly outlined in 1937 by Jacob Furth and Morton Kahn who showed that single leukaemic cell was able to transmit cancer when transplanted into a mouse[46]. However, only in mid 1990-ies these cells comprising a minor population in the tumour bulk were identified and characterised in human acute myeloid leukaemia[47,48] and later on in many solid tumours such as breast[49] and lung cancer[50], brain tumours[51] and others.
Cancer stem cell model is the most discussed hypothesis at the moment[52]. This model, also known as hierarchical model of tumourigenesis, gives some answers to questions that remained unanswered by the long-standing stochastic model where all neoplastic cells are believed to contribute equally to malignancy. Considering the estimated mutational rate, the likelihood that a differentiated somatic cell could acquire defects during its lifespan to become neoplastic is nearly impossible[53], whereas stem cells are long-living and therefore are more prone for the risk of transforming events required for the carcinogenesis. Besides, stem cells are already endowed with critical capabilities of self-renewal and proliferative capacity, partial resistance to apoptosis[54] as well as limited immune recognition[55] – important hallmarks of cancer[1]. Besides, the plasticity of stem cells that is characterised by the ability to give rise to different cell types explains the functional heterogeneity of tumour cells, a common feature of carcinogenesis. The main difference between CSCs and their normal counterparts is that the latter follow strict rules of regulation while CSCs do not, which is believed to occur due to acquired cell-intrinsic alterations; however, a role of aberrant signals derived from surrounding cells also has been pointed – stem cell homeostasis is regulated by their interaction with stromal cells that form a special microenvironment called "niche", which provide stem cells with critical extrinsic signals needed for triggering defined signalling pathways that direct specific programmes in stem cells[56]. Accumulating evidence points to the significance of tumour-stroma interactions in epithelial tumourigenesis – it has been shown that tumour cell niche support survival and self-renewal of tumour cells, which has been supported by genetic studies[57,58]; however, mechanisms of this phenomenon are still poorly understood[56].
There is an ongoing debate on the exact origin of these cancer initiating cells. Initially, it was thought that epithelial tumours originate from transformed tissue-specific stem cells. Later, the discovery that bone marrow can give rise also to non-haematopoietic tissues[59,60] lead to the idea that bone marrow-derived cells might be the *bona fide* origin of CSCs as they migrate and home in the sites of tissue damage and become tissue-specific stem cells. In support of this, the first evidence was gained from the study of Houghton *et al.* in gastric cancer[61], and conceivably it could be the case for multiple solid cancer types[62]. Alternatively, evidence for circulating bone marrow-derived stem cell fusion with pre-existing transformed cells exists[63] that could lead to the acquisition of stem cell properties in the neoplastic cells thus favouring further tumour development. Yet, the CSCs could also originate from more differentiated cells that have (re)acquired stem cell characteristics due to spontaneous mutations[64]. It is not clear whether CSCs and normal stem cells exploit the same molecular pathways to achieve the same phenotypic features; however, aggressive and poorly differentiated human tumours demonstrate embryonic stem cell-like gene expression signatures and are associated with poor prognosis[65]. Noteworthy, if only a minor fraction of tumour bulk (i.e. CSCs) is responsible for tumour outgrowth, cancer treatments should fight CSCs. Many cancer treatment approaches are being revised in order to target CSCs, and significant efforts are focused on the identification of specific CSC markers and possible therapy targets[66].

1.1.2. THE PHYSIOLOGICAL HALLMARKS OF CANCER

Today the development of cancer is seen as a monoclonal growth of a single progenitor cell, most likely a cancer stem-like cell, which has acquired multiple important capabilities through stepwise accumulation of genetic and epigenetic changes and multiple rounds of selection. These capabilities are the hallmarks of cancer that enable the transformed cell to overcome the homeostatic mechanisms that govern normal cell proliferation. Douglas Hanahan and Robert A. Weinberg have proposed six major physiological changes acquired in the majority of cancer cells[1]: (i) self-sufficiency in growth signals, (ii) insensitivity to growth-inhibitory signals, (iii) evasion from apoptosis, (iv) limitless replicative potential, (v) sustained angiogenesis and (vi) tissue invasion and metastasis (Figure 1).

Self-sufficiency in growth signals

In order to move from the quiescent state to the active proliferative state normal cells require mitogenic growth signals from their tissue microenvironment. These signals include diffusible growth factors, extracellular matrix (ECM) components, and cell-cell adhesion/interaction molecules[1]. While most normal cells are completely dependent upon growth signal stimulation *in vitro* and *in vivo*, their neoplastic counterparts demonstrate significantly reduced dependence on exogenous signals to proliferate. They tend to achieve this independence by several means. Many cancer cells are able to produce and release soluble growth factors to which they are responsive, creating a positive feedback loop[67]. Alternatively, tumour cells may induce their normal neighbouring cells to abundantly produce growth factors. Cancer cells are also known to overexpress their growth factor receptors thus augmenting the proliferative signals transmitted to the cell even when their ligands are met at average levels in the tumour stroma[1]. For instance, overexpression of wild-type epidermal growth factor receptor (EGFR/ERBB1) and HER-2/neu (ERBB2) is shared by several types of human carcinomas[68]. Cancer cells may also acquire the ability to switch ECM receptor types, favouring those which transmit growth signals[69].

Downstream intracellular pathways that receive and process the signals received by growth factor receptors and integrins are frequently deregulated in cancers as well. The SOS-Ras-Raf-MAPK cascade appears to be the key player in mediating responses to extracellular mitogens. For example, Ras proteins are found to be mutated in about 15-20% of human tumours[70]. Similarly, somatic point mutations in *BRAF* are detected in nearly 70% of malignant melanomas and at lower frequency in other tumours[71]. These mutations lead to constitutive or elevated activity of the respective protein resulting in an inappropriate activation of the downstream growth signalling pathway without ongoing receptor stimulation by the corresponding ligand.

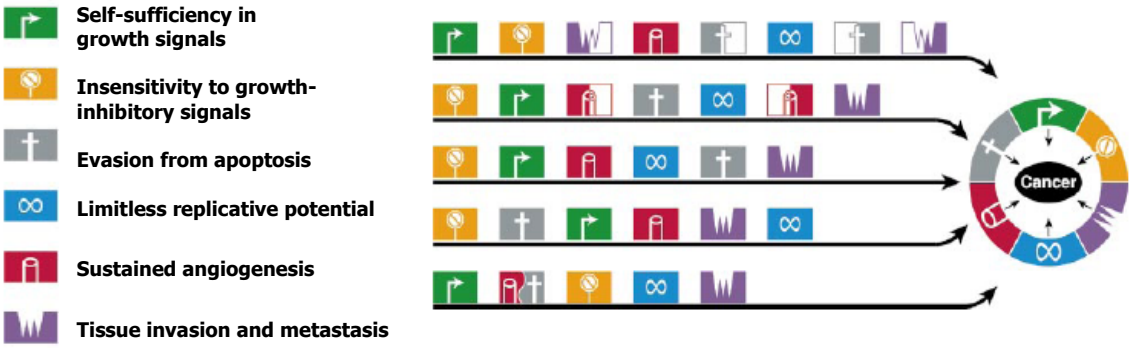


Figure 1. Six capabilities acquired by neoplastic cells during tumourigenesis. The sequence in which the capabilities are acquired can vary widely, both among tumours of the same type and certainly between different tumour types. Adapted from Hanahan & Weinberg, 2000[1].

Insensitivity to growth-inhibitory signals

In order to proliferate extensively, tumour cells have to bypass another obstacle. Within normal tissues there are soluble factors and immobilised molecules as a part of ECM, which act on the surrounding cells providing antigrowth signals in order to maintain tissue homeostasis. These signals act through two distinct mechanisms – either the cell is guided into the transient quiescent state (G_0) or they may be induced to step into postmitotic states of terminal differentiation[1]. Decisions whether to proliferate, remain quiescent or enter a postmitotic state are made during transition through G1 phase of the cell cycle. If self-sufficiency of growth signals is mainly achieved by the mechanisms leading to the activation of proto-oncogenes, the loss of sensitivity to growth-inhibitory signals often results from the loss of tumour suppressor protein expression and functions, particularly retinoblastoma protein and its upstream and downstream associates (the RB-p107-p130 pathway)[30,72].

Tumour cells are also known to elaborate various strategies to avoid their terminal differentiation. One apparent example is the deregulation of *MYC (c-myc)* proto-oncogene expression reported in a wide range of human malignancies. The MYC protein is a transcription factor that regulates a variety of cellular processes including cell growth and proliferation, cell-cycle progression, transcription and differentiation[73] and its overexpression or functional deregulation leads to impaired cell differentiation and promoted growth. Noteworthy, it has been shown that the predominant consequence of *MYC* activation in tumour cells is widespread repression of miRNA expression with the exception of pro-tumourigenic miR-17-92 cluster that is directly upregulated by MYC[74] and recently has shown to be a cell cycle regulated locus[75].

Evasion from apoptosis

Not only the ability to extensively proliferate but also the enhanced chance to survive in the milieu of different intracellular and extracellular stress signals is important for neoplastic cells to increase their numbers. Apoptosis (or programmed cell death) machinery is inherent for virtually all cell types throughout the human body, and it can be bisected into two major pathways – extrinsic and intrinsic[1]. The intrinsic pathway is triggered in response to DNA damage, signalling imbalance provoked by oncogene activation, oxidative stress and others while the extrinsic pathway is activated due to the abrogation of cell-matrix and cell-cell adherence-based survival signals and responses from activated immune system[76,77], all of which are prominent players in tumourigenesis. Indeed, tumour cell apoptosis, first reported in 1972 by Kerr, Wyllie and Currieet[78], is commonly seen during tumour progression and thus it is another barrier to overcome.

According to conventional textbooks, both intrinsic and extrinsic apoptotic pathways are funnelled to the activation of intracellular protease (caspase) cascades that accomplish neat breakdown of cellular components. Extrinsic apoptotic pathway is triggered by the engagement of death receptors (Fas, TNF-α and TRAIL receptors) on the cell surface then followed by the activation of procaspase 8 thus starting up the caspase cascade. The intrinsic mechanism is mediated by mitochondria where series of biochemical events are induced leading to the permeabilisation of mitochondrial membrane, the release of cytochrome c and the cleavage of initiator procaspase 9[23]. And again, tumour cells exploit different ways to acquire resistance to apoptosis. Both, molecules that trigger apoptotic processes and the key regulators are targets for alterations and deregulated expression in cancer.

The *TP53* tumour suppressor gene encoding the pro-apoptotic key sensor of DNA damage, p53 protein, is commonly seen to be lost or mutated in more than a half of human cancers[79,80], while in cancers expressing wild type p53 the p53 pathway is inactivated by alterations in other components[81]. For example, a new group of p53-binding proteins – apoptosis stimulating proteins of p53 (ASPP) – have emerged recently as potent regulators of p53 activity, and mutations and alterations in ASPP protein expression in a variety of human cancers are linked to tumour progression[81].

With regard to the intrinsic apoptotic pathway, it is regulated by the members of Bcl2-like protein family, which include both anti-apoptotic (Bcl2, Bcl-x_L, Bcl-w, A1 and Mc11) and pro-apoptotic (Bax and BH3-only proteins) molecules, deregulated expression and functions of which are seen in various tumour types in humans and mouse models[42,82]. For example, in B-lymphoid tumours, chromosomal translocation results in the juxtaposition of *BCL2* oncogene to enhancer elements in the immunoglobulin heavy chain locus leading to constitutively activated *BCL2* expression[83,84].

The constitutive activation of central survival PI3K-Akt pathway (also linked to alternative cell death mechanism autophagy), the loss of the PTEN tumour suppressor that normally attenuates PI3K-Akt pathway, and the down-regulation of cell surface death receptors are other evident examples of how

tumour cells acquire resistance to apoptosis[1,77]. Noteworthy, deregulation of non-apoptotic cell death pathways including autophagy, necrosis, senescence and mitotic catastrophe also have been reported in cancer[85].

Limitless replicative potential

It is thought that almost all cell types in the human body have limited proliferative capacity – after ~60-80 doublings cells, although remaining alive and metabolically active, enter an irreversible growth arrest called senescence[86]. This inherent physiological feature thus represents another obstacle to be overcome by neoplastic cells to grow into enormously large tumour bulk. Majority of the observations regarding the cell replicative potential come from cultured normal and neoplastic cells, which must overcome two inherent cell-autonomous replicative barriers to replicate indefinitely – the senescence and the crisis[87].

The inherent program of replicative senescence can be induced through various pathways[88], nevertheless, generally it is driven by the shortening of telomeres – the repetitive DNA structures that protect the chromosome ends during each cell division[1]. This ultimately results in unprotected telomere ends perceived as double strand breaks that potently induce p53-mediated responses[89]. The senescence state can be overcome through the inactivation of p53 and RB pathways (hallmark features of tumour cells) by certain oncogenes leading to further telomere attrition that eventually results in crisis – widespread end-to-end chromosomal fusion, karyotypic chaos and cell death[90].

To overcome crisis, cancer cells have to resolve the telomere shortening problem. In about 90% of human tumours irrespective of their origin and histological type the maintenance of telomere length and replicative immortality are achieved through acquired expression of telomerase reverse transcriptase (TERT) that together with the ubiquitously expressed RNA component (TERC) of the telomerase holoenzyme maintain telomere length[91,92]. Telomerase activation is thought to be rather late event in cancer development (see Figure 1), this is supported by observations that neoplastic cells often have shorter telomeres than surrounding normal tissues and that human cancer cells often harbour telomeric associations and anaphase bridges[90]. Noteworthy, TERT expression in normal somatic tissues has been evidenced in activated lymphocytes, germ cells, and tissue stem cells. Convincing evidence about the mutations, amplifications or rearrangements of *TERT* gene locus in cancer is lacking. However, several upstream pathways by which TERT expression might be negatively regulated have been identified, including TGF-β-Activin, MAD1-Myc and MEN1 pathways – all of which are involved in carcinogenesis[93]. Apart from that, an alternative mechanism for the maintenance of telomere length is utilised by human tumours – the alternative lengthening of telomeres (ALT), which involves lengthening of telomeres by homologous recombination-mediated replication of telomere DNA[94].

Sustained angiogenesis

Cells could unlikely survive if they lacked adequate supplies of oxygen and nutrients or were unable to dispose of toxic molecules. As a tumour enlarges, these factors may significantly retard its growth. The ability of tumours to induce angiogenesis – another biological property commonly observed among diverse tumours – was first reported in 1939 by Gordon Ide and co-workers[95] and is believed to be an early to midstage event in many human cancers (Figure 1).

The process of angiogenesis – the formation of blood vessels – is orchestrated by composite and dynamic signals received from a variety of pro-angiogenic and anti-angiogenic factors[96]. Although multifactorial, the process used to be dominantly viewed through the facet of vascular endothelial growth factors (VEGF-A, B, C and D), mainly VEGF-A, and their receptors expressed on the surface of endothelial cells. When activated, these receptors, especially VEGFR-2, trigger downstream growth, survival and cell mobility pathways resulting in the growth of new blood vessels[97]. Induced and increased VEGF expression in tumours is evidenced nearly ubiquitously and it can be triggered by numerous factors such as hypoxia, low pH, inflammatory cytokines, growth factors, sex hormones and chemokines. Besides, alterations occurring in tumour cells may facilitate VEGF expression, including inactivating mutations in tumour suppressor genes and activated oncogenes[97]. Tumour cells are known to secrete another pro-angiogenic factor, basic fibroblast growth factor (bFGF) and, as a consequence of p53 inactivation, down-regulate the expression of anti-angiogenic proteins, such as thrombospondin-1[98,99]. Many more examples could illustrate the complex process of tumour vasculature formation.

With regard to the current focus on cancer stem cells – they have been suggested to be strongly pro-angiogenic. For instance, putative CSCs in brain tumours reside in close proximity of blood vessels and were shown to express high levels of VEGF[100,101]. Besides, aggressive tumours also exert vasculogenic mimicry, or neovascularisation – a process that differs from angiogenesis and may occur simultaneously with angiogenesis, and is characterised by the formation of functional microvascular networks with red blood cell perfusion. The ability to manifest endothelial cell-like properties is observed in many tumour types and it is attributed to aggressive cells with plastic, multipotent tumour cell phenotype[102].

Tissue invasion and metastasis

Sustained angiogenesis is important not only because of blood supply – newly formed vasculature provides the route for tumour cell dissemination. Sooner or later tumour cells – more likely those that have acquired above described physiological properties – gain the ability to metastasise to distant sites of the body where they form secondary tumours. Metastatic spread is an extremely complex process involving multiple successive steps – invasion of surrounding tissue, migration through the ECM, intravasation into the systemic circulation (blood and/or lymph vessels), surviving during the transport, extravasation and pre-metastatic niche conditioning, the final growth of macrometastasis and the late colonisation process of metastases[103].

From a very simplified point of view, tumour cells possessing invasive or metastatic capabilities express altered proteins that are involved in cell-cell adhesion, integrins that link cells to ECM, and extracellular proteases that are able to degrade a path through ECM and stroma[1]. An example apparent in many epithelial cancers is the loss-of-function of the metastasis suppressor E-cadherin, a homotypic cell-to-cell interaction molecule ubiquitously expressed on epithelial cells. The coupling between E-cadherin molecules on nearby cells transmits antigrowth and other signals into the cell through β-catenin to various intracellular signalling circuits[104]. The loss of E-cadherin function is achieved by different means, including mutations, proteolytic cleavage of its extracellular domain, methylation of the *CDH1* gene (encoding E-cadherin) promoter as well as mutations in β-catenin gene CTNNB1[104,105], and this leads to an impairment of cell-cell adhesion, which allows detachment of cells and thus facilitate the epithelial-to-mesenchymal transition (EMT) of carcinoma cells. Noteworthy, the activation of the complex EMT program has been shown to coincide with the expression of stem cell markers in breast cancer[106]. In support of this, a metastatic stem cell model has been proposed (Figure 2, A), which states that cancer stem-like cells rather than tumour cells with limited proliferative potential are responsible for metastasis formation[107], these tumourigenic cells are endowed with higher metastatic potential and they are more frequently detected in circulation and in micrometastases[108].

With regard to the timing of neoplastic cell dissemination during tumourigenesis – it has been hitherto considered as a late event in cancer progression (Figure 1). However, new findings suggest that tumour cell dissemination might occur earlier during carcinogenesis (which is supported by parallel progression model of metastasis, see Figure 2, B), particularly in breast cancer[109], and that early disseminating tumour cells (DTCs, that may be represented by any tumour cell from primary lesion) set up the basis of metastatic disease[107,110].

Intriguingly, an alternative explanation for the nature of metastatic progression has been recently proposed by John Pawelek and Arun K. Chakraborty[111]. The long-standing view on metastatic progression has been essentially Darwinian, but this concept fails to fully explain the phenomenon of metastatic competence[112]. At least to some extent, the metastatic competence has been explained by the identification of master regulatory genes (i.e. metastatic switches) that control multiple pathways and initiate pro-metastatic cascades, such as *SATB1*[113], *SNAIL*, *SLUG* and *TWIST*[114]. The gene expression programmes they initiate often remarkably resemble that from migratory bone marrow-derived cells (BMDCs). Authors suggest that at least in some cases metastasis could be initiated by the fusion of cancer cells with migratory BMDCs, a phenomenon recently evidenced in human cancer. The metastatic ability thus might be acquired through co-expression of both fusion partner genomes[111]. This proposed model thus supplements the current vision of the metastatic models shown in Figure 2.

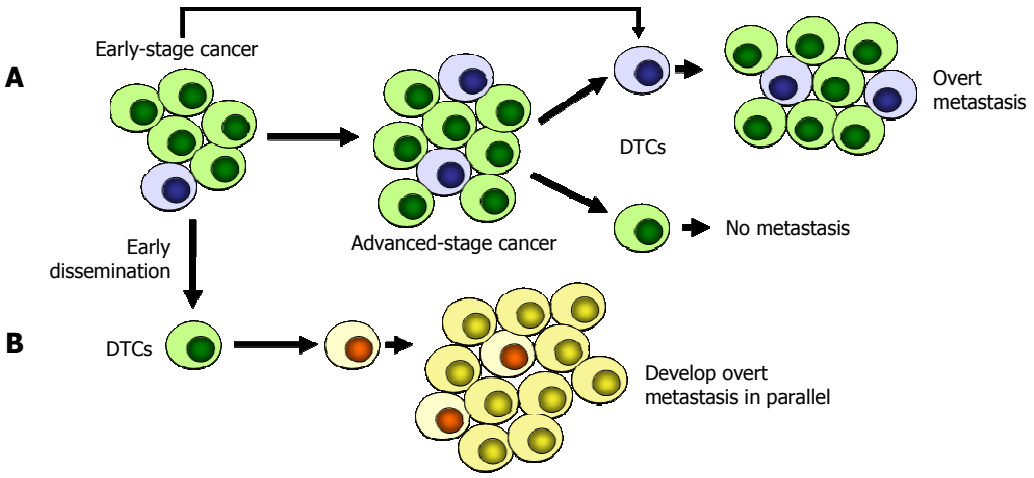


Figure 2. Basic models for systemic cancer progression. A | Metastatic stem cell model. In this model only self-renewing CSCs (blue) are believed to have a capacity to generate overt metastasis on dissemination while DTCs without stem cell properties (green) have only limited proliferative capacity. There, CSCs may disseminate from early stage tumours, or follow the **linear progression mode**l of metastasis where tumour cells proceeds to full malignancy within the primary tumour microenvironment, which is followed by tumour cell dissemination. **B** | Emerging experimental and clinical data increasingly favours **parallel metastatic progression model**, which states that early DTCs may progress independently from primary tumour (red) and acquire ability to develop overt metastasis in parallel. Although these models seem to be contradictious, experimental data from multiple human studies indicate that in fact they might be complementary[107,110]. DTCs denotes disseminating tumour cells. Adapted from Pantel, Brakenhoff & Brandt, 2008[107].

Genetic instability

Genetic instability – either at DNA sequence or karyotype level – is a general feature of almost all epithelial cancers and it is driven by either inherited mutations or somatic alterations in the genes that monitor genome integrity[115], including those encoding various proteins involved in DNA reparation, proper chromosome segregation, and mitotic checkpoint control. This trait of cancer cells results in the increased mutability that significantly contribute to the acquisition of such an array of mutant alleles and physiological deflections outlined above[1]. This may easily be exemplified by many inherited cancer syndromes caused by genetic defects in genes responsible for proper DNA reparation. *Xeroderma pigmentosum* is caused by an inherited defect in nucleotide excision repair leading to the hypersensitivity to the sun and a 1000-fold increased risk of skin cancer. Another example is hereditary non-polyposis colorectal cancer (HNPCC), one of most common cancer syndromes in human. A half of all HNPCC patients carry a germline mutation in *MLH1* or *MSH2* genes participating in mismatch repair[23]. There are also examples related to somatic events – e.g., in cells with mutant p53 uncapped telomeres promote genome instability and progression to cancer[90]. Besides, progressive DNA hypomethylation due to defects in DNA methyltransferases is shown to contribute to the generation of chromosomal instability through the reactivation of repetitive and endoparasitic genetic elements[31]. Importantly, although experimental evidence yet is little[116,117], it has been proposed that genetic instability acquired in self-renewing stem cell population might represent the best driving force for tumourigenesis[56].

1.1.3. MOLECULAR PATHOGENESIS OF DEFINED CANCER TYPES

Significant attempts have been made to reveal and to understand the cellular, molecular, genetic and epigenetic underpinnings of specific oncotypes in order to reveal novel therapeutic targets and strategies. It is next to impossible to shortly overview defined types of cancer in the face of the expanding amount of information driven by advances in modern molecular techniques. Nevertheless, next chapters will try to summarise the disease mechanisms and the interplay of well-known and emerging players in the molecular pathogenesis of malignant melanoma, prostate cancer and gastric cancer.

Malignant melanoma

Melanoma has neuroectodermal origin and most often it stems from epidermal melanocytes (or melanocyte precursors – e.g. melanoma CD20+ stem cells[118]), the pigment melanin producing cells found in skin. It can be also diagnosed in the eye, internal mucosal areas, and the meninges. Melanoma incidence has reached 5.5 cases per 100,000 of European population in the year 2007 and there is a overall tendency for melanoma incidence to increase by 3-8% a year in the Caucasian population[119], including Latvia (data from Cancer Registry of Latvia, Dr. A. Stengrevics, personal communication). Although it is relatively rare in comparison to other skin cancers (i.e. <5%), it is responsible for over 80% of skin cancer deaths[120]. Melanoma differs from the majority of solid tumour types with its high metastatic rates, the low survival of patients with the metastatic disease, the resistance to conventional treatment and a higher incidence among younger people. Among the risk factors of malignant melanoma the strongest appears to be a family history of the disease, while immunosuppression, skin phototype, multiple nevi and exposure to ultraviolet radiation are additional risk factors. With regard to the latter, it is the only known environmental risk factor for melanoma, which results from an intense rather than cumulative sun exposure[121,122].

Malignant melanoma is one of the most studied cancers. The inherited and somatic genetic and epigenetic defects in multiple molecular pathways have been identified in melanoma, enabling the acquisition of the above described six cancer hallmark features[1]. These pathways almost invariably include RAS-RAF-MEK-ERK pathway, the INK4A-CDK4-RB pathway, the ARF-p53 pathway, the PI3K-Akt and less frequently the canonical WNT signalling pathway[123]. Individual genes mutated in melanoma (the main ones) are summarised in Table 1.

There are two genes found to be associated with high penetrance susceptibility to melanoma: *CDKN2A*, the most prevalent one in melanoma families, and *CDK4*. Besides, polymorphic genetic determinants of low penetrance that are associated with sensitivity to UV light have been linked to the predisposition to the disease[121]. For example, people with light skin and red hair often carry germline polymorphisms in the melanocortin receptor 1 gene *MC1R* resulting in its reduced activity[124,125]. Stimulation of MC1R by α-melanocyte stimulating hormone (α-MSH) triggers intracellular signalling circuits responsible for melanin biosynthesis and when impeded, can considerably increase melanoma risk[126]. In general, familial melanomas represent about 8-12% of all melanoma cases; however, not all families with multiple melanoma cases have detectable *CDKN2A* and *CDK4* mutations thus indicating that other melanoma susceptibility genes yet have to be identified[64].

According to the Clark model, the sequential histological changes occurring during melanoma progression are summarised in the five distinct stages: (1) benign nevus, (2) dysplastic nevus, (3) radial-growth phase melanoma, (4) vertical-growth phase melanoma, and (5) metastatic melanoma[127]. The most frequent sporadic changes attributed to the premalignant phases include mutations in *BRAF* (stage 1), loss of CDKN2A and PTEN (stage 2). The radial growth phase is associated with a decreased cell differentiation and the reduced expression of melanoma markers regulated by MITF transcription factor, while the progression form stage 3 to 4 is marked by striking changes in the control of cell adhesion. These include the switch from epithelial E-cadherin to neural N-cadherin, the expression of $\alpha V\beta 3$ integrin and matrix metalloproteinase 2 (MMP-2)[121]. Noteworthy, tumour thickness also called Breslow's depth, is a clinically applied prognostic factor, which indicates the risk of metastasis and the prognosis of survival[128].

Interestingly, in comparison to the majority of other tumours where mutations in p53 are common, in melanoma inactivation of p53 is a relatively rare event[129]. An obvious explanation for this would be that the p53 function is abrogated due to the sporadic loss-of-function of suppressor protein ARF, the positive p53 regulator (Table 1).

Table 1. Genes altered in melanoma.

Gene	Encoded protein	Most frequent type of alteration	Frequency (%)*	Physiological hallmark
Proto-oncogenes				
NRAS	N-RAS, small guanidine binding protein	Mutation	15-25	Cell proliferation
BRAF	BRAF, serine-threonine kinase	Mutation (90% accounts for V600E)	50-70	Cell proliferation Senescence induction
KIT	KIT, stem cell growth factor receptor tyrosine kinase	Mutation	2-10	Cell proliferation
CDK4‡	CDK4, cyclin-dependent kinase 4	Mutation, amplification	0-9	Cell proliferation
CCND1	CD1, G1–>S specific cyclin D	Amplification	6-44	Cell proliferation
MITF	MITF, a transcription factor for melanocyte-specific genes	Amplification	10-16	Melanocyte lineage survival and progenitor cell maintenance
CTNNB1	β-catenin, cadherin-associated protein	Mutation	2-23	Cell proliferation Invasion & metastasis
PIK3CA	Catalytic subunit of PI3K, phosphatidylinositol 3 kinase	Mutation	<5	Increased survival Invasion & metastasis
AKT3	AKT, protein kinase B	Amplification	40-60	Increased survival Invasion & metastasis
Tumour suppressor genes				
INK4A‡ *(CDKN2A#)*	INK4A or p16, inhibitor of G1–>S cyclin-dependent kinase CDK4	Deletion (9p21), mutation PH	40-87 (25-40*) 10-33	Cell proliferation Evasion from senescence
ARF‡ *(CDKN2A#)*	ARF or p14, positive regulator of p53, MDM2 inactivator	Deletion (9p21), mutation	40-70	Delayed senescence Evasion form apoptosis
APC	APC, adenomotous polyposis coli	PH	19	Cell proliferation
PTEN	PTEN, phosphate and tensin homologue	Deletion (10q23-24), mutation PH	5-40 0-62	Increased survival Invasion & metastasis
RB1	RB, retinoblastoma protein, negative cell cycle regulator	Deletion, mutation	NA (rare)	Cell proliferation
TP53	Tumour protein p53	Mutation	0-25	Evasion from apoptosis and senescence
CDH1	E-cadherin, epithelial cell adhesion molecule	SNAIL-induced transcriptional repression	NA (frequent)	Transition from radial to vertical growth phase

* For complete reference set see recent reviews[64,121,123]
‡ Genes carrying also germline mutations
# Transcription of the *CDKN2A* gene may result in either INK4A or ARF (denoting alternative reading frame) proteins due to the combination of alternative splicing and reading frames.
PH denotes promoter hypermethylation, NA – not assessed.
Summarised from the comprehensive reviews[64,121,123].

Melanomas originating at particular sites in the human body and thus having differences in the sun exposure seem to exhibit different genetic alterations[130]. It has been shown that melanomas arising in the skin regions without chronic solar exposure had frequent mutations in *BRAF* together with losses of chromosome 10 containing *PTEN,* or mutations in *N-RAS* alone, whereas melanomas from chronic sun-induced damage rarely have mutations in *BRAF* and have frequently higher levels of CCND1 due to the gene amplification[130] (Figure 3). This is consistent also with the observation that mutations in *BRAF* do not contain typical ultraviolet "fingerprint" mutations (CC–>TT transitions)[71].

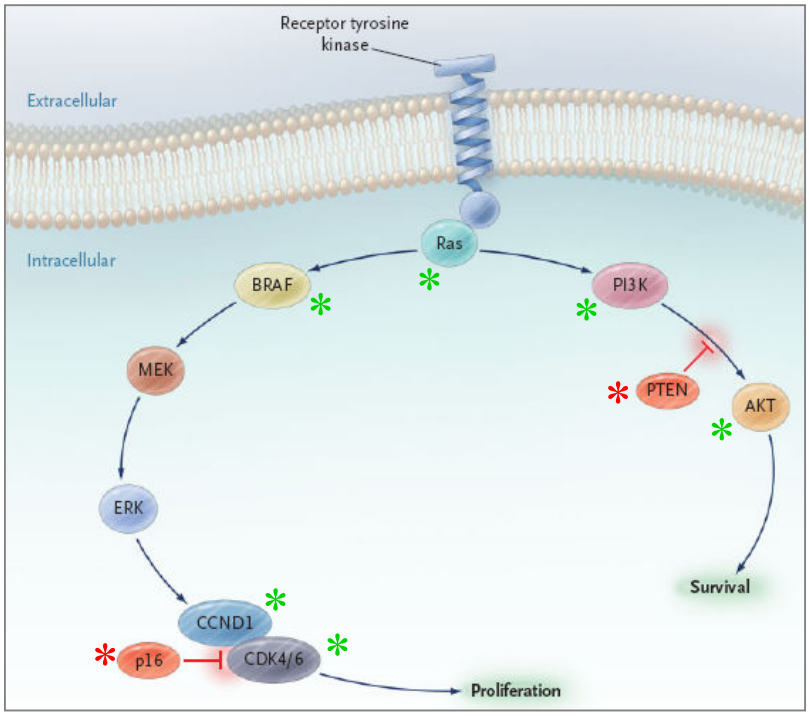


Figure 3. RAS-RAF-MEK-ERK and PI3K-Akt pathways in melanoma. Signals from receptor tyrosine kinases can promote cell proliferation and survival through the two different signalling pathways. There is a set of mutually exclusive mutations in melanoma, for example mutations in *PTEN* occur together with those in *BRAF*, but not *N-RAS*, suggesting the PI3K-Akt pathway as an independent somatic target. As well, mutated NRAS and BRAF are mutually exclusive. Further, *CDK4* and *CCND1* are also indicated as independent oncogenes in melanoma without mutation in either *BRAF* or *N-RAS*. Red stars denote loss-of-function, green stars – gain-of-function (see also Table 1). Supplemented from Curtin *et al.*, 2005[130].

The relevance of melanocyte stem cells in the aetiology of malignant melanoma is uncertain. There is a growing body of evidence suggesting that during melanomagenesis aggressive melanoma cells acquire a multipotent, plastic phenotype much alike embryonic stem cells[102]. An apparent example of this plasticity is the vasculogenic mimicry, a feature of aggressive melanoma cells expressing endothelial-associated genes[131]. Cells with stem cell characteristics have been isolated from different melanoma cell lines and tissues[132], and amongst the characterised ones CD20+[133] and drug resistant ABCB5+/CD133+ cells can be named[134]. In addition, a case study has demonstrated the evidence strongly supporting the role of a common progenitor cell in the initiation of multiple recurrences of malignant melanoma[117].

Prostate cancer

This disease is the leading cause of illness and cancer-related death among men over 65 years of age in United States and Western Europe[135]. According to Cancer Registry data of Latvia (Dr. A. Stengrevics, personal communication), dramatic increase in prostate cancer incidence and mortality has been experienced in Latvia (Figure 4), and this, although at lesser extent, is similar to that seen in other European countries and USA – the estimated incidence of prostate cancer in year 2008 reaches ~140 cases per 100,000 in the United States[136]. Death rates from the disease generally are 3-5 times lower. In Latvia, prostate cancer is responsible for ~13% of all cancer-related deaths holding the second position (after bronchi/lung cancer).

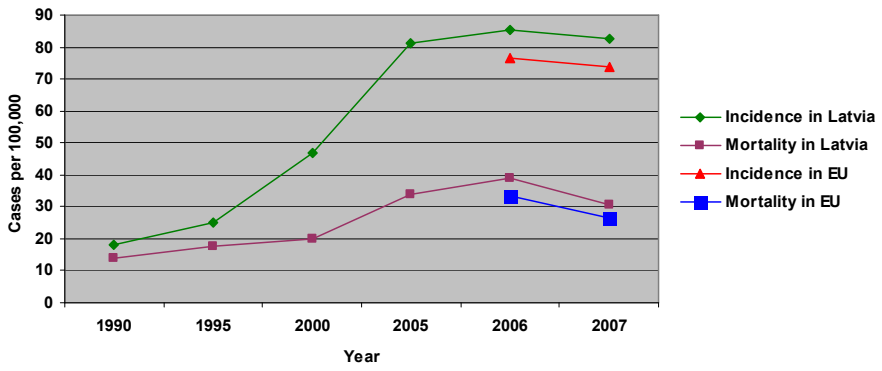


Figure 4. Prostate cancer statistics in Latvia. Prostate cancer incidence and mortality rates have increased dramatically in Latvia for last ten years. Data representing age-standardised rates per 100,000 European population in years 2006 and 2007 are given for comparison. Data were obtained from Cancer Registry of Latvia (Dr. A. Stengrevics, personal communication).

Prostatic adenocarcinoma originates from prostate epithelial cells or – in accordance with the cancer stem cell model – from epithelial stem cells embedded in the epithelium of prostate, a gland in the male reproductive system. The list of the factors contributing to prostate cancer risk includes diet and lifestyle-related factors, androgens, inflammation, environmental carcinogens and ineritance[135]. The stages and steps of the molecular pathogenesis of prostate cancer are depicted in Figure 5.

Genetic studies in Danish and Swedish twins revealed that the hereditary component in predisposition to prostate cancer is likely to be stronger than in any other type of human cancer – the results allowed to anticipate that ~42% of the risk of the disease could be due to heritable factors[137]. Familial prostate cancer studies have lead to the identification of several candidate susceptibility genes. *RNASEL* gene encodes a latent endoribonuclease that participates in an interferon-inducible RNA decay pathway and it has a suggested role in viral and cellular RNA degradation. Various inactivating mutations affecting *RNASEL* have been found by analysing familial prostate cancer cases likely leading to the diminished IFN-α antiviral activity[135]. The expression of MSR1, the macrophage scavenger receptor, appears to be restricted to macrophages in the prostate, particularly at the sites of inflammation. Germline *MSR1* mutations have been linked to familial prostate cancer and detected in ~3% of non-hereditary prostate cancer cases[138]. Both proteins encoded by *RNASEL* and *MSR1* genes have critical functions in host defences against infections supporting the role of inflammation in the aetiology of this tumour type. Further, polymorphic variants of androgen receptor gene (*AR*), the cytochrome P-450c17 gene (*CYP17*), the steroid-5-α-reductase type II gene (*SRD5A2*) and others have been associated with increased prostate cancer risk in genetic epidemiology studies[135]. However, despite large number of studies aimed to identify the prostate cancer susceptibility loci, there is no major susceptibility gene for prostate cancer identified yet.

Apart from inherited alterations, genes affected by somatic defects have been found in prostate cancer (for their onset during prostate cancer progression see Figure 5). *GSTP1* encodes a π-class glutathione S-transferase – an enzyme rarely present in prostate cancer cells due to transcriptional repression attributed to hypermethylation of *GSTP1* promoter[139]. Since this protein functions in xenobiotic metabolism, it may represent a link between the exposure to genome damaging stress and increased genomic instability[140].

NKX3.1 encodes a prostate-specific homeobox protein, which represses expression of the prostate-specific antigen (PSA) gene thus being essential for normal prostate development and it is considered as the candidate "gatekeeper" in the aetiology of prostate cancer. The NKX3.1 protein expression is partially lost at the early stage prostate cancer and it continues to decrease during the progression of the malignancy due to the loss of 8p21 DNA sequence[135]. Moreover, a recent study indicated that certain germline sequence variants in *NKX3.1* may play a role also in the susceptibility to hereditary prostate cancer[141].

The somatic allelic loss of *PTEN* tumour suppressor gene and reduced levels of p27, a cyclin-dependent kinase inhibitor encoded by *CDKN1B* gene, are common in prostate cancer. Both proteins are linked via the PI3K-Akt signalling pathway – PTEN inhibits the PI3K-Akt survival pathway and leads to elevated levels of p27. As the molecular basis of low p27 levels has not been revealed yet, it has been supposed as an indirect effect of the PTEN function loss[135].

Androgen receptor (AR), a member of the nuclear receptor superfamily, is a critical effector of the prostate cancer development and progression. Testosterone is the most abundant androgen found in men's circulation and it is converted to a more potent form of dihydrotestosterone (DHT) in the prostate epithelium. The binding of DHT to AR results in the receptor translocation to the nucleus where ARs as homodimers bind DNA on androgen responsive elements leading to the transcriptional activation of target genes (including PSA) involved in development, growth and survival[30,142]. Although usually the treatment of patients with androgen suppression and/or antiandrogens is initially successful, sooner or later androgen-independent tumour cell variants emerge. These variants are characterised by (i) somatic mutations in the ligand-binding region of AR enabling its activation by other ligands, (ii) increased expression of AR resulting from the gene amplification, and (iii) ligand-independent AR activation by growth factor signalling pathways[142]. As well, proteins involved in signalling downstream of AR are altered – e.g. RB protein is lost or inactivated in 30-60% of prostate adenocarcinomas through different mechanisms[142,143] sustaining cell proliferation.

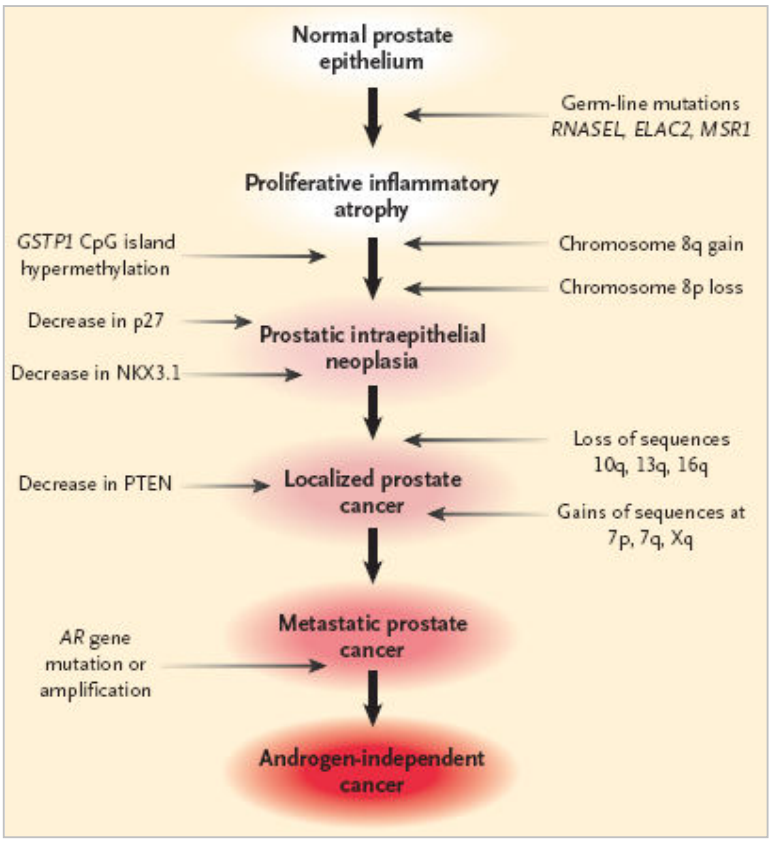


Figure 5. The molecular pathogenesis of prostate cancer. Proliferative inflammatory atrophy is a new precursor lesion of prostate cancer, which represents a link between prostatic inflammation and cancer. From Nelson *et al.*, 2003[135]. Abbreviations: RNASEL – ribonuclease L, ELAC2 - elaC homolog 2 (*E. coli*), MSR1 - macrophage scavenger receptor 1, GSTP1 - glutathione S-transferase pi 1, NKX3.1 - NK3 homeobox 1, PTEN - phosphatase and tensin homolog, AR – androgen receptor.

The existence of stem cells in prostate and prostate cancer has also been investigated recently. Prostate epithelial stem cells have been identified in human and mice, whereas evidence on prostate CSCs is limited[144]. It has been suggested that putative prostate cancer stem cells exhibit CD133+/CD44+/$\alpha_2\beta_1$−integrin^{high}, CD44^{high}/SMO+/β-catenin and CD44^{high}/CD133+ phenotypes[66,145]. Nevertheless, there is some doubt that the characterised cell subpopulations are indeed of cancer origin, since direct confirmation of this was lacking[144,146]. However, prostate cancer treatment has the advantage over many other cancer types – the normal counterparts of prostate CSCs are not vitally important and thus could be simultaneously targeted when treating the disease with yet to be emerging prostate stem cell-targeted therapy thus avoiding the need for highly specific CSC targets.

Gastric cancer

According to WHO data, gastric cancer is the second most common cancer in men and the third one in women[147]. Although gastric cancer incidence is decreasing worldwide, it still accounts for 3-10% of all cancer related deaths[148] largely due to its late diagnosis. Incidence of gastric cancer varies greatly across different geographical locations. In year 2007 it reached 36 and 23 new cases per 100,000 of the European male and female population, respectively, while in Latvia incidence of gastric cancer is generally of ~9-10% higher in comparison to that seen in Europe. In Latvia gastric cancer is third most common cancer in men and fifth in women and it is responsible for 10% of all cancer-related deaths holding third position (after bronchi/lung cancer and prostate cancer) in men and second (after breast cancer) in women in Latvia (data from Cancer Registry of Latvia, Dr. A. Stengrevics, personal communication).

The risk factors of gastric cancer, amongst others, include the *Helicobacter pylori* infection, diet habits (most likely responsible for the differences in the worldwide cancer incidence), chronic atrophic gastritis, bile reflux, gastric polyps, obesity, and family history[148]. Familial gastric cancer comprises 8-10% of total stomach cancer cases and the underlying genetic event is not defined in most of them[149] – while two thirds of all gastric cancer families remain without molecular diagnosis, one third accounts for a single autosomal-dominant syndrome called hereditary diffuse-type gastric carcinoma (HDGC)[149] (Figure 7). HDGC is specifically associated with high penetrance germline mutations in *CDH1* gene most frequently leading to the expression of truncated E-cadherin, rarely seen in sporadic cancer[150]. Besides, gastric carcinoma occasionally is seen in families with germline mutations in p53 (Li-Fraumeni syndrome) and *BRCA2*[151].

In the aetiology of gastric cancer (and MALT lymphoma arising in stomach[152]) an important role is allocated for the persistent *H. pylori* infection, which is the strongest known risk factor for non-cardia gastric cancer[153]. *H. pylori* is a gram negative, flagellated microaerophilic bacterium, a parasite of human gastric and duodenal mucosa that is well known causative agent for ~90% of gastric and duodenal ulcers. However, the assessment of the causal relationship between the infection and the progression from gastritis to gastric cancer has been a difficult task[154]. The worldwide prevalence of *H. pylori* infection reaches ~80%, yet only a minority develop gastric cancer. Moreover, patients with duodenal ulcers almost invariably are infected with *H. pylori*, but rarely develop gastric cancer[154]. These discrepancies have been associated with (i) inherent properties of distinct *H. pylori* strains, (ii) inflammatory responses governed by host genetics and (iii) interactions between host and microbial determinants, which collectively are involved in fate determination for the disease caused by the *H. pylori* infection (Figure 6)[154]. Studies in human and mice have lead to the identification of multiple targets in the host cells affected by the bacterial toxins. For example, the oncogenic factors like vacuolating toxin (VacA) targets mitogen activated protein kinases (MAPKs) and lead to elevated cyclic adenosine monophosphate (cAMP) while cytotoxin-associated antigen (CagA) activates the c-Met receptor tyrosine kinase, thus promoting acquisition of the hallmark properties of cancer cells. Moreover, *Helicobacter* spp. are also known to alter cell cycle control and apoptosis[153]. Regarding host genetics, individuals with defined polymorphisms in the *IL1B* (interleukin 1-β) gene promoter region are at significantly increased risk of developing hypochlorhydria, gastric atrophy and cancer[155]. Besides, chronic *H. pylori* infection also promotes cancerogenesis via inflammatory mechanisms, e.g. the production of pro-inflammatory cytokines, the stimulation of cyclooxygenase-2 (COX2) expression, and the production of reactive oxygen species (ROS) and reactive nitric oxide species (RNOS)[156], with the two latter being implicated in increasing rates of DNA damage and post-transcriptional alterations[157,158]. Thus, the bacteria encourage the stomach epithelial cells to acquire the important hallmark capabilities involved in carcinogenesis[1].

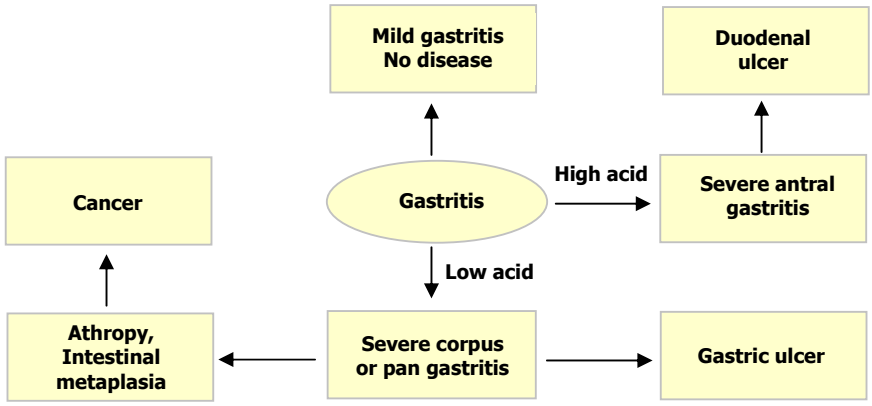


Figure 6. Potential outcomes following *H. pylori* infection. Gastritis-associated hypoacidity is associated with an increased risk of gastric malignancy, which has been linked to rapid bacterial colonisation of stomach. Patients developing gastric or duodenal ulcers, which result from inflammation associated with high acid secretion thus are more likely protected from neoplastic lesions[159]. The factors determining the fate of the *H. pylori* infection include the bacterial strain variations and host responses to the infection. Adapted from Axon, 2007[154].

According to the WHO system, gastric cancer is categorised in five subtypes – adenocarcinoma, papillary, tubular, mucinous and signet-ring cell cancer. Approximately 85% of the cases of neoplastic lesions in stomach are adenocarcinomas that occur in the lining of the stomach mucosa and histologically these are classified into two major subtypes – intestinal and diffuse (i.e. Lauren classification)[148], both of which are accompanied by the key molecular alterations (Figure 7)[158].

The intestinal type adenocarcinoma is often preceded by a prolonged precancerous phase of well defined histological changes starting from chronic gastritis and subsequently leading to atrophic gastritis, intestinal metaplasia, dysplasia and eventually carcinoma (Figure 7)[158]. The transition from atrophic gastritis to intestinal metaplasia is characterised by an inappropriate activation of the intestine-specific transcription factor CDX2 (caudal type homeobox transcription factor 2), expression of which is absent in normal stomach epithelial cells[160]. The mechanisms responsible for its ectopic expression are unclear, although the BMP/SMAD pathway has been proposed to have a role[161]. The ectopic CDX2 expression results in the transcription of intestine-specific proteins (e.g. MUC2, sucrase/isomaltase, carbonic anhydrase) and the upregulation of WAF1 (known also as p21 or CIP1), a cyclin-dependent kinase inhibitor, and itself. Thus, this transcription factor participates in the induction of intestinal-like differentiation and cell cycle arrest in the intestinal metaplasia. As the transformation progresses to the neoplastic state, cells inevitably tend to become less differentiated and this is accompanied by downregulated CDX2 expression, possibly through the promoter hypermethylation mechanism[158].

The transition from intestinal metaplasia to the adenoma/dysplasia is known to be accompanied by other somatic events (Figure 7). Mutations in adenomatosis polyposis coli (APC) gene are frequently found in adenomas leading to the β-catenin stabilisation through the activation of the WNT-TCF signalling pathway. Nevertheless, *APC* mutations in gastric cancer are rare indicating that they might be involved in only small subset of adenoma-carcinoma sequences[162]. Besides, the genes encoding β-catenin and KRAS are to be independent somatic targets; however, both of them are rarely mutated in gastric cancers[158]. About 10% of gastric cancers have been associated with genetic changes in TGF-β type II receptor (TGFBR2) leading to the resistance of TGF-β-induced growth suppression. Interestingly, these changes, which most frequently affects the polydeoxyadenine tract of the gene, are highly associated with microsatellite instability resulting from the transcriptional silencing of the epigenetic mismatch repair gene *MLH1*[163,164].

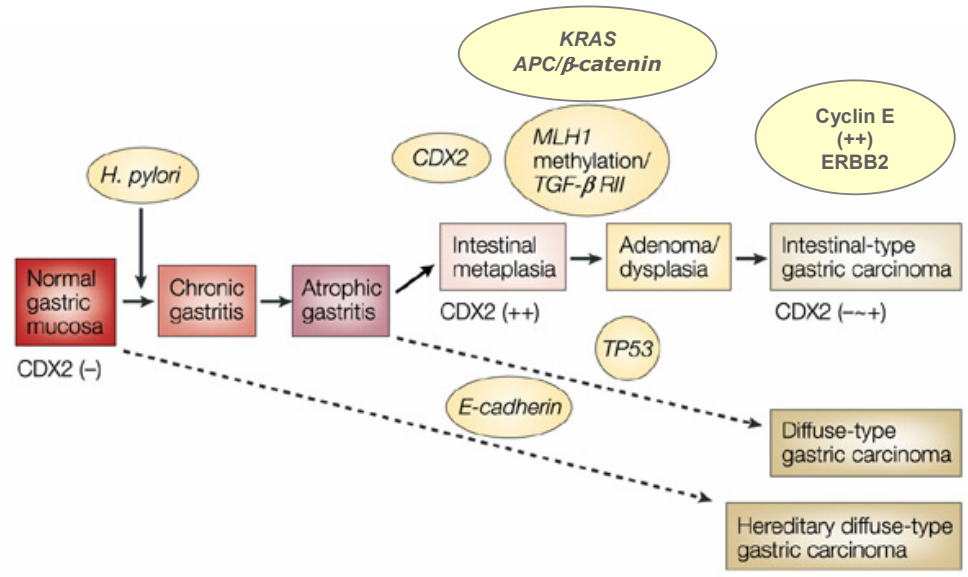


Figure 7. Models for the pathways of gastric carcinogenesis. The three main types of carcinomas are known to arise from gastric epithelium. The sporadic carcinogenic pathways are associated with *H. pylori* infection, subsequent inflammation and tissue regeneration. During the process of tissue regeneration, the differentiated intestinal-like phenotype and histologically undifferentiated phenotype is acquired resulting in the intestinal-type and diffuse type gastric carcinomas, respectively. The (-~+) indicates no or low expression of the protein. Supplemented from Yuasa, 2003[158].

Up to 80% of dysplastic lesions may progress to invasion, the key diagnostic factor for carcinoma[148]. Amongst the changes associated with the intestinal-type gastric cancer, there can be mentioned a downregulation of RUNX3, a runt-domain transcription factor, which is evidenced in 45-60% human gastric cancer cells, which results in cell proliferation and apoptosis suppression[158,165]; the amplification of ERBB2 (HER-2/neu) and cyclin E genes, as well as increased telomerase activity[158]. Besides, *TP53* mutations and/or loss have been detected in more than a half of both gastric adenocarcinoma subtypes[166].

The molecular pathogenesis and sequential steps of histological changes leading to the diffuse type gastric adenocarcinoma are less well defined (Figure 7). This neoplasia arises less frequently than intestinal type cancers and is believed to originate from the sites of gastric atrophy, and usually is histologically undifferentiated. These tumours are formed of non-cohesive neoplastic cells diffusely infiltrating the stroma of the stomach, and show no or little glandular structure formation. In contrary, intestinal-type carcinomas usually show the formation of recognisable glands similar to intestinal ones, and have an expanding rather than infiltrative growth pattern[148,158]. Alterations in the genes encoding E-cadherin and p53 through different mechanisms seem to have crucial roles in the aetiology of this tumour subtype[158].

Normal gastric stem cells yet have to be defined and characterised. Nonetheless, they seem to be good candidates for gastric CSCs since the induction of tissue repair due to the *H. pylori* infection-driven atrophy likely could lead to hyper-proliferation and an increased rate of mitotic errors in gastric stem cells. Recent studies by Takaishi, Okumura and Wang have pointed the existence of gastric CSCs, which were characterised by the expression of a single cell surface marker (name undisclosed), the formation of spheroids *in vitro*, the ability to induce tumours *in vivo* and resistance to chemotherapy[167]. In parallel, another mechanism of the emergence of gastric CSCs has been proposed. Circulating bone marrow-derived cells that home in the sites of tissue injury resulting from chronic *Helicobacter*-induced infection were shown to contribute to gastric carcinogenesis[61].

1.2. BASIC TUMOUR IMMUNOLOGY

The human immune system historically has been seen as an expert able to fulfil the task to properly discriminate between self and non-self (foreign) – to fight infections in order to maintain tissue homeostasis and to repair damaged tissues[168]. Tumour cells in this context lay in the middle since are expressing both, self and altered self molecules (or foreign in the case of virus-induced tumours), and in certain circumstances are perceived by the immune system as dangerous. Many lines of evidence suggest that the immune system, apart from many cell-intrinsic surveillance mechanisms, may take part in the tumour cell eradication through the process known as cancer immunosurveillance (Figures 8 and 9). Paradoxically, but chronic human immune system-driven responses (especially by its innate part) are increasingly becoming recognised as the promoters of tumour development[169]. Despite enormous efforts paid to unravel the complex nature of tumour-immune system relationships, it has to be stressed that the field still remains in a great flux, with even the basic concepts being a matter of extended debate.

At present, the recently proposed general concept outlined in the Figure 8 suggests that the multistep development of tumours results from the crosstalk between cancer-intrinsic factors and the effects of host immune system (cell-extrinsic factors) [170]. The above described and generally accepted six hallmark capabilities are cell-intrinsic properties acquired by malignant cells during tumour progression[1]. In this context, the ability of tumour cells to avoid immune surveillance has been named as the seventh hallmark of cancer[171]. Neoplastic cells tend to escape protective innate and adaptive immune responses (i.e. immunosurveillance) by immunoselection, a process of non-immunogenic tumour cell variant outgrowth, and by immunosubversion – the active suppression of immune responses[170].

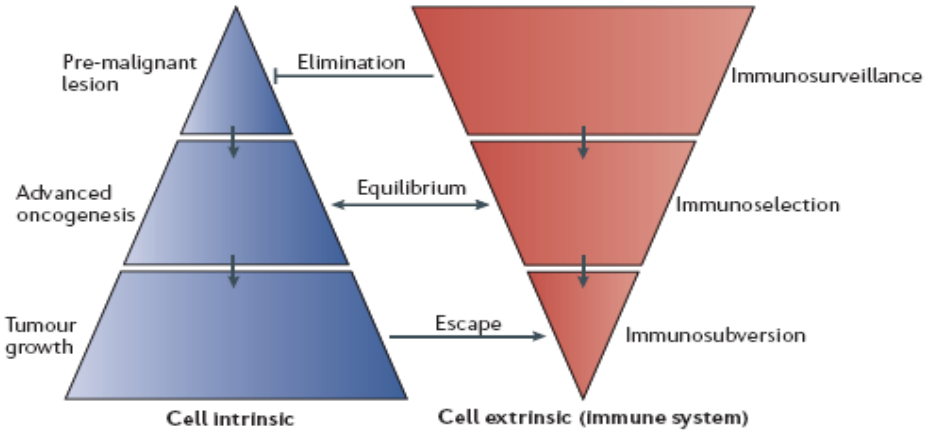


Figure 8. The proposed general concept of the involvement of the immune system in tumour development. Emerging data supports the existence of numerous links between tumour cell-intrinsic and cell-extrinsic (immune system-mediated) mechanisms underlying the process of multistep carcinogenesis. From Zitvogel, Tesniere & Kroemer, 2006[170].

1.2.1. CANCER IMMUNOSURVEILLANCE

In 1909 Paul Erlich proposed that tumour growth can be suppressed by the host's immune system[172], and later this concept was termed "cancer immunosurveillance" by McFarlane Burnet and Lewis Thomas[173]. Nevertheless, only recently it has gained definitive approval through experimental knock-out mouse models[174]. Although it has been difficult to examine natural immunosurveillance in human cancers, the studies revealing early tumour infiltration with immune effector cells and the generation of immune responses to tumour-derived proteins (i.e. tumour antigens), both of which have been found to correlate with positive disease outcome, as well as the increased risk of cancers seen in immunocompromised individuals have provided convincing evidence[175]. Cancer immunosurveillance is driven by various effectors participating in innate and adaptive immune responses leading to the recognition and elimination of incipient cancer cells in premalignant lesions. It is believed that

immunosurveillance keeps many initial tumours from growing into clinically apparent bulk[170,176] (Figure 8). Immunosurveillance mechanisms described herein are exactly those that cancer immunotherapy, at least initially, has attempted to use and enhance to achieve tumour eradication.

Initiation of anti-tumour immune responses

As tumour growth progresses, the death of tumour cells by means of apoptosis or necrosis is inevitable due to multiple cell-intrinsic and extrinsic stresses. It has been widely accepted that apoptosis is a physiological type of cell death, which remains immunologically silent. One of the mechanisms is well known – the dying apoptotic cells are rapidly and specifically recognised by phagocytic cells through sensing phosphatidylserine residues (an "eat me" signal) on the plasma membrane surface of the apoptotic cell, normally exposed only on the inner leaflet of the plasma membrane bilayer[177]. The clearance of these cells remains silent since phagocytic antigen presenting cells (APCs) in these conditions do not express costimulatory molecules (such as B7) on their surfaces[178]. Besides, this process is accompanied by the release of anti-inflammatory mediators in order to avoid local inflammatory reactions[179]. However, recent studies have revealed that at certain circumstances apoptosis can be immunogenic, as it is evidenced in tumours[180,181]. The decision to initiate an immune response to apoptotic cells or not is likely made by dendritic cells (DCs) and it is guided by the interrogation of the dying cells through different proteins on their plasma membrane, released inflammatory cytokines or end-stage degradation products with immunogenic properties[180].

By contrast, necrotic cell death is generally considered to be immunogenic, since it is accompanied by the release of their contents as soluble molecules into the extracellular space, the induction of pro-inflammatory cytokines and the expression of costimulatory molecules on APCs[182]. Considering all this, the endogenous "danger signals" (also known as damage-associated molecular patterns, or DAMPs), such as uric acid[183], high-mobility group box 1 (HMGB1), heat shock proteins (HSPs) 70 and 90 are inevitably released by tumour cells in the microenvironment leading to the recruitment of immune system components to the tumour site[180]. Besides, when tissue homeostasis is perturbed, sentinel tissue residing macrophages and mast cells immediately release soluble mediators (e.g. cytokines, chemokines, histamine and matrix remodelling proteases) in the milieu through which additional leucocytes are attracted to the damaged tissue. This results in the initiation of a process known as inflammation[168].

The signs of inflammation are seen in most pre-cancerous and cancerous lesions[184]. Although immunogenic tumour cell death can trigger inflammatory responses themselves, it is important to mention that unresolved inflammation often precedes cancer. Over 15% of all human cancers are believed to be initiated by infections that induce chronic inflammatory conditions[185]. Apart from infections, the genetic predisposition of the host as well as an unceasing exposure to ongoing chemical or physical irritation contributes to unresolved inflammatory states[169]. This illustrates the paradoxical role of the immune system in tumourigenesis since instead of protecting the host from transformed cell outgrowth, it may contribute to tumour initiation or promotion via indirect mechanisms such as the induction of DNA damage through the production of free radicals, enhancing tumour growth by releasing factors stimulating tissue architecture renewal during tissue repair, promoting angiogenesis etc[169]. These mechanisms are largely mediated by the innate immune system components (e.g. neutrophils, macrophages and mast cells)[169]. Importantly, unresolved inflammation facilitates tissue-specific stem cell hyper-proliferation and/or the recruitment of bone marrow-derived cells[61] implicated in cancer aetiology.

Innate immune system and cancer immunosurveillance

The innate immune system is thought to act as the first line of defence against tumours. The innate response to tumours is invariable and acts through germline-coded pool of specific receptors, called pattern recognition receptors, which recognise a broad range of different antigens in a major histocompatibility complex (MHC*) molecule-independent manner[182]. As well, it provides the means towards the generation of the adaptive anti-tumour immunity[168]. For example, cellular components of the innate immune system, namely natural killer (NK) cells, natural killer T (NKT) cells, γδT cells[186] and eosinophils[187], contribute to the immunogenic tumour cell death thus augmenting tumour antigen

* The abbreviation MHC holds groups of cell surface antigen presenting proteins found in all jawed vertebrates, and it is used throughout in the text since a large part of the studies referred herein have been done in rodents. In humans, these molecules are also known as human leukocyte antigens (HLA).

uptake by tissue-residing APCs, most importantly – immature dendritic cells (DCs)[188]. After the phagocytic uptake of a tumour-derived cargo, DCs are activated and migrate to the draining lymph node, where they can present tumour antigens to naïve antigen-specific T cells in the context of MHC class I and class II molecules – the process known as cross-presentation[189,190] thus initiating the adaptive immune response (Figure 9).

In general, there are three types of antigen presenting cells – DCs (the most professional ones), macrophages and B cells[168], but it has to be kept in mind that many cell types may acquire APC phenotype in the inflammatory environment as it has been reported for γδT cells[191] and tumour cells themselves[192]. **DCs** are crucial cells because they play a central role in the initiation as well as regulation of the innate and adaptive immunity[193]. These professional antigen presenting cells are found to be infiltrated in many types of human tumours[194,195] and the nature of DC-induced immune responses is largely dependent on the balance between different positive and negative signals received from surrounding cells in the tumour microenvironment[193]. Besides, different DC subsets exist (plasmacytoid and myeloid DCs, the latter includes Langerhans cells and interstitial DCs), each displaying different physiological phenotypes and biological functions[193].

Macrophages are phagocytes involved in dying tumour cell clearance. These cells also have important roles in the initiation, propagation and resolution of inflammation[196]. They stem from blood-derived monocytes, which enter tumour tissue and differentiate either into immature dendritic cells or tissue macrophages depending on the cytokines present in the microenvironment. Tissue macrophages can further differentiate into M1 or M2 cells, each of them secrete different patterns of cytokines and exert different functions. Tumour-infiltrating macrophages have been implicated in the local modulation of immune responses since M1 cells (also known as classically activated macrophages) promote acute inflammation, cellular immunity and tumour cell death, but M2 cells (also known as alternatively activated macrophages) downmodulate immune responses and provide growth and angiogenic factors for tissue repair and thus also supporting tumour growth[195,197].

Eosinophil infiltration in tumours has been documented in many studies, and high numbers have been associated with increased survival in many cancer types[198-200]. Recently it has been proposed that eosinophils may play an active role in cancer immunosurveillance through Th2-mediated adaptive anti-tumour immunity. Cytokines secreted by Th2 cells, particularly interleukin (IL)-4, IL-5 and IL-13, have been shown to have a role in the eosinophil recruitment to the tumour site and their activation for tumour cell killing[187]. However, the mechanisms of eosinophil-mediated tumour cell clearance still remain to be elucidated.

NK cells comprise ~15% of all circulating lymphocytes, and are also found in peripheral tissues, such as liver, placenta and peritoneal cavity[201]. These cells are defined as CD3-CD56+ lymphocytes, which can be divided into two major subtypes – $CD56^{high}$ and $CD56^{low}$. The former have immunoregulatory functions while the latter exert cytotoxic functions[202]. NK cells are activated in response to type I interferons and macrophage-derived cytokines and are known to help in protecting host against infections[168] and tumour transformation by eliminating affected cells[201]. The main mechanism of target cell killing is the induction of membrane damage and/or apoptosis through the release of cytotoxic granules (containing perforin and granzymes) onto the surface of a bound target cell – much like it is seen in cytotoxic T lymphocyte (CTL)-mediated killing[168]. Besides, NK cells express molecules of the TNF superfamily that can trigger death receptors on target cells[202], and activated NK cells secrete interferon gamma (IFN-γ), a cytokine with a pivotal role in promoting host anti-tumour immunity[203]. Apart from this, NK cells express an activating receptor for IgG constant regions, for example FcγRIII (i.e. CD16), and this enables NK cells to recognise and kill antibody-coated target cells through the antibody-dependent cell-mediated cytotoxicity (ADCC) mechanism[168]. Extensive NK cell infiltration in tumours has been linked to good prognosis, and the involvement of all the above listed mechanisms have been documented in anti-tumour immune responses[201]. NK cells are especially good on targeting tumour cells that have lost MHC class I molecule expression on their surfaces (which is a frequent event during tumourigenesis[204]), since these molecules deliver inhibitory signals to NK cells[205].

γδT cells are lymphocytes belonging to the unconventional T cells expressing T-cell receptors (TCRs) composed of γδ heterodimers instead of αβ heterodimers, which form the TCRs expressed on the surface of the majority of T cells. γδT lymphocytes encompass multiple cell subsets, most of which are characterised by CD4-CD8- phenotype and generally comprise 1-10% of all T cells[206]. These γδT cells are particularly enriched in epithelial surfaces, show a much more restricted TCR diversity[168], and

exhibit characteristics that place them on the border between innate and adaptive immunity[206]. γδTCRs may recognise an antigen in an MHC-independent manner in its natural unprocessed conformation, as well as those presented by the non-classical MHC-like proteins[168,206]. Antigens recognised by these receptors include those of protein and non-peptide ligands, such as phosphorylated ligands and lipid antigens[168]. γδT cells have important roles in antimicrobial immunosurveillance and several lines of evidence indicate their role in cancer immunosurveillance as well[206]. In TCRδ$^{-/-}$ mice, chemically induced cutaneous tumours occur with shorter latency, and tumours form more readily after the injection of tumour cells[207]. The most well known mechanism of γδT cell induced cytotoxicity is NKG2D-mediated lysis. NKG2D (denoting NK group 2, member D) is an activating receptor expressed in NK cells, CD8+ αβT cells, macrophages and γδT cells[206]. The identified ligands of NKG2D include MHC class I chain-related A and B (MICA and MICB), which are expressed on a variety of tumour cells, and their binding to NKG2D provide a costimulatory effect and triggers tumour cell destruction[208,209]. Besides, γδT cells have also been shown to secrete IFN-γ early in tumour development[210].

NKT cells are true T cells bearing TCRs and CD3 molecules, but they also have receptors specific to NK cells that allow them to respond more rapidly in an innate fashion[211]. Thus, NKT cells, like γδT cells, lay in the middle between innate and adaptive immunity. These cells are found in particularly large numbers in the lung and liver, and they recognise foreign and self glycolipid and lipid antigens that are presented by the MHC class I-like molecule, Cd1d[168]. Different NKT cell subsets have inherited ability to produce Th1 and/or Th2 cytokines upon activation. The choice of secreted cytokines depends upon the surrounding stimuli received by NKT cells, and the NKT cell type, and it leads to a corresponding bias in the induced adaptive T cell responses[211] indicating a strong regulatory role of NKT cells. There are two major subsets of NKT cells – type I and type II NKT cells, which seem to have opposing functions. Type I NKT cells have semi-invariant TCR repertoire (hence they are known also as invariant, or iNKT cells[212]) and these cells are known to contribute to cancer immunosurveillance through the direct lysis of tumour cells expressing defined molecules (such as α−galactosylceramide, αGalCer), the production of IFN-γ that leads to the recruitment of NK and CD8+ T cells to the tumour site, and the stimulation of DCs to secrete IL-12 that enhances protective Th1 responses[211]. On the other hand, type II NKT cells, which have more diverse TCR repertoires, have been implicated in the suppression of CD8+ T cell-mediated cancer immunosurveillance via the secretion of IL-13[213]. With regard to other major myeloid cell subsets of the innate immune system, namely mast cells, basophils and neutrophils, these have been shown to have a lesser impact on cancer immunosurveillance and rather exert tumour promoting effects[169,214].

The humoural arm of the innate immune system encompasses **natural (auto)antibodies** secreted by B-1 B cells, and the plasma protein complex – the **complement system**[168]. The role of natural autoantibodies in cancer will be discussed in detail thereafter. With regard to the complement, it may contribute to tumour cell killing through the antibody-initiated complement-dependent cell lysis (i.e. CDC mechanism)[170]. Nevertheless, compelling evidence of the direct complement system involvement through complement-dependent cytotoxicity (CDC) and complement-dependent cellular cytotoxicity (CDCC) mechanisms in cancer immunosurveillance in its natural course is missing.

Generation of adaptive immune responses

The adaptive arm of the immune system involves different subsets of T and B lymphocytes, each bearing unique antigen-specific cell surface receptors. Mature lymphocytes recirculate continuously from the bloodstream through the secondary lymphoid organs and return to the bloodstream through the lymphatic vessels until they meet the corresponding antigen[168]. As already mentioned, tumour cells may become APCs in the inflammatory environment, however, generally it is believed that the initiation of an adaptive anti-tumour immunity depends on the proper cross-presentation of tumour antigens (detailed overview on tumour antigens see in section 1.2.3.) by mature professional APCs, namely DCs, to the naïve antigen-specific TCR-bearing T cells in the tumour draining lymph node[192], although it may happen also within the tumour tissue[215,216]. In a rather simplified manner, the generation of protective tumour-specific adaptive immune responses are overviewed in Figure 9.

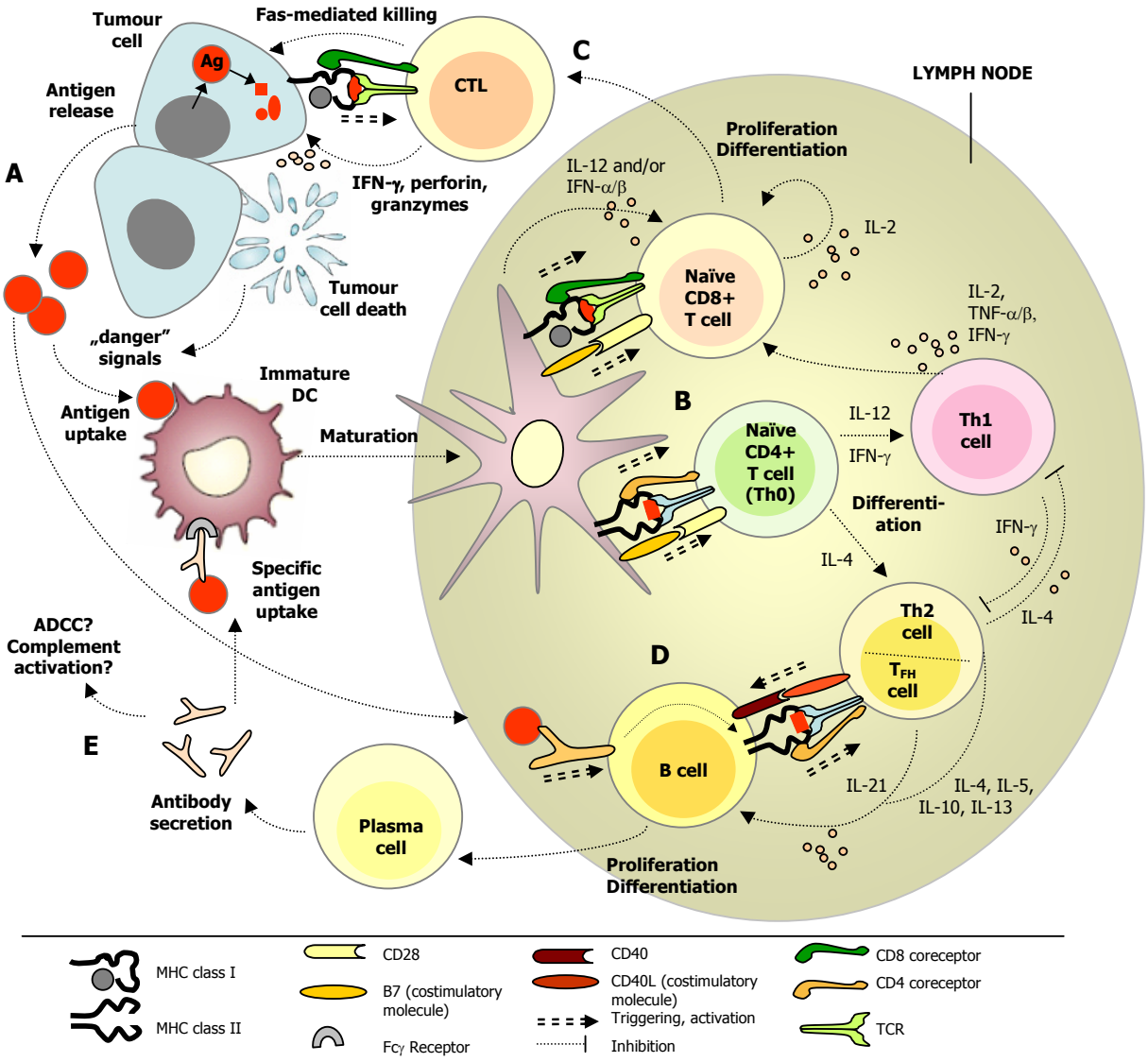


Figure 9. The generation of protective adaptive immune responses to tumours. A | Anti-tumour adaptive immune responses are believed to be initiated through the uptake of antigens from dying or live tumour cells by tissue residing immature DCs, which then migrate to the tumour draining lymph node, which provides the most optimal conditions for naïve T cell priming. DCs undergo maturation characterised by the elevated expression of MHC class I and II molecules and costimulatory molecules, the formation of dendrites and ceased antigen uptake[168]. **B |** The internalised antigen cargo is processed and presented on the DC surface in the context of MHC class I and II molecules that allows the antigen recognition by T cells bearing antigen-specific TCRs coupled to the coreceptors CD8 and CD4, respectively. Apart from the first signal received from triggered TCRs, it is crucial for T cells to receive the second signal from costimulatory molecules, and third one via the cytokines[217]. In a lymph node, naïve CD8+ T cells may be triggered to differentiate into CTLs directly by mature DCs with high intrinsic costimulatory activity[168]. However, help from antigen-specific CD4+ Th1 cells by means of cytokines or cell contact-dependent costimulation is often crucial[217]. **C |** After CD8+ T cell activation, expansion and differentiation, CTLs migrate to the tumour site and exert their cytotoxic activity through the release of IFN-γ, perforin and granzymes, and the expression of Fas ligand, leading to the apoptosis of tumour cells that bear cognate MHC-Ag complexes on their surfaces. **D |** Naïve B cell differentiation generally is dependent upon antigen-specific CD4+ T cell (follicular B helper T cell (T_{FH}), Th2 or Th1 cell (not shown)) help[168]. While T cells are able to recognise the antigen only in its processed form, B cells recognise molecules in their native conformation through antigen-specific B cell receptors (membrane immunoglobulins, namely IgM) (first signal) and this leads to the antigen internalisation, processing and presentation to the antigen-specific CD4+ T cells in the context of MHC class II molecules. The DC-activated CD4+ helper cell responds to such B cells by costimulation thus ensuring the second signal needed for B cell proliferation and differentiation into antibody secreting plasma cells. **E |** Secreted IgG antibodies are thought to contribute to cancer immunosurveillance through augmenting specific antigen uptake by APCs, as well as antibody dependent cell-mediated cytotoxicity (ADCC) and antibody-induced complement-mediated lysis of tumour cells[169].

Many different factors are involved in the choosing the appropriate effector arm of the adaptive immune system as well as tuning of the adaptive immune response, including the nature and amount of antigen or antigenic epitopes, the DC subset that predominates in the tumour-affected tissue[218], the composition of often conflicting signals in the given tumour microenvironment, which are integrated by DCs[193], and others. From the protein antigen stand point, it may encode epitopes suitable for the presentation in the context of MHC class I or II molecules, or both[168]. MHC class I molecules present 8-11 amino acid long peptides derived from cytosolic proteolysis while MHC class II molecules found on the surface of APCs are known to carry longer peptides of 11-17(-24) amino acids, which are generated from engulfed antigens by endosomal and lysosomal proteases[168]. Sometimes integrated T and B cell responses might be elicited to an antigen bearing also B cell epitopes. The excellent example of a tumour-associated antigen able to elicit integrated immune responses *in vivo* after vaccination is so called Cancer-Testis (CT) antigen NY-ESO-1 that encodes epitopes for CD4+, CD8+ T cells and B cells spanning the same immunodominant region[219].

Important role in the generation of adaptive immune responses is addressed to CD4+ helper T cells – cytokine signals integrated from the surrounding milieu guide the differentiation of naïve CD4+ T (Th0) cells into one of at least five CD4+ helper T cell subsets with different phenotypes (i.e. Th1, Th2, Treg, T_{FH} and Th17). This is achieved through the activation of particular transcription factors, often simultaneously inhibiting other differentiation pathways. The effector role of helper T cells is addressed to their secreted cytokines, which serve to skew an immune response in a particular direction[220].

According to the conventional text books, there are two main functionally distinct types of immune responses, which may be elicited through the differentiation of naïve CD4+ T helper cells (Th0) of the same antigenic specificity into either Th1 or Th2 cells after the antigen encounter, proper costimulation and the particular cytokine signals (Figure 9, B). Th1 cells secrete IFN-γ, IL-2, TNF-α/β and largely help to activate cellular effectors (CTLs and macrophages) that mediate acute inflammatory states (type 1 response)[168,187]. However, IFN-γ secreted by Th1 cells may facilitate humoural responses of a particular IgG subtypes (e.g. IgG1 and IgG3) as well – these B cell responses are known as the type 1 antibody responses[219]. In contrary, Th2 cells are known to secrete IL-4, IL-5, IL-13 and IL-10 and predominantly ensure the help for the generation of B cell responses and granulocyte activation that often leads to chronic inflammation (type 2 response)[168,187]. Th1 and Th2 cell-mediated responses are thought to crossregulate each other by the means of the cytokines they secrete (Figure 9) – if one subset becomes dominant, it is often hard to shift the response to the other[168,221]. In general, type 1 responses are recognised as protective against tumours while type 2 responses traditionally have been viewed as favouring tumour growth through various indirect mechanisms[169]. Accordingly, most of the concepts in tumour immunology and immunotherapy concepts have been focused on the type 1 immune responses, since the major effector lymphoid cell subset contributing to the cancer immunosurveillance is activated antigen-specific CD8+ lymphocytes or CTLs. Tumour infiltrating CD8+ T cells have been seen in many types of cancer, including breast, ovarian and colorectal cancers, and this was found to correlate with improved survival[222-224]. A study analysing large cohorts of colorectal cancers (n=490) by gene expression profiling and *in situ* immunohistochemistry revealed an inverse correlation of Th1-mediated immunity and tumour recurrence and indicated that the beneficial effect of the cellular adaptive immunity may persist throughout tumour progression[224].

However, it has been demonstrated lately that Th0 cells, apart from the Th1 and Th2 lineages, may give rise to another CD4+ T cell effector populations. These include (i) different subsets of regulatory T (Treg) cells, which ensure peripheral tolerance against self-antigens (and which have important role in the tumour evasion from protective CTL responses as discussed hereinafter), (ii) follicular B helper T (T_{FH}) cells, which has become acknowledged as the true helpers for B cells in germinal centre reactions, and (iii) the most recently discovered IL-17-secreting CD4+ T cell subset – Th17 cells[220]. In humans, the differentiation of Th17 cells is induced by means of TGF-β and IL-6, and the specific effector functions of Th17 cells are attributed to the in the induction of organ-specific autoimmunity and tissue inflammation responses[225]. Th17 cells have been found in various tumours, including prostate cancer and cutaneous T-cell lymphomas[226,227], as well as tumour-draining lymph nodes[228], however, there is still no clear indication of either a pro-tumoural or anti-tumoural role of this cell subset.

The existence of immune-mediated cancer dormancy

Cancer dormancy may occur through different mechanisms including angiogenic dormancy, cellular dormancy (G0-G1 arrest), and immune-mediated dormancy[229] that is also known as equilibrium (Figure 10)[230]. Cancer dormancy is believed to occur during primary tumour development or during metastasis outgrowth[231]. However, the existence of the equilibrium phase has not been proved, and the involvement of the immune system in this process has been elusive and poorly understood, and it has been suggested that the phase of tumour latency exists due to the long-term and multi-step process of carcinogenesis rather than actively involves immune responses[7].

In humans, some indirect evidence of the existence of this phase has come from clinical observations – cancers of unknown primary origin occur in immunosuppressed recipients following transplantation of organ with undetected cancer[232,233]. For example, two kidney donors developed fatal melanomas that were transferred together with donated organs from a single patient, which had undergone a surgery of primary melanoma 16 years ago[232].

However, the experimental evidence supporting the existence of the equilibrium phase has been gained recently. Robert D. Schreiber with collaborators exploited mouse models of chemically induced primary carcinogenesis to study whether the equilibrium state can occur during primary tumour development, and to try to asses the underlying mechanisms[234]. To make a long story short, the study design involved mice treated with a low dose of carcinogen 3'-methylcholanthrene (MCA) and which had not developed progressively growing sarcomas by over a period of 200 days. These mice carrying small, non-expanding tumours were treated with either control immunoglobulins or a mixture of monoclonal antibodies (mAb) that lead to the depletion of CD8+ and CD4+ T cells, and the neutralisation of IFN-γ. Sixty percent of the mAb-treated mice developed apparent tumours while control mice did not. The experiment was repeated several times and in two different mice strains with similar results. At the same time, depletion of NK cells did not trigger tumour growth indicating the crucial role of the adaptive system-mediated responses for keeping tumours in the equilibrium[234].

The results of these and additional experiments included in the study guided the authors to several important findings. They showed that the equilibrium occurs, and that it is mechanistically distinguishable from the elimination and escape phases since the equilibrium is maintained solely by adaptive immune cells, whereas the elimination is achieved through the action of both innate and adaptive immunity. The tumour cells found in the equilibrium state are transformed, highly immunogenic (i.e. unedited), but proliferate poorly *in vivo*, but those cells that have spontaneously escaped the equilibrium state have attenuated immunogenicity – they become edited and grow into clinically apparent tumours. As well, authors point that the equilibrium state may represent a relevant goal of cancer immunotherapy. However, the molecular mechanisms through which the adaptive immunity holds cancer in equilibrium yet have to be unravelled[234].

The experimental evidence of tumour immunosurveillance is growing and, although many tumour immune rejection models share common effector cell subsets and molecules, there are also often many unique or seemingly contradictious aspects involved and therefore it has been pointed that there is much to be learned about the immune system-mediated eradication of tumours[231]. Taken together, cancer immunosurveillance is recognised to be a multivariable process in which various factors influence the immune response. These factors include the cellular origin of the tumour, anatomic location, mode of transformation, stromal responses, cytokine production profile, and inherent immunogenicity[235].

1.2.2. CANCER DESPITE IMMUNOSURVEILLANCE

Cancer immunosurveillance concept, although supported through different and independent lines of evidence, still does not explain why tumours occur in immunocompetent individuals at such a significant level. Currently there is a strong polarisation in views with regard to how developing tumours evade from immunosurveillance, and each of these opinions is supported by considerable experimental evidence.

How tumours bypass immunosurveillance – proposed scenarios

In 2002, considering the accumulated experimental evidence apparently driven by the availability of different knock-out mice models, the team of Robert D. Schreiber proposed a more general concept termed "cancer immunoediting". This concept gives broader view on the cancer-immune system

interrelation and acknowledges the host-protecting as well as tumour-sculpting roles of the immune system on developing malignancies, and it suggests that a significant proportion of tumours develops through three phases occurring in a sequential manner: elimination (i.e. immunosurveillance), equilibrium or cancer persistence phase, and escape[230] (Figure 10). The cancer immunoediting concept predicts that tumours loose their immunogenicity due to the immunogenic cancer cell variant death through immunosurveillance mechanisms, which is accompanied by non-immunogenic cell variant outgrowth – a process also referred elsewhere as "immunosculpting" or "immunoselection"[170] (Figures 8 and 10). Although the immunoselection of non-immunogenic tumour cell variants has been emphasised in the cancer immunoediting process, it does not exclude the immunosubversion – an active suppression of the immune effector functions by tumour cells, which is thus perceived as integrative to tumour escape phase[203].

Nevertheless, recent studies by Thomas Blankenstein and co-workers have reasonably questioned the role of cancer immunoediting in spontaneous carcinogenesis *in vivo*. These researchers have developed and exploited a sporadic tumour model in mice where the dormant oncogene SV40 T antigen is activated by rare stochastic events in individual cells, and thus it better resembles the physiological tumour development (in comparison to chemical-induced primary carcinogenesis model that has been used as a central model to document the cancer immunoediting process). Collectively, these studies have shown that tumour cells do not loose their immunogenicity (i.e. – they do not become edited), but they instead induce early CTL tolerance that is firstly attributed to the tumour-specific antigen and later becomes generalised. Moreover, it was concluded that the more immunogenic is the target antigen, the higher is the immune suppression. This tolerance occurred almost simultaneously with the IgG antibody responses against SV40 T antigen and it was shown to be an early (i.e. premalignant) event in the tumour development. As well, it was associated with high serum levels of TGF-β1 and large numbers of immature myeloid cells (also referred to as myeloid-derived suppressor cells (MDSC) elsewhere) in the tumour and spleen while no local regulatory T cell involvement was detected. However, the molecular mechanisms through which the general CTL unresponsiveness is mediated still remains an open question[7,236].

Tumour immune escape – Immunoselection and Immunosubversion

The tumour immune escape phenomenon is recognised as the seventh hallmark of cancer[171]. There is a plenitude of scientific papers suggesting many different strategies used by tumour cells to escape their recognition and/or destruction by the immune system. Broadly, these can be divided into two categories – (i) the changes in the tumour cells themselves, which lead to the loss of their immunogenicity and which are driven by the pressure of the immune system (i.e. immunoselection), and (ii) tumour-induced immune tolerance and active suppression of the immune responses (i.e. immunosubversion, see Figures 8 and 11)[170,203,236,237].

The adaptive immune system is blinded to the tumour cells that have lost the corresponding antigen/epitope expression or the ability to properly present antigens to the activated effector cells. The loss or downregulation of the expression of tumour antigens or MHC class I molecules has been widely documented in a large range of different cancers and such changes usually are associated with poorer outcomes[170,175]. However, it has been shown in breast cancer patients that the MHC class I molecule complete loss rather than downregulation is associated with better prognosis[238], which probably could be explained by tumour cell death mediated by NK cells, to which MCH class I molecules are inhibitory. Apart from this, a broad array of data suggests the selection for tumour cell variants that have acquired alterations leading to the downmodulation of molecules important for proper antigen processing and presentation, such as β2-microglobulin and transporter associated with antigen processing 1 (TAP1), and others[239,240].

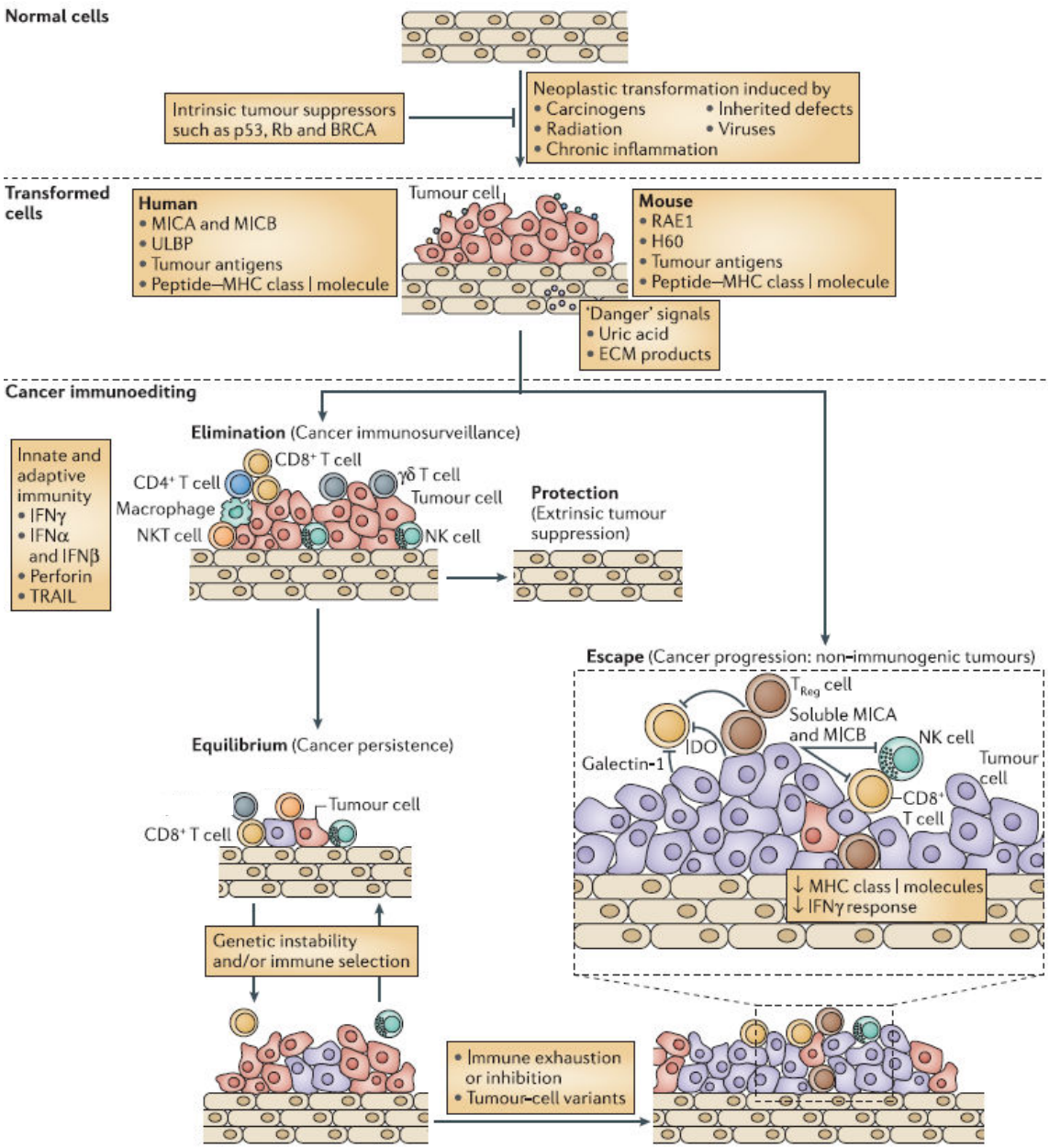


Figure 10. The overview of the cancer immunoediting concept. Three phases are involved in cancer immunoediting – elimination, equilibrium and escape. In the elimination phase, incipient transformed cells are killed my means of multiple innate and adaptive immune system effectors. If the cancer cells stay alive despite immunosurveillance, the process can progress to the equilibrium phase – a subclinical phase in which tumour, although is persistent, is prevented from expanding by the pressure of the adaptive immune system. When tumour cells succeed to overcome the immune pressure, begins the third, escape phase, which results in a clinically apparent tumour bulk. However, non-immunogenic tumour cell variants enter the escape phase directly. The tumour escape phenomenon involves multiple different mechanisms. BRCA – breast cancer, early onset, ECM – extracellular matrix, IDO – indoleamine 2,3-dioxygenase, IFN – interferon, MIC – MHC class I polypeptide-related sequence, NK – natural killer, NKT – natural killer T cell, p53 – tumour-suppressor protein p53, RAE1 – retinoic acid early transcript 1, Rb – retinoblastoma protein, TRAIL – tumour necrosis factor-related apoptosis-inducing ligand, T_{reg}, - CD4+CD25+ regulatory T cell, ULBP – cytomegalovirus UL16-binding protein. From Dunn, Koerbel & Schreiber, 2006[203].

All the mechanisms just described are suggestive to immunoediting; however, it is important to determine whether the immunosurveillance drives the phenotypic changes – one can argue that the antigen loss or the loss of critical components of antigen presentation might be simply due to the increased genetic instability since it has been estimated that, for example, chromosomal instability that is frequently seen in many common cancers significantly contribute to the loss of tumour cell alleles[29,175]. As well, loss of tumour cell differentiation may be accompanied by antigen loss – for example, downregulation of melanin biosynthesis pathway-related protein expression (e.g. MLANA, tyrosinase – both are known melanoma-associated antigens) is observed in aggressive melanomas[241]. Nevertheless, there are experimental proofs that, for example, antigen/MHC class molecule loss variants are not just a favourable side-effect for a tumour cell that accompanies genetic instability, but indicates to the immunoselection of such tumour cell variants. For example, there is evidence that a patient treated with a therapeutic vaccine containing antigenic peptides from melanoma antigens gp100, MART-1 and tyrosinase experienced dramatic initial response and an almost complete tumour regression, which was associated with the presence of antigen-specific CTL clones. Nevertheless, this successful response was followed by several recurrent lesions, and the analyses of the tumours revealed the loss of either the melanoma antigens or MHC molecules strongly indicating the role of immunoselection in the process of repeated tumour outgrowth[242]. Apart from this, mutations in the antigenic epitopes rather than the antigen loss *per se* may be responsible for the tumour cell escape from specific cytotoxic T lymphocyte-mediated killing[243,244]. This phenomenon, also known as the antigenic drift, results in neo-epitopes that severely reduce tumour cell recognition through modulation of MHC-peptide interactions and TCR binding to MHC-peptide complex. *In vivo* such immunoselection has been demonstrated in the P1A antigen-expressing tumour model, in which recurrent tumours have mutated the P1A-specific CTL epitope after passive immunotherapy with high doses of P1A TCR transgenic T cells[244].

With regard to the **immunosubversion** – although the antigen-specific CTL tolerance has been demonstrated to occur in early stages of spontaneous tumour development[7,245], there is also evidence, which indicate that the suppression of anti-tumoural immune responses emerges together with advanced carcinogenesis and is preceded by initial protective immunity[246,247]. The induction of T cell anergy and the active suppression of other immune effector cells has been monitored in cancer patients and murine cancer models – these effects are generated through many different pathways (many of which are depicted in Figure 11) and can be seen at tumour sites, in tumour-draining lymph nodes and in the peripheral circulation and lymphoid organs[248,249].

Tumour cells are known to secrete soluble factors to recruit immunosuppressive cells to their microenvironment (Figure 11). One apparent example is the release of granulocyte-macrophage colony-stimulating factor (GMCSF), granulocyte CSF (GCSF) and IL-3 by tumour cells, which contribute to the recruitment of myeloid-derived suppressor cells (MDSCs) to the tumour site[250]. MDSCs comprise a heterogeneous population of CD11b+GR1+ myeloid cells including monocytes, granulocytes and immature myelomonocytic cells with considerable plasticity to differentiate into several cell types, for example DCs or macrophages, depending on the cytokine signals they receive. These cells induce activated T cell unresponsiveness through the overproduction of nitric oxide and an increased arginase-1 activity. The resulting inhibition of T cell functions is characterised by an impaired TCR signalling or the induction of T cell apoptosis, or the downregulation of IL-2 signalling, all through MHC-independent mechanisms[251-253]. In this context, tumour cells are also known to secrete factors (e.g. VEGF, MCFS and IL-6) that inhibit the immature myeloid-derived stromal cell differentiation into DCs[254]. In support of the above described, a study by Hans Schreiber and co-workers indicated that the growth of solid tumours depends on tumour stroma – depletion of MDSCs cross-presenting tumour antigens resulted in sustained CTL cell activity, prevented neovascularisation and restrained tumour growth. Authors point to the importance of targeting stromal MDSCs by T cells as a new therapeutic approach[255].

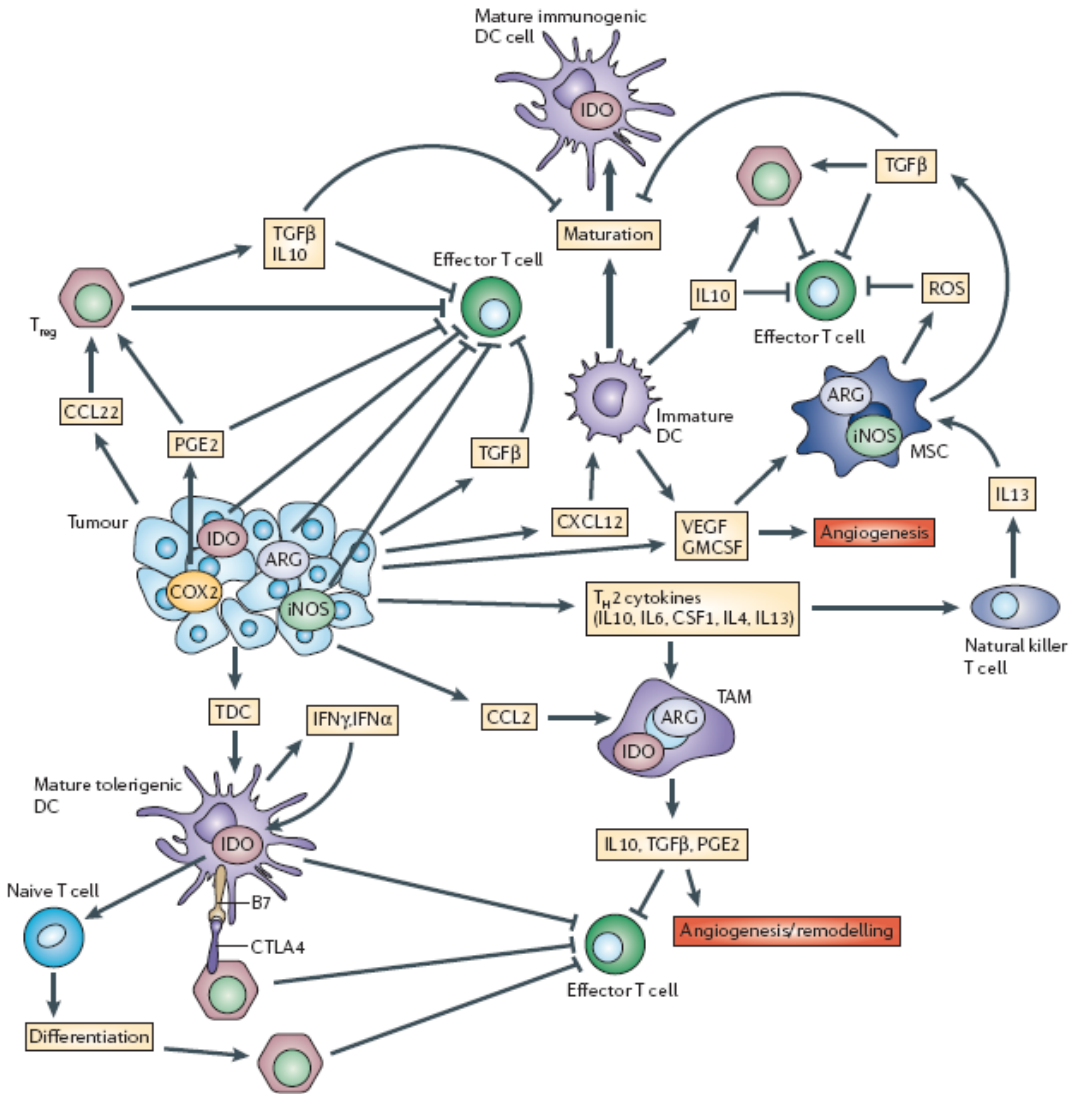


Figure 11. The interactions of tumour and immune system cells contributing to immunosubversion. Tumour cells produce and release cytokines (IL10, transforming growth factor-β (TGF-β), IL13 and vascular endothelial growth factor (VEGF)) and chemokines (such as CCL22, CCL2 and CXCL12), that can promote the migration and expansion of cells that negatively regulate effector cells of the immune system. Among these cells regulatory T cells (Treg), myeloid-derived suppressor cells (MDSCs), immature dendritic cells, natural killer T cells (NKTs), and tumour-associated macrophages (TAMs) can be highlighted. Tregs may block effector T cells directly or through the production of immunosuppressive cytokines such as TGF-β and IL10 while the others suppress effector cell proliferation, the production of IFN-γ and IL2, and the cytotoxic activity. Apart from that, tumour cells as well as host cells can express immunosuppressive enzymes – arginase (ARG), indoleamine 2,3-dioxygenase (IDO), cyclooxygenase 2 (COX2) and inducible nitric oxide synthase (iNOS)). Besides, tumour-derived antigen presentation by tolerogenic DCs in tumour-draining lymph nodes and Treg interaction with them through cytotoxic-T lymphocyte-associated antigen-4 (CTLA4) induces IDO activity in these DCs thus facilitating further Treg expansion and differentiation. From Muller & Scherle, 2006[249].

Other proven examples of immunosuppressive cells that mediate tumoural immune tolerance include tumour-associated macrophages (TAMs, known also as M2 cells), immature DCs, mature toleragenic DCs and regulatory T cells (Tregs)[249,256] (Figure 11). Special emphasis should be laid on Tregs, formerly known as suppressor T cells – the immunosuppressive T cells that have a crucial role in the maintenance of peripheral immune tolerance. Their role in the tumour escape has been increasingly recognised and extensively studied in the last decade. Two major subsets of Treg cells can be named – naturally occurring Tregs and induced or adaptive Tregs; the former represents a discreet lineage of self-reactive thymus-derived CD4+CD25+FoxP3+ T cells while the latter stem from conventional T cells through alternative differentiation during normal immune response and comprise different subsets of CD4+ (e.g. Tr1 and Th3) and CD8+ T cells with regulatory function[249,256,257]. These cells in their natural course are involved in the shutting down of solved immune responses and keeping in check immune responses to self molecules to prevent autoimmunity. In cancer patients, both of these subsets – i.e. natural and adaptive Tregs – are known to be implicated in the suppression of effector immune cells[256]. Moreover, it has been shown that tumours actively induce Treg trafficking, differentiation and expansion thus actively suppressing tumour-associated antigen-specific immunity[257]. The elevated proportion of CD4+CD25+ Treg cells among tumour infiltrating or circulating CD4+ T cells has been associated with tumour aggressiveness and progression[258,259].
Basic strategies used by Tregs include (i) the secretion of cytokines (Figure 11) that inhibit effector T cell function, (ii) the induction of effector T cell death through different cell-cell contact-dependent and independent pathways, and (iii) immunosuppression achieved through inhibition of DC maturation and function[260]. The induction of Treg-mediated tolerance in cancer patients has largely been accounted for the failure of cancer immunotherapy strategies – newest immunotherapy strategies that include the depletion of Treg cells in cancer patients (e.g. anti-CTLA4 antibodies) have lead to the elevated immune-mediated tumour clearance, however, it also brings the awareness of harmful side effects in the form of autoimmunity[257,261,262].
Presumably, the above described mechanisms outline only a part from a broad array of ways tumours dare to bypass immunosurveillance[263], and the prevalence of the individual mechanisms in the *in vivo* carcinogenesis in humans is not known. Nonetheless, it surely gives an explanation why spontaneous rejection of progressively growing tumours is observed only rarely. Thus, the understanding of the mechanisms of tumour immune evasion may lead to more effective therapeutic strategies aimed to prevent and/or reverse these immune alterations and improve the outcomes of immunotherapy.

1.2.3. HUMAN TUMOUR ANTIGENS

The idea that tumours might express their own unique antigens has come from Paul Erlich in the early 20th century [172], but only after ~50 years the existence of specific immune-mediated tumour rejection was shown experimentally in mouse models by Richmond T. Prehn and Joan M. Main and reported in their article "Immunity to methylcholanthrene-induced sarcomas"[264]. Since then, the identification of potential targets for the immune attack on cancer has become increasingly important as it represents an important step towards the generation of cancer vaccines. Nonetheless, the first human tumour antigen MAGE-1 in melanoma was described only in 1991 by Thierry Boon's group[265]. A few years later in 1994 Steven Rosenberg and colleagues succeeded in the identification of another melanoma antigen MART-1[266]. The identification of these first tumour antigens was based on the approach exploiting tumour-infiltrating lymphocytes (TILs) isolated from cancer patients. In parallel, approaches for the interrogation of B cell targets evolved[267]. To date, more than 250 HLA class I and class II-restricted T cell epitopes and more than two thousands of serologically reactive proteins have been identified by interrogating almost every tumour type[13,268-270]. Nevertheless, not all of them have been well characterised.

Approaches for tumour antigen identification

Multiple strategies have been exploited in order to reveal antigens and epitopes to which tumour-bearing hosts have generated adaptive immune responses. First human tumour antigens were identified by a strategy based on the **genetic approach**[265,266]. In order to identify antigens, cDNA expression libraries from tumour cells are constructed and transfected into target cells expressing appropriate HLA molecules. Then, TILs that have been isolated from tumours undergoing regression *in vivo* are tested *in vitro* for their ability to lyse specific target cells from which the antigen encoding gene can be traced down. The identification of the antigen nucleotide sequence enables the search for putative

HLA epitopes, which could be tested for their reactivity with the original tumour-specific CTL clone. The major obstacle of this approach is the need for the establishment and characterisation of tumour and TIL cell lines that is a difficult task with regard to the majority of human tumours. However, the application of this approach has led to the identification of several melanoma antigens including MAGE-1[265], MART-1[266], gp100[271], tyrosinase[272] and others. In order to identify specifically CD4+ T cell targets, this approach was modified through the construction of cDNA libraries where the recombinant protein is expressed as a fusion protein with invariant chain polypeptide directing them to the class II pathway. APCs expressing appropriate HLA class II molecules are used for the transfection and tested for the reactivity with tumour-reactive CD4+ T cells[273].

Another group of methods applied for the tumour antigen identification is based on the **biochemical approach**. Generally these methods involve growing large numbers of tumour cells, cell lysis and purification of HLA class I molecules. Afterwards, HLA-bound peptides are acid-eluted and fractioned by using high-performance liquid chromatography (HPLC), and the fractions are then analysed by loading them onto APCs that are tested with known tumour-reactive CD8+ T cells. Those fractions that are able to elicit T cell responses are further analysed by mass spectrometry (MS) to identify the epitopes[274]. Alternatively, similar approach has been used to identify HLA class II-restricted antigens through the analyses of HLA class II+ tumour cells through the use of autologous CD4+ TIL cell line[275,276]. The application of his approach has contributed to the identification of several novel tumour antigens, including CT (cancer-testis) antigens, e.g. HAGE[277], however, it has met some technical difficulties associated with sequencing of very small amounts of peptide recovered from the tumour cell surface[274].

Reverse immunology is another approach that involves several methods in a consecutive order in which the identification of putative antigens is the initial step. Candidate genes are selected by gene expression analyses (e.g. search for overexpressed antigens), the large array of candidates may further be scaled down by selecting genes with crucial involvement in carcinogenesis or those expressed early in tumour development or metastatic lesions etc. The selected candidate genes then are analysed to deduce their potential immunodominant epitopes and this process involves several steps including (i) prediction of HLA binding peptides, (ii) antigen processing prediction, (iii) *in vitro* testing of the HLA binding and antigen processing and (iv) the analyses of T cell repertoires, which may include CTL activation assay, ELISPOT assay and tetramer analysis[278]. The examples of tumour antigens identified by applying this approach are hTERT, a catalytic subunit of human telomerase reverse transcriptase[279], PRAME[280], tyrosinase-related protein 2 (TRP-2)[281] and others. The potential of this approach has been enormously facilitated through the availability of human genome sequences, improved bioinformatic prediction tools and optimised methods for high-throughput gene expression profiling.

Tumour antigen identification has been done also by performing ***in silico*** **transcriptomics** that is based on screening the available human genome and Expressed Sequence Tag (EST) sequences computationally rather than in laboratory. Candidate genes are selected through comparing arrays of annotated EST sequences originating from hundreds of tissues including tumour tissues from various types and stages in order to reveal candidates that have tumour-associated expression. Yet, putative antigens then have to be tested for specific expression in tumour and normal tissues as well as for their immunological relevance[282,283].

Another branch of tumour antigen discovery stems from the interrogation of humoral responses (i.e. antibodies) to tumour-associated proteins. The development of the approaches for **serological tumour antigen identification** was fostered by the technical difficulties faced in defining antigens by T cell responses. The major advancement in the field of serological tumour antigen identification was the development of the SEREX approach by Ugur Sahin and Ozlem Tureci in 1995 that stands for serological identification of antigens by recombinant expression cloning[11,12]. This approach is based on the construction of phage cDNA expression libraries from tumour tissues, the transfection of the libraries in *E. coli* and immunoscreening of the recombinantly expressed proteins with patient's serum (Figure 14). Serum-reactive clones then are sequenced and encoded antigens identified[11]. Many hundreds of different tumour-associated antigens have been identified in nearly all tumour types by applying this approach – the antigen sequences and information are available on the public Cancer Immunome Database[13]. Among the SEREX-identified antigens there is NY-ESO-1, a cancer-testis antigen, which is one of the most immunogenic tumour antigens identified so far pointing to the potential of this approach to identify relevant immunotherapy targets[284,285]. Apart from the conventional SEREX, more recently several other approaches making use of tumour-induced

humoural responses have emerged, overview of which will be given in the next section (1.3. Autoantibodies in Cancer Patients).

Categories of human tumour antigens

Many classifications of human tumour antigens exist. However, they roughly can be classified into antigens of protein and non-protein nature. The latter include lipids, glycolipids and glycoproteins that are expressed predominantly on the surfaces of tumour cells[286] and most often the carbohydrate structures are the ones of aberrant nature. Among them there are O-glycans[287], gangliosides (i.e. GM2, GD2 and GD3), mucins (MUC1 and MUC4), heat shock proteins (HSPs)[286] to name but a few. Although such antigens represent considerable proportion of human tumour antigens and may serve as targets for innate immune system, they most likely are not presented to the adaptive immune system in contrast to protein antigens, which thus may be recognised by T cells and/or follicular B cells. Human tumour antigens eliciting adaptive immune responses may be classified into three major categories – foreign, mutated self and non-mutated self (Table 2)[192].

Foreign antigens mainly represent proteins encoded by viruses that establish persistent infection and induce transformation of the host cells, such as herpes, hepatitis B, papilloma viruses and others. Although the immune response is triggered against foreign antigen, the transformed cells are the target cells for the immune system. To date, the only available prophylactic cancer vaccines are generated to mount an immune response to viral (human papilloma virus and hepatitis B virus) antigens[288].

The second category encompasses neo-antigens encoded by genetic information, which has undergone any type of mutation (e.g. point mutations, translocations, inversions, deletions, insertions) including the expression of proteins translated in non-natural reading frames (Table 2) leading to the changes in the protein structure. These structurally altered proteins bear potential neo-epitopes and might trigger an immune response – the "potential" and "might" is because not all mutations guarantee the generation of neo-epitope that can bind MHC molecules, and binding does not necessarily guarantee T cell stimulation[192]. However, such neo-antigens are recognised as altered-self by the immune system and are called **tumour-specific antigens**. From the vaccination stand point these antigens represent ideal targets because the CTL responses mounted to these antigens would be of high specificity against tumours and with function often critical for tumourigenicity. In addition, T cell responses generated against these antigens would not be dampened down through tolerance mechanisms since the immune system has not been previously exposed to these antigens[168,287]. On the other hand, the use of such antigens would be highly limited since usually they are patient-specific.

The antigens included into the third category are unaltered self proteins, which are rendered immunogenic due to different reasons (Table 2). These antigens are called **tumour-associated antigens** (TAAs) since their expression in not restricted solely to the tumour cells but is found also in normal tissues either in ubiquitous levels or in the specific compartments or specialised cell types in the body.

A substantial group of TAAs are the so called **overexpressed antigens** – proteins expressed in tumour cells in much higher levels (up to 100 times) in comparison to the normal tissues due to the gene amplification, elevated transcription, or increased stability of corresponding mRNAs or proteins. It is known that low avidity self-reactive T cells escape the negative selection in thymus while T cells bearing high-avidity TCRs against self proteins are deleted[289]. There exists a connection – the less the functional avidity (e.g. combined binding strength of TCR-peptide-MHC complex) of TCR, the more the TCRs are needed to be triggered to mount further T cell proliferation and differentiation. Thus, the underlying reason for the immunogenicity of such proteins is thought to be the overrepresentation of these proteins and their derived MHC class I and class II peptides at sufficiently high levels to reach the threshold of activation of these low-affinity TCR bearing CD8+ T cell and CD4+ T cell, respectively[290-292].

There are antigens known to be expressed in particular sites in human body known as the sites of immune privilege (testes, foetal ovary, retina, brain and pregnant uterus) and which are aberrantly activated in cancer. A substantial group of these antigens that are **shared between immunoprivileged tissues and cancer** is represented by the so called cancer-testis (CT) antigens. More than 150 CT genes are known to date, many of which elicit cellular and humoural immune responses in cancer patients[293,294]. Although traditionally CT antigen expression has been attributed to germline and cancer[285,295], results of a recent study have revealed that the expression of CT genes exhibit heterogeneous profiles allowing to classify them into (i) testis-restricted, (ii) testis/brain-restricted and

(iii) testis-selective group that encompasses genes, which expression is highly biased to germ cells, however, is detectable also at variable levels in other adult somatic tissues[293]. Proteins expressed in testis were thought to be hidden from the immune system[296]; however, the expression of some CT antigens has been demonstrated in medullary thymic epithelial cells thus indicating that they have been displayed to developing T cells[297], and thus some level of tolerance might be expected against these antigens. The immunogenicity for such antigens thus may be complementary attributed to their overexpression commonly seen in particular types of cancer[298]. More than a hundred CT antigens/antigen families are known to date[294] and their (co)expression at variable levels is evidenced in many tumour types and has been linked to the cancer-related epigenetic deregulation[285,299]. Noteworthy, recently the expression of several CT antigens including SSX, NY-ESO1 and N-RAGE was reported in undifferentiated mesenchymal stem cells adding a strong support to the concept of the stem cell origin of cancer[300]. Accordingly, the expression of CT antigens thus would not be aberrantly activated in cancer cells but rather inherited from cancer stem cells.

Table 2. The categories of human tumour antigens.

Antigen category	Examples*	Immune recognition#	Refs
Foreign			
Viral	HPV-encoded antigen E7	CD8+ and CD4+ T cell, and antibodies	301-303
	EBV-encoded antigen LMP2	CD8+ T cell	304
Mutated self			
Point mutation	CDC27	CD4+ T cell	273,305-307
	p53	CD8+ and CD4+ T cell, antibodies	308,309
Alternative reading frame	CAMEL (CTAG2)	CD8+ and CD4+ T cell	310
Splicing defects	GnT-V (MGAT5)	CD8+ T cell	
Non-mutated self			
Overexpressed	TERT	CD8+ T cell	279
	HER-2/neu (ERBB2)	CD8+ T cell and CD4+ T cell, antibodies	311-313
Shared between cancer and immunoprivileged tissues			
Cancer-Testis	NY-ESO-1 (CTAG1B)	CD8+ and CD4+ T cell, and antibodies	284,314
	MAGEA-1		11,265,315
Cancer-Retina	Recoverin (RCVRN)	Antibodies	316
Onconeural	NOVA-1	Antibodies	317
Oncofoetal	CEA‡ (PSG2)	CD8+ and CD4+ T cell, and antibodies	318,319
	AFP	CD8+ T cell	320,321
Differentiation			
Melanocyte	Tyrosinase (TYR)	CD8+ and CD4+ T cell, and antibodies	322-324
	gp100 (SILV)		325-327
Mammary gland	NY-BR-1 (ANKRD30A)	CD8+ T cell, antibodies	328,329

* The official gene symbols are given in brackets (the abbreviations are not disclosed herein).
# The immune recognition means the identified CD4+ or CD8+ epitopes in the protein sequence of the antigen and/or the serological response (either spontaneous or vaccine-induced).
‡ CEA is oncofoetal antigen referred elsewhere also as differentiation antigen since its expression is activated in colon cancer and very low expression levels are also detectable in normal colon epithelium.
Abbreviations: HPV – human papilloma virus, EBV – Epstein-Barr virus, LMP2 – latent membrane protein 2, CAMEL – CTL-recognised antigen on melanoma, CDC27 – cell division cycle 27 homolog (*S. cerevisiae*), GnT-V – N-acetylglucosaminyltransferase V, TERT – telomerase reverse transcriptase, MAGEA-1 – melanoma antigen family A-1, NOVA-1 – Neuro-oncological ventral antigen 1, CEA – carcinoembryonic antigen, AFP – alpha-fetoprotein.

Differentiation antigens represent another group of non-mutated self antigens that are known to elicit immune responses in cancer patients. These antigens are molecules expressed on the same cell lineage as the tumour. The vast majority of the identified antigens belonging to this group are melanocyte differentiation antigens that are expressed during melanocyte differentiation, in normal melanocytes and in melanomas[278]. However, the expression of such antigens can also be found in prostate carcinoma, breast cancer and gut carcinomas[330]. The reason why these antigens become immunogenic is not clear, but it is supposed that distinct lineages of specialised cells bear unique antigens, which have not been expressed in thymus during T cell selection and thus the corresponding T cell clones can be met in the peripheral T cell repertoire.
Apart from the above described, there are other mechanisms through which tumour-associated proteins might become immunogenic. For example, in a study aimed to identify the CTL epitope for a tumour antigen fibroblast growth factor-5 (FGF-5) in renal cancer it was shown that the antigenic peptide was generated by the posttranslational protein splicing mechanism[331]. In the case of B cell responses, also altered posttranslational modifications[332-334], aberrant subcellular localisation resulting for instance from virus infection[335] or altered binding partners of normal self-proteins, their cleavage during apoptosis[336] or their localisation on the surface of apoptotic tumour cells as shown in the case of β-actin in neoplastic cells from medullary carcinoma of the breast[181] may render cellular proteins immunogenic.

Tumour antigens and the immunotherapy of cancer

From the immunotherapy point of view, ideal tumour antigen (i) should elicit an immune response that is clinically beneficial (here the usage of the term "tumour rejection antigen" fits in) and do not cause serious side effects in the patient[337], (ii) should be shared between tumour types and patients[278], (iii) would be induced early in tumour progression or even in pre-malignant lesions when tumour-induced immune suppression is thought to be absent, however, good tumour antigen would be expressed also in metastatic lesions to treat secondary tumours after surgical removal of primary lesions, should (iv) have a crucial role in carcinogenesis thus avoiding possible antigen escape variants[278] and (v) encode both MHC class I and II epitopes since it has been shown that CD4+ T cells are crucial for the maintenance of CD8+ T cell survival and long term memory[338]; moreover, (vi) more than one high-affinity epitope would be of great advantage in order to induce responses from multiple T cell clones[278].
The identification of novel tumour antigens has contributed to cancer vaccination enormously. The recent progress in the immunotherapy of cancer has largely been driven by a greater understanding of immune system-tumour interactions and the immune system itself. While prophylactic vaccination against cancer is aimed to generate an immune response against an antigen(s) prior to any appearance of malignancy in order to protect the host from the onset of cancer, therapeutic vaccination or immunotherapy focuses on boosting pre-existing anti-tumour responses as well as sustaining large numbers of effector cells at the tumour site and, more recently, on the depletion of immunosuppressive cells. Various approaches of cancer immunotherapy have been established and currently are undergoing from early to late phase clinical trials. Broadly, these approaches can be categorised in the following groups:
(i) **Peptide vaccines.** These vaccines make use of novel immunogenic tumour antigen identification and characterisation since they focus on the immune responses to specific epitopes. Synthetic peptides corresponding to the epitopes presented on the common human HLA molecules (e.g. HLA-A2) are generated, and they can easily be modified to enhance their binding to the HLA molecule or the TCR – a process known as "epitope enhancement"[339]. They are used in different vaccination modalities – pulsed within autologous DCs or combined with cytokines, chemokines, and costimulatory molecules to increase the desired immune response. As well, there is a need to include a T helper epitope of the antigen to obtain an optimal CTL response[340].
(ii) **Adoptive T cell transfer** is defined as the transfer of T cells (autologous or allogeneic) with anti-tumour properties that can mediate therapeutic effects through direct and/or indirect modes of action[341]. Briefly, T cells (most frequently TILs) are isolated from tumour-bearing host and individual T cell clones with a high avidity for a tumour antigen are then selected and grown to large numbers, and afterwards administered back to the tumour patient. The efficacy of this type of therapy has been improved significantly by the introduction of non-myeloablative, lymphodepleting preconditioning of a patient prior to infusion of autologous TILs, which was shown to involve, apart from other factors,

elimination of Tregs and repopulation of the patient with anti-tumour T cells[342,343]. Moreover, the ability to enhance tumouricidal properties through genetic modifications of adoptively transferred T cells has opened new perspectives for cancer treatment[344].

(iii) **DC-based vaccines** – these vaccines aim to immunise cancer patients with their own DCs loaded *ex vivo* with tumour antigens. Immature DCs are isolated, cultured *in vitro* to increase their numbers and to induce their maturation, loaded with an antigen and then are administered back to the cancer patient in order to increase the efficacy of antigen-specific T cell priming. The DC antigen loading may be done in different fashions – exogenously as peptides or the whole protein, tumour lysates or apoptotic debris, or in complexes with antibodies. Alternatively, antigen loading can be endogenous through transfecting DCs with antigen-encoding cDNA or mRNA[345]. Importantly, there have not been reports about serious adverse effects or autoimmune reactions induced by this type of vaccination[346].

(iv) **Undefined antigen vaccines** initially were applied in the form of irradiated autologous or allogeneic whole tumour cell or cell extract vaccines, however, many other undefined antigen preparations in combination with different vaccination modalities are used today to boost the anti-tumour immune responses (reviewed in [347]). The rationale for the usage of undefined antigens is that tumour cells express many TAAs as well as tumour-specific antigens, which are not yet fully identified or well characterised in the majority of common malignancies. In general, these vaccines are well-tolerated and the exists an evidence of immunologic activity and examples of impressive clinical responses[347].

Despite the advances in the field, the clinical realisation of effective therapeutic vaccines for solid tumours has not yet been convincingly achieved. Between February 1995 and April 2004, 541 different cancer vaccines were used for the treatment of 440 metastatic cancer patients (from which 422 had metastatic melanoma) in the Surgery Branch of National Cancer Institute (USA), and the overall objective response rates (using conventional oncologic criteria for clinical tumour response) were low reaching only 2.6%, and this was comparable to the results obtained by other studies[348]. Nonetheless, in the last years more encouraging results have been gained from several II/III phase trials. Immunosubversion and immunoselection are recognised as the major obstacle to cancer immunotherapy and new generation therapy approaches are conducted to break host immune tolerance. For example, administration of anti-CTLA4 monoclonal antibodies and TLR9 agonists, which prevent inhibitory signals that downregulate T cell activation and stimulate DC activation, respectively, have been shown to activate acute inflammatory responses in patients with advanced inoperable melanomas, reaching more promising, although not complete, objective response rates (reviewed in [349]).

1.3. ANTIBODIES IN CANCER PATIENTS

1.3.1. B CELL RESPONSES AGAINST TUMOUR-ASSOCIATED ANTIGENS

There are various distinct subsets of B cells in human. However, two general classes have to be mentioned. **B-1 B cells** are the predominant lymphocytes in the peritoneal and pleural cavities and they have been ascribed to innate immunity as they carry BCRs of limited diversity and produce natural antibodies and autoantibodies without cognate T cell help. Antigens eliciting such antibody responses are known as the thymus-independent (TI) antigens. B-1 B cells can be distinguished from other B cells by the CD5+/CD11b+ or CD5-/CD11b+ phenotype depending on the subset (i.e. B-1a and B-1b B cells, respectively), and by the ability of self-renewal[350,351]. **B-2 B cells** represent conventional follicular B cells that are involved in the generation of adaptive humoural immune responses and require CD4+ T cell help – hence the antigens eliciting such responses are known as thymus-dependent (TD)[168,350].

Natural autoantibodies and cancer

Spontaneous or natural antibodies (NAs) are found in the serum of almost all jawed vertebrates and are addressed to the innate immunity. The major part of NAs is comprised of low-affinity polyreactive antibodies[352] and belong mainly to IgM, but may also include IgA and IgG isotypes encoded by germline genes with no or few mutations. These antibodies are reactive to a range of different and structurally unrelated foreign as well as self antigens (hence the designation natural autoantibodies – NAAs), usually short-living, and found in healthy non-immunised individuals and newborns[353]. The

physiological role of NAs has been attributed to early protection against bacterial infection[354]. On the contrary, NAAs recognise a limited set of evolutionary conservative circulating, intracellular and cell surface self proteins, carbohydrates, nucleic acids and lipids[355].
While NAs seem to function in the early stages of bacterial infection, the function of NAAs remains elusive. Various physiological roles, including the clearance of dying cells, senescent erythrocytes and platelets have been ascribed to NAAs. Apart from that, NAAs also have been suggested to be implicated in first line of defence against cancer[356] – a significant subset of tumour-specific antigens are carbohydrate antigens (e.g. glycolipids and glycoproteins) found on the surface of tumour cells, with carbohydrate structures being the ones of aberrant nature[286]. Among them there are O-glycans referred elsewhere to also as tumour-associated carbohydrate antigens (TACA)[287], gangliosides (i.e. GM2, GD2 and GD3), mucins (MUC1 and MUC4), heat shock proteins (HSPs) and others, and to some antigens the IgM subclass NAA responses are shown to correlate with improved survival[357]. However, the prevalence of protective NAA-mediated responses in cancer immunosurveillance in oncology patients remains to be determined.

Generation of adaptive humoural responses

Follicular B cells are the central components of adaptive humoural responses and their effector functions are attributed to antibody production, antigen presentation and secretion of pro-inflammatory cytokines[168]. The generally accepted model of the generation of antibody responses to antigens has been introduced in Figure 9, yet, in an incomplete and oversimplified way. The generation of adaptive humoural responses involves several multistep phases. As discussed above, the specific T cell response is initiated when naïve circulating T cells in draining lymphoid tissues meet their corresponding antigens brought and presented there by immunogenic DCs, and become activated. Recirculating naïve follicular B cells, in their turn, engage their specific antigens in their native conformation and the meeting place is thought to be either in the liquid phase of the circulation (in optimal conditions) or in the secondary lymphoid tissues[358]. It has been shown that simple diffusion from afferent lymph may operate as a mechanism of antigen transport to lymph nodes[359]; however, it might work only for soluble antigens present in high levels. Nevertheless, it is known that antibody responses can be induced also by minute quantities of antigen[358]. In this regard, a recently identified mechanism reveals that an antigen in the form of immune complexes is trapped in lymph node by specialised macrophage-like cells (i.e. subcapsular sinus macrophages) that are located in the migration pathway of recirculating B cells, which then can engage the trapped antigen[360,361].
In more detail, the processes in lymph node after B cell has encountered an antigen, internalised it, processed and presented on its surface in the context of MHC class II molecules (Figure 9) are overviewed in Figure 12, B. While lymph nodes obtain antigens from lymphatic-drained tissues, B cells can be activated in other secondary lymphoid tissues as well – in the white pulp of spleen where an antigen is accessed from blood, and in Peyer's patches to respond to antigens in the gut[168].
There are two possibilities for follicular B cells after they encounter the TD antigen and receive T cell help – some of them are induced to develop through extrafollicular pathway (Figure 12, c) while some follow follicular pathway (Figure 12, d, e) of differentiation. The **extrafollicular pathway** typically occurs in medullary cords of lymph nodes and in foci in the red pulp of spleen, while it is not common in the secondary lymphoid tissue of the mucosal areas. This pathway is associated with immunoglobulin class switching but, most frequently, only limited hypermutation and gives rise to short-lived plasma cells[362]. In parallel, a few B cells from the primary focus migrate to the primary follicle and follow **follicular pathway** of differentiation that is accompanied by germinal centre (GC) formation and include clonal expansion, and somatic hypermutation that enable the affinity maturation of the BCRs, immunoglobulin class switching from IgM to other classes, and the selection of most appropriate B cell clones. GC response reaches maximum in day 10-14 after immunisation and then diminishes. GC B cells become either circulating memory B cells or antibody-producing cells that leave lymphatic tissue as pre-plasma cells and home in the sites of infection, epithelial surfaces or bone marrow where they differentiate into plasma cells that have a life span of months to years. Thus, antibodies produced by these cells can be found in circulation for years after the initial immune response[168].

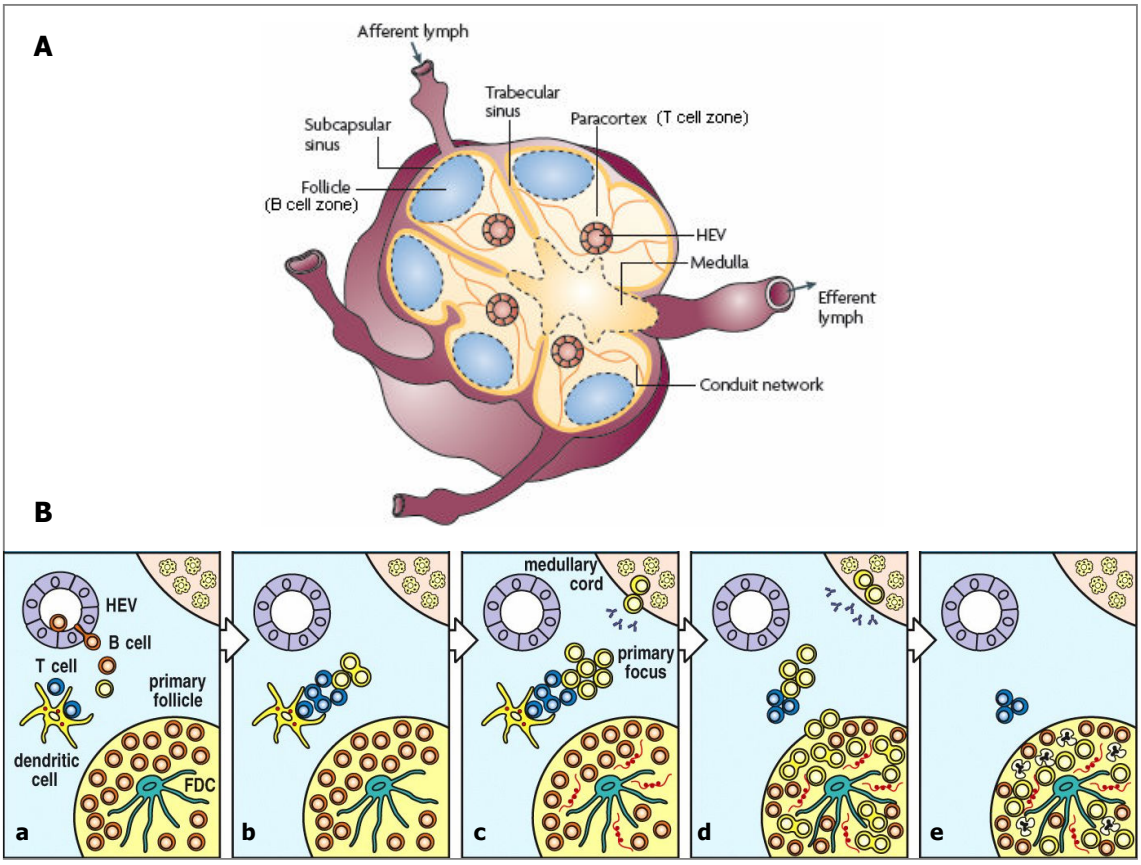


Figure 12. A | The structural organisation of a lymph node. B | The generation of adaptive antibody responses in lymph node. a |Recirculating B cells enter lymphoid organs through high endothelial venules (HEV) and migrate to the primary follicle. **b |** If a B cell meets a DC-primed CD4+ T cell, which is specific for one of the epitopes derived from the antigen, this B cell is trapped at the border between the T cell zone and the follicle and is stimulated to proliferate. **c** | Proliferating B cells (i.e. plasmablasts) form a structure called primary focus; in the mean time, some activated B cells migrate to medullary cords where they divide, differentiate into short-living plasma cells and secrete low-affinity antibodies, which provide early protection while high-affinity antibodies are generated. As well, these early antibodies engage with the antigen enabling increased antigen trapping on the follicular DCs (FDCs) surface in the form of immune complexes. **d** | Few B cells migrate into a nearby follicle where they undergo clonal expansion and form the germinal centre (GC) – this is the structure where the B cells undergo somatic hypermutation and immunoglobulin class switching. **e** | High-affinity B cells are selected to differentiate further into plasma cell precursors and memory cells while those with low-affinity BCRs dye by T cell-induced apoptosis. These processes require contact with the intact antigen stored on the surface of FDCs and local helper T cells (i.e. T_{FH})[168,351,363]. From Batista, 2009[361] (panel A) and Janeway *et al.*, 2005[168] (panel B).

With regard to the **T cell help**, Th2 cells have long been stereotypically cited as the key helpers of B cell responses. However, it has become increasingly evident that different helper T cell subsets including Th1, Th2 and follicular B helper T cells (i.e. T_{FH} cells) may participate in different stages of antibody responses. Fore example, both Th1 and Th2 cells may be equally crucial to provide help for B cells, inducing the immunoglobulin class switching and sustaining their differentiation in the extrafollicular pathway[363]. In GCs there are several functionally different T cell subsets identified and characterised, which are able to either sustain or suppress B cell differentiation. Although both Th2 and more rarely Th1 cells are found among GC helper T cells, the predominant helper T cell subset here is T_{FH} cells, which represent a recently identified effector T cell subset and have been shown to provide the most efficient help for immigrating B cells. The differentiation of T_{FH} cells is induced by IL-21 and IL-6 and they are characterised by a cell surface phenotype and gene expression/cytokine profile that differs from those seen in Th1 or Th2 cell subsets and thus represent a distinct (although it is not entirely clear whether independent) lineage of helper T cells[220,363]. These cells are known to

express a CD28-like costimulatory molecule ICOS, CD40L and IL-21 – crucial molecules for B cell help[220], and moderate levels of IL-10, but they are poor producers of IL-4 that is consistent with their ability to induce class switching to IgG1-3 and IgGA1, but not IgE[364,365]. The effector function of T_{FH} cells is suppressed by IFN-γ and TGF-β indicating that Th1, Th2 and Treg cells present in the GC may control the function of T_{FH} cells.

The central **tolerance** is less strict for B cells as for T cells. During different phases of development in the bone marrow and later in the spleen, immature B cells undergo several rounds of negative selection to avoid self-reactivity[351]. However, mature B cells circulating in healthy individuals still include 5-20% of autoreactive B cells[363]. The expansion and differentiation of self-reactive follicular B cells in periphery is precluded by T cells, which generally follow stricter rules of central tolerance – the B cells do not receive adequate help and undergo arrest in the T cell zones of secondary lymphoid organs. Yet, there is another source of self-reactive B cells – in GCs, the somatic hypermutation of immunoglobulin variable region gene segments is accompanied by changes in the BCR affinity against the specific antigen and this may potentially result in *de novo* development of self-reactive B cells[366]. These B cells are kept in check by censored T_{FH} cells, and those rare T_{FH} cells that exhibit self-reactivity are supposed to be restricted by local regulatory T cells[367]. Apart from such a mechanism, it has been shown that CD4+CD25+ Tregs may suppress B cell activity in GCs directly without the need to suppress T cell help, and that the suppression is dependent upon cell-cell contacts and is accompanied by the inhibition of immunoglobulin class switch recombination[368].

Antibody-mediated effector mechanisms

While CTLs are the most effective in the elimination of localised tumour masses, antibodies are ideally suited for the eradication of free circulating tumour cells and distant micrometastasis. It has been shown that ongoing genetic changes in established tumours, which result in the generation of novel tumour antigens, may rapidly trigger IgG responses despite the downregulation of T cell responses, which often accompany advanced cancers. This phenomenon is thought to occur via the help of pre-existing CD4+ T cell repertoires activated by other, possibly cross-reactive antigen epitopes during early tumour develpment[369].

Antibodies secreted by plasma cells and B-1 B cells may participate in immune responses through different mechanisms. A simple binding of an antibody to the specific antigen may contribute to the protection. For example, circulating antibodies against a tumour cell surface antigen encoded by an oncogene may have suppressive impact on tumour cell growth through the impairment of the biological function exerted by the oncoprotein (i.e. preventing receptor dimerisation or ligand binding)[370,371]. However, the most crucial mechanisms are only mediated by antibodies, which ensure the specificity while the effector function is attributed to the secondary "players" – the complement system and various cellular effectors of the immune system that eventually may lead to the tumour cell death (Figure 13).

Naïve B cells can alter their effector function through switching the isotypes of their immunoglobulin molecules. For example, IgM can participate in complement-mediated reactions, but not in ADCC while IgG1 has the most favourable properties – it binds complement, many classes of cellular FcγRs and has a long half-life (Figure 13). Among the IgG antibody subclasses, the effector capacity may be ranked as IgG1~IgG3>>IgG2~IgG4[221]. The antibody isotype switching is regulated by the prevailing cytokine environment that depends on the nature and amount of the antigenic stimulus as well as the route of antigen entry and host genotype[168,221]. However, there is a significant degree of discrepancy with regard to the switch factors and their cellular origin for the four IgG subclasses (i.e. IgG1-4). Although Th1 cell-secreted IFN-γ is known to promote switching to IgG1 and IgG3 subclasses and Th2 cell-secreted cytokine IL-4 promotes the production of IgG4 and such responses are referred elsewhere as the type 1 and type 2 antibody responses, respectively[363,372], it seems to be an oversimplification. All helper T cells can induce the IgG class switching in B cells, especially those found in GCs, and among known switch factors there are IL-21, IFN-γ and IL-4 as well as CD40L[364].

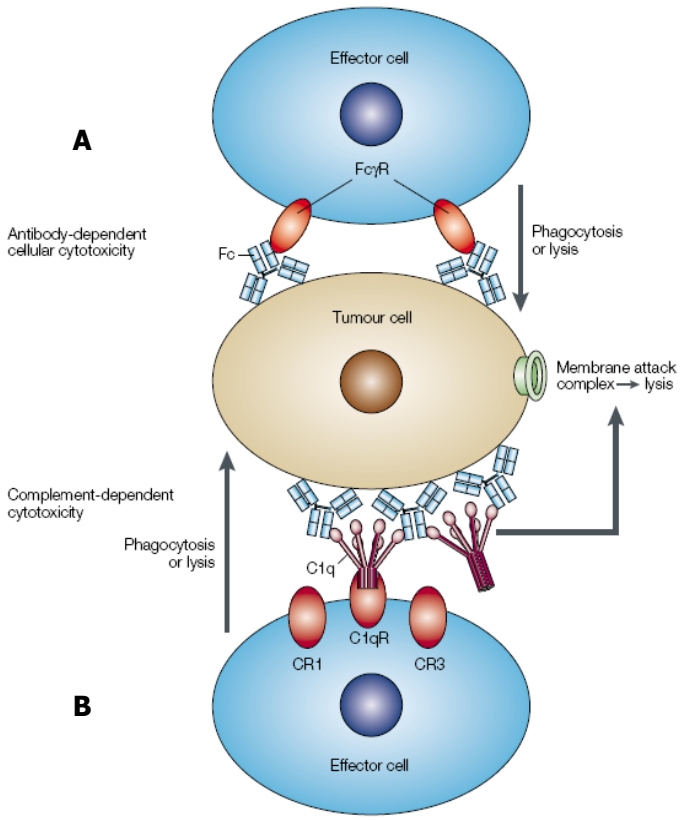


Figure 13. Antibody-mediated effector mechanisms. A | Antibody-dependent cellular cytotoxicity (ADCC) mechanism enables killing of antibody-coated tumour cells by cytotoxic cells carrying activating Fcγ receptors (FcγRs, e.g. receptors recognising IgG class heavy chain constant region). This mechanism is accomplished by the means of various immune effector cells including NK cells and macrophages and it results in tumour cell lysis or phagocytosis depending on the type of the engaged effector cell (the term "opsonisation" is used in the case of phagocytic destruction) **B** | Complement-dependent cytotoxicity (CDC) mechanism depends upon target cell coating with IgG (especially IgG1 and IgG3 subclasses) or IgM antibodies, which are known to bind complement component C1q via their heavy chain constant regions. This triggers the proteolytic cascade that activates complement that may lead to the formation of membrane attack complex resulting in tumour cell lysis. Apart from that, C1q component may attract the effector cells which express complement receptors (CR1, C1qR, CR3) and this can result in tumour cell lysis or phagocytosis[168]. From Carter, 2001[373].

With regard to the IgG subclasses and their FcγR-mediated effector functions, studies by Nimmerjahn and Ravetch have investigated how the affinity of different IgG isotypes to different FcγRs affects the outcome of cellular responses[374,375]. Briefly, in humans there are three types of FcγRs – FcγRI, FcγRII (and its subtypes A, B and C) and FcγRIII, predominantly expressed on the surfaces of cells of innate immune system. From these only one, FcγRIIB, is inhibitory while others are activating receptors and upon IgG-antigen binding they trigger specific effector functions – e.g., oxidative bursts, cytokine release, phagocytosis by macrophages, ADCC by NK cells and mast cell degranulation[376], while FcγRIIB triggering inhibits these reactions. It is known that immune cells can co-express both activating and inhibitory receptors and thus the ultimate response is determined by the balance of these conflicting signals, also known as the activation/inhibition (A/I) ratio. The authors showed that different IgG isotypes exert different A/I ratios in mice through their preferential binding to one or another FcγR with IgG2a (which is functional analogue to human IgG1) having the highest A/I ratio that is followed by IgG2b, IgG1 having lowest ratio, and IgG3 demonstrating no activating FcγR binding. The functional significance of such A/I ratios was demonstrated *in vivo* – tumour antigen-specific IgG2a antibodies (and not other isotypes) were able to efficiently clear lung metastasis of melanoma in mice through ADCC mechanism[374] suggesting that the induction of particular IgG subclass switching may significantly influence the outcome of the immune response (here, the switching of IgG2a isotype in mice is induced by IFN-γ, a Th1 cytokine).

Conflicting roles of antibody-mediated responses in tumour development

The generation of antibody responses against tumour antigens has nearly ubiquitous occurrence in cancer patients (see also Table 2)[6]. As well, cancer patients have elevated levels of circulating immune complexes[377-379]. There is controversial experimental evidence regarding the involvement of antibody responses in tumour development – both, host-protective as well as tumour-promoting roles have been addressed to the antibodies recognising tumour antigens. In this vein, the presence of specific IgG antibody responses against particular tumour antigens has been correlated with both, a better and poorer prognosis. For example, patients suffering from small cell lung carcinoma and having IgG1

antibodies (Th1 mediated) against SOX2 have a better prognosis[380] while colorectal cancer patients with p53 antibodies have poorer prognosis in comparison to those without the humoural responses[381].

A host-protective effect of HER-2/neu-specific antibodies has been demonstrated in spontaneous autochthonous breast cancer model in HER-2 transgenic mice. Here, the antibody responses of IgG2a subclass were elicited by adenoviral HER-2 vaccination, and they were shown to protect mice from tumour growth as well as to inhibit tumour cell growth in HER-2-expressing mammary tumour cell lines. Concordantly, tumour development was not inhibited in B cell deficient immunised mice. Neither complement-mediated nor FcγR-mediated mechanisms were involved indicating that the inhibitory effect was achieved through deprivation of growth signals mediated through direct antibody binding to HER-2. Importantly, protective responses were mediated solely by antibody responses, which required IFN-γ and Th1 cells in the immediate post-vaccination period while CD8+ T cell responses were not required at all[370]. Nevertheless, there is no such protective antibody response observed in breast cancer patients – it has been discussed that unlike the vaccine-induced antibody responses, spontaneously occurring antibody levels against this immunogenic protein might be subtherapeutic and therefore may not contribute to the protection[382].

However, the blocking effect alone, although may stabilise established tumour growth, does not confer to the tumour eradication. Current cancer treatments in humans exploiting monoclonal antibodies against cell surface antigens (e.g. anti-HER-2/neu (Trastuzumab/Herceptin) and anti-CD20 (Rituximab)) is considered to partially act through the ADCC mechanism[373,383]. CDC mechanism, in its turn, is believed to have a minor effect since many tumour cells and some normal cells express cell surface molecules (e.g. CD46, CD55, and CD59) that interfere with the complement cascade activation thus enabling the escape from CDC[384]. Nevertheless, during spontaneous carcinogenesis *in vivo* the level of productive ADCC is not known and should be interpreted with caution because although IgG antibody responses are readily (and possibly predominantly) mounted to cytosolic tumour-derived proteins[382] due to their release via immunogenic tumour cell death, such antigens could not serve as targets for the direct actions of antibodies, nor for the antibody-mediated effector mechanisms.

Apart from the above described mechanisms, antibodies may contribute to protective T cell-mediated immunity indirectly – e.g., through augmenting antigen-specific cross-priming of the T cells, which results from the increased antigen uptake and presentation by DCs that is mediated by the FCγRs recognising antigen-IgG complexes (Figure 9, E). Indeed, it has been demonstrated that the FcγR-mediated uptake of immune complexes by DCs dramatically lowers the dose of antigen, which is required for T cell activation[385]. The usage of this mechanism of promoted cross-priming has been supported by several studies[386-388] including a study where patients were vaccinated against recombinant CT antigen NY-ESO-1. The antigen has been shown to encode B cell as well as HLA class I and II epitopes enabling the generation of integrated Th1-mediated immune responses, and the priming of specific CTL responses was shown to correlate with the availability of adequate levels of antigen-specific IgG1 and IgG3 antibodies[219].

However, it is obviously inconsistent with other studies, which have demonstrated that **B cells limit tumour immune surveillance** by disabling Th1 help in the priming phase thus impeding protective T cell-mediated responses. It seems that the immunological effect of the antibody is dependent on the type of the induced B cell responses that result in the switching to specific IgG isotypes and the secretion of specific cytokines by B cells (e.g. IFN-γ or IL-4) that sustain the polarisation of the CD4+ T cell differentiation pathways resulting in either the inhibition or promotion of protective CTL responses[389,390].

It has been shown in different mouse models that B cell depletion or their complete absence lead to the prevention of tumour growth, reduction of tumour burden or loss of tumour invasion and metastasis[390-394]. However, there is no consistent opinion on how B cells contribute to tumour promotion, and all B cell effector functions, including antibody production, cytokine secretion and antigen presentation have been suggested to have a role in the tumour promotion[169,395,396].

With regard to the antibody responses, the formation of immune complexes is a common feature of tumourigenesis and in certain autoimmune conditions they are known to have pathological role through potentiating chronic inflammation[376]. In cancer patients circulating antigen-antibody complexes have been associated with an increased tumour burden and poor prognosis in different malignancies[377-379]. Apart from the circulation, antibody deposition has been reported in pre-malignant and malignant human prostate and breast tissues[169]. It has been suggested by several studies that the

immune complexes that accumulate in the tumour tissue contribute to chronic inflammatory states through recruiting innate immune cells, for example granulocytes and macrophages. It results in elevated levels of VEGF and matrix metalloproteases (MMPs) that induce angiogenesis and augment tissue remodelling, respectively, thus promoting tumour progression[392,394]. In another study exploiting epithelial HPV16-driven carcinogenesis model, mice depleted for B and T cells showed marked delay of early tumourigenesis associated with reduced inflammation, while mice that received adoptive transfer of B cells or sera from wild type transgenic mice restored inflammatory cell infiltrates, angiogenesis, epithelial hyperplasia and tumour progression in premalignant lesions[394].
Besides, early antigen-specific antibody responses have been correlated with the induction of antigen-specific T cell tolerance in premalignant lesions. The causal relationship between antibody production and CTL tolerance was not ascertained, however, these processes appeared to closely coincide, and the CTL tolerance was associated with the involvement of myeloid suppressor cells and high serum levels of TGF-β1[7].

Taken together, both natural autoantibodies and adaptive B cell responses can be mounted by tumour-derived proteins and exert different effector functions through the particular immunoglobulin classes and subclasses and the secretion of particular cytokines, the induction of which is multilaterally regulated. Both, host protective and tumour promoting roles have been attributed to humoural immunity in tumour-bearing hosts. However, there are controversial issues, which need to be addressed in future studies to clarify the involvement of these roles in the spontaneous tumour development in humans.

1.3.2. SEROLOGICAL IDENTIFICATION OF HUMAN TUMOUR ANTIGENS

The cancer serology has a long history and first attempts to identify tumour antigens by antibodies run into early 1960-ies. The immunisation of experimental animals with human tumour tissues and the removal of antibodies reactive to normal tissue antigens by serial absorption techniques represents the first approach of the serological recognition of tumour antigens[397]. This resulted in the identification of two useful antigens – alpha fetoprotein (AFP)[398] and carcinoembryonic antigen (CEA)[399]. In 1970s Lloyd Old and co-workers established another technique called **"autologous typing"** that was intended to restrict the analysis to autologous reagents from the same patient – autologous serum and cell lines established from the autologous tumour were used to detect cell-surface antigens. The antigens were identified biochemically using extensive absorption analyses[400,401]. The team succeeded to identify some potential tumour targets; however, it was clear that most antigens remained undetected.
Next important step in cancer serology was introduced by the development of the **SEREX approach** (serological analysis of antigens by recombinant expression cloning) by Michael Pfreundschuh's group in 1995[11,12], which originally was based on the generation of tumour tissue-derived cDNA expression libraries and their immunoscreening with autologous patients' sera (an overview can be seen in Figure 14). To date, a number of **modifications on the original SEREX design** have been introduced to overcome some of the inherent limitations of this approach (Table 3). Sources of mRNA other than autologous tumour have been exploited. For example, the use of tumour cell lines instead of tumour biopsies has been particularly useful in the case of analysing tumours from which it is difficult to obtain specimens (e.g. small cell lung cancer)[402-404]. Alternatively, tumour-derived libraries frequently have been screened with (i) allogeneic cancer patients' sera (often pooled) to broaden the antigen repertoire and to identify shared tumour antigens[405-407]; (ii) sera derived from patients after standard treatment or immunotherapy to assess the induced humoural responses[408,409]; (iii) sera from rabbits immunised with a plasma membrane enriched fraction of cutaneous T cell lymphoma (CTCL) cell line have been used to specifically search for cell surface antigens[410]; (iv) it was shown that urine is suitable for the SEREX to monitor high-titre antibody responses (i.e. 1:10,000)[411].
Since 1995 the application of SEREX approach has resulted in the identification of a plenitude of genes encoding novel potential tumour antigens through the interrogation of nearly all tumour types[6,13]. In 1996, a branch of the Ludwig Institute of Cancer Research established the SEREX Collaborative Group comprising multiple laboratories worldwide, and it aimed to contribute to the definition of complete repertoire of immunogenic gene products in human cancer that is becoming known as the **cancer immunome**. The SEREX database was established in 1997 and later it became

incorporated into the publicly accessible Cancer Immunome Database (CIDB)[13]. Currently CIDB deposits 2743 sequences derived from 2316 SEREX-defined clones (May 2009), most of them contributed by the SEREX Collaborative Group. Considering the identified antigen numbers, SEREX has taken the leading place among all the approaches that have been developed for serological identification of TAAs so far.

Table 3. Examples of conventional SEREX approach modifications.

Variable	Modification	Refs
mRNA source for cDNA libraries	Pooled tumours of the same oncotype	412
	Allogeneic normal testis	413,414
	Tumour cell lines	402-404
Antibody source	Allogeneic sera from cancer patients	405-407
	Sera from cancer patients after vaccination and standard treatment	408,409
	Sera from animals immunised with specific tumour cell fractions	410
	Urine	411
Vector system	Yeast surface display system (RAYS)	14,15
	Filamentous phage (M13) surface display expression system	16,17
	Lytic phage (T7, λ) surface display expression systems	16,18-20

Abbreviations: RAYS – recombinant antigen expression on yeast surface, RDA – representational difference analysis, SAGE – serial analysis of gene expression.

Apart from the above listed modifications of the conventional SEREX approach, recombinant antigens have been expressed onto the surface of yeast *Saccharomyces cerevisiae*[14]. The application of this approach (called **RAYS**, recombinant antigen expression on yeast surface) is aimed to enable more natural protein folding and PTMs, and to date have resulted in the identification of several known overexpressed tumour antigens as well as mutated protein variant of small breast epithelial mucin in breast cancer[15]. Besides, phage surface display vector systems have been introduced in the original SEREX design (called **phage display-based SEREX**), which enable tumour-derived cDNAs expression in fusion to one of the phage coat proteins and exposure on the surface of the phage – this allows the selection of serum-reactive phage clones by biopanning and thus represent much faster as well as cost and labour-effective alternative to the conventional immunoscreening[18,20,415,416].

More recently on the basis of the advancements in molecular techniques an array of new approaches has been developed. The **SERPA approach (or PROTEOMEX)** is based on Western blot analysis of two-dimensional electrophoresis separated tumour proteome that is independently probed with sera from cancer patients and healthy individuals, followed by antigen excision from the preparative gel and identification by mass spectrometry (MS)[417,418] (see Figure 1, A in the review paper **V**). Application of this approach has resulted in the identification of several, mostly overexpressed TAAs, including Tim, MnSOD[419] and alpha-enolase[420] in lung squamous carcinoma, THIO in renal cell carcinoma[418] etc. Although proteins with PTMs can be detected by this method, they are resolved in denatured conditions thus loosing their native conformations; besides, this method is biased towards the identification of abundant proteins.

Another approach called **AMIDA** (autoantibody-mediated identification of antigens) makes use of parallel immuno-precipitation of cancer cell-derived lysates by purified cancer patient serum-derived IgGs and IgGs from healthy donors, which is followed by two-dimensional electrophoresis, comparison of spot patterns between the two groups, relevant spot excision and MS analysis[421]. Such approach has been successfully applied to head and neck cancer, which resulted in the identification of 39 TAAs with cancer-specific serum reactivities including large proportion of hitherto unknown antigens[421,422]; however, the application of this approach faces the same biases as described for SERPA.

The **MAPPing** (multiple affinity protein profiling) approach combines two-dimensional immunoaffinity chromatography (AC) with MS (see Figure 1, B in the review paper **V**). In the first AC step tumour-derived proteome is depleted for antigens recognised by healthy donor's sera while in the second AC antigens reactive with cancer patient sera are selected[423]. The selected protein pools undergo enzymatic digestion, fractionation by nano-RPLC (nano-reverse phase liquid chromatography) and identification by a tandem MS technique. This approach enables the analysis of proteins in their native conformations. The application of MAPPing approach to colon cancer has

resulted in the identification of a single tumour-specific antigen (name undisclosed) and a comprehensive list of antigens recognised by cancer-free individuals hence pointing out the significance of the first depletion step.

Recently, Philip *et al.* developed an immunoprecipitation/MS-based technique for the identification of autoantibody-based serum biomarkers and combined it with the identification of endogenous peptides presented by MHC class I molecules[424]. This potentially **theranostic approach** was applied to ovarian cancer resulting in the identification of more than 50 antigens that can serve as therapeutic and diagnostic targets.

Protein microarrays represent promising technique for the discovery of novel TAAs; besides, it have become most widely applied approach for the selection and validation of autoantibody biomarker candidates (see Figure 1, C in the review paper **V**). **Recombinant phage microarrays** are produced by arraying phage particles that have been selected from phage displayed tumour-derived cDNA libraries via several rounds of biopanning with sera from cancer patients. The microarrays consisting of hundreds to thousands of phages displaying different proteins encoding for potential antigens can be probed with as little as a few microliters of serum, and antigen clones with cancer-specific sero-reactivities then can be identified through direct sequencing of the phage clone inserts[425-428]. An alternative to the phage-displayed protein microarray is generation of **natural protein microarrays**, which should allow the identification of TAAs derived from altered PTMs and conformational epitopes. Such an approach has been implemented by Hanash *et al*. – two-dimensional liquid chromatography was used to separate lysates of cancer cell lines into 1760 to 1840 fractions that were used for the production of microarrays. These platforms were analysed to search for autoantibody biomarkers in colon, lung and prostate cancer, resulting in the detection of 39, 63 and 38 fractions, respectively that showed enhanced reactivity with sera from cancer patients in comparison to controls[429,430], and from which TAAs may be deduced. Another version of natural protein microarrays is **"reverse capture" autoantibody array**, in which native proteins from cancer cells are captured by highly specific monoclonal antibodies arrayed onto a microarray slide and tested with cancer and control sera labelled with CyDyes. To demonstrate a proof-of-principle, it was applied to search for autoantibodies that distinguish prostate cancer sera from sera derived from individuals with benign prostate hyperplasia, which resulted in the identification of 48 proteins reacting preferentially with sera from cancer patients[431]. Utilising of **known protein microarrays**, such as Invitrogen's ProtoArray containing up to 8000 proteins, represent another valuable way of TAA discovery. Currently seems that the use of this approach is limited by the cost of the production of recombinant proteins and presumably by the variations due to differences in conformations and PTMs in different platforms; nonetheless, taking into account rapid developments in the field, this may become a very promising tool in the nearest future.

AIMS OF THE PRESENT STUDY

The overall **aim of the current study** was to evaluate the potential of the conventional and phage display-based SEREX approaches for the assessment of antigen repertoires eliciting B cell responses in cancer patients and to identify a comprehensive set of antigens associated with melanoma, prostate and gastric cancer that further would be searched for novel autoantibody biomarkers as well as for potential immunotherapy targets.

To achieve these goals, **following tasks were proposed**:

(i) to apply the conventional SEREX approach for the tumour-associated antigen identification in gastric cancer patients;
(ii) to compare the ability of T7 and λ phage-based surface display vector systems for their ability to display known tumour antigens, and
(iii) to test the suitability of the two phage display vector systems for the generation of a phage-displayed antigen microarray and serum autoantibody profiling;
(iv) to elaborate and optimise the phage display-based SEREX approach for large-scale tumour-associated antigen identification, and
(v) to use this approach to identify a comprehensive set of antigens in melanoma, prostate cancer and gastric cancer patients;
(vi) to develop the identified antigen microarray that would be suitable for high-throughput survey of autoantibody repertoires.

2. MATERIALS AND METHODS

2.1. MATERIALS

All the clinical material used in the current study was received after the patients' informed consent was obtained and it was done in accordance with the regulations of local ethics committees.

2.1.1. SERUM SAMPLES

Serum specimens used in the conventional SEREX study were derived from 56 gastric cancer, 23 colon cancer, 11 breast cancer and three prostate cancer patients and were collected at Latvian Oncology Center; in addition sera were withdrawn from 35 healthy donors.

Paired pre- and post-vaccination serum samples that were withdrawn from patients enrolled in three ongoing immunotherapy trials were used in the phage display-based SEREX study and were received from EU 6th Framework program ENACT partners:

(i) 24 melanoma patients undergoing Tyrosinase-based immunotherapy (designated Mel-TYR); collected at the Skin Cancer Unit, German Cancer Research Center;

(ii) 31 prostate cancer patients enrolled in a DC-based immunotherapy trial (designated Pr-ONY); collected by Onyvax Vaccine Therapies Ltd, UK;

(iii) 24 prostate cancer patients undergoing a DC-based immunotherapy (designated Pr-NRH); collected at Norwegian Radium Hospital.

In addition, serum samples from 90 melanoma patients (22-23 from each stage) and sera from 80 healthy donors were received from the Skin Cancer Unit, German Cancer Research Center. Four melanoma, 27 gastric cancer, 15 gastritis patient-derived serum specimens and sera from 56 healthy volunteers were received from Latvian Genome Project database. All the serum samples were aliquoted and stored at -80^{o}C.

2.1.2. TUMOUR TISSUES

Forty-eight stomach tumour tissue specimens were collected at Latvian Oncology Center, from 36 of the 48 gastric cancer patients paired normal stomach tissue specimens were also collected and used in the study. Melanoma tumour tissues (and matched serum samples) were collected at the Skin Cancer Unit, German Cancer Research Center and Latvian Oncology Center. Three prostate cancer cell lines OPCT1-3 (designated elsewhere in the text collectively as OPCT) were received from Onyvax Vaccine Therapies Ltd, UK. Tumour tissues were collected intraoperatively and snap-frozen in liquid nitrogen or preserved in RNALater (Ambion) solution immediately after biopsy and stored at -80^{o}C. Clinical characteristics for the tumour tissue specimens used for the construction of cDNA expression libraries are summarised in Table 4.

Table 4. Clinical characteristics of tumour tissue samples used for cDNA expression library construction.

Tissue code		TNM	Histological type	Grade	Localisation	Gender
Melanoma						
Mel-T2		410	Cutaneous, pigmented	NA	Shank	F
Mel-T4		43X	Cutaneous, non-pigmented, nodular	NA	Thigh	F
Mel-1816		NA	NA	NA	NA	NA
Mel-525		NA	NA	NA	NA	NA
Mel-445		NA	NA	NA	NA	NA
Gastric cancer						
Ga-1*		400	Adenocarcinoma	GII	Corpus	M
Ga-445	T7-GaCaP5	200	Signet-ring cell	GIV	Pylorus	M
Ga-446		310	NA	GIV	Corpus	M
Ga-449		300	Tubular	GII	Fundus	M
Ga-450		210	Intestinal adenocarcinoma	GII	Antrum	F
Ga-451		320	Intestinal adenocarcinoma	GIII	Total	F
Ga-454	T7-GaCaP6	310	Tubular	GIII	Cardia	M
Ga-455		100	Intestinal adenocarcinoma	NA	Antrum	F
Ga-460		NA	Signet-ring cell	NA	Fundus	M
Ga-464		311	Diffuse adenocarcinoma	GIV	Total	F
Ga-465		300	Diffuse adenocarcinoma	GIII	Corpus	F
Ga-473		200	Signet-ring cell	GIII	Pylorus	M

* Tissue specimen analysed in conventional SEREX study;

Abbreviations: NA – information not available, M – male, F – female.

2.2. METHODS

2.2.1. APPLICATION OF THE CONVENTIONAL SEREX TO GASTRIC CANCER

Construction and immunoscreening of gastric cancer cDNA expression library

The construction of gastric cancer cDNA expression library was done as described in the original paper **I**. Briefly, a moderately differentiated, ulcerated gastric adenocarcinoma tissue specimen (Ga-1, Table 4) was used for the construction of a cDNA expression library in λ Uni-ZAP XR vector (Strategene) according to the manufacturer's protocol. The primary library of size 2 x 10^6 pfu was obtained, amplified once and immunoscreened with 1:250 diluted autologous serum according to Sahin *et al*[11]. To omit the IgG encoding cDNA clones from further analyses, nitrocellulose membranes with plaque lifts were pre-screened with alkaline phosphatase (AP)-conjugated anti-human IgG secondary antibody and developed, and reactive plaques were marked. In parallel, 1:250 diluted serum derived from cancer patient suffering from poorly differentiated infiltrative gastric adenocarcinoma was used for immunoscreening of the cDNA expression library. Serum-reactive clones were subjected to additional round(s) of immunoscreening, obtained as monoclonal phage stocks and converted to pBluescript phagemids through *in vivo* excision procedure. The sera were absorbed with *E. coli* and λ phage lysate prior to immunoscreening to reduce background signals.

Characterisation of serum-reactive clones

To identify the encoded cDNAs, the excised plasmid DNAs were purified and clone inserts sequenced. The DNA sequences were analysed using BLAST tool at www.ncbi.nlm.nih.gov, Translate tool at www.expasy.org and compared against sequences available at Cancer Immunome Database (http://ludwig-sun5.unil.ch/ CancerImmunomeDB).

The frequency of allogeneic autoantibody responses against the identified antigen phage clones was determined by a plaque assay – *E. coli* were transfected directly on gridded agar plates, by spotting 20-30 pfu of monoclonal serum-reactive phage side by side with non-recombinant phages. The phage arrays were screened with 1:200 diluted allogeneic sera from cancer patients and healthy donors, excluding the IgG pre-screening step. All the sera used for serological analyses were pre-absorbed with *E. coli* and λ phage lysates.

In parallel, mRNA levels of identified antigens in a panel of different normal tissue RNA from whole brain, liver, heart, kidney, lung, trachea (Clontech), spleen, colon, stomach, testes, ovary (Ambion), PBLs and 20 paired gastric cancer and matched normal tissues were compared by applying comparative RT-PCR analysis as described in the original paper **I.**

Molecular characterisation of NUCB2

The molecular characterisation of the SEREX-defined antigen NUCB2 has been accomplished as described in the original paper **II.** Briefly, NUCB2 mRNA expression pattern was analysed by real-time RT-PCR in a panel of total RNA from normal tissues – e.g. whole bone marrow, brain, colon, heart, kidney, lung, pancreas, skin, spleen, stomach, testis, thymus and trachea (BioCat, Ambion) and compared between paired tumour/normal stomach tissues. First-strand cDNA was synthesised from 4 μg of total RNA by random hexamer priming using First-Strand cDNA Synthesis Kit (MBI Fermentas). In all experiments 1/30 of each RT mixture was amplified exploiting 7500 Real Time PCR System (Applied Biosystems). All reactions were performed in duplicates. TUB3A, ACTB and POLR2A were used as reference genes for normal tissue panel and ACTB, TBP and POLR2A for the gastric cancer/normal tissue panel.

NUCB2 protein levels in corresponding gastric cancer and adjacent normal tissue specimens (that were used for the mRNA expression level comparison) and AGS cell line were analysed by Western blot. Total protein was isolated from tissue samples using TRI reagent (Sigma) simultaneously with RNA according to manufacturer's instructions and 10 μg analysed in 10% SDS-PAGE with 1:200 diluted mouse anti-NUCB2 monoclonal antibody, kindly provided by Dr. Shitsu Barnikol-Watanabe (Gottingen, Germany). To assess the phosphorylation status, protein samples were treated with 1 U of SAP (MBI Fermentas) for 1 h at 37 ^{o}C and then subjected to Western blot analysis as described above. Deglycosylation was carried out using Enzymatic deglycosylation kit (Glyko) according to the manufacturer's instructions.

mRNA expression analyses of TACC1

TACC1-A and TACC1-F transcript variant distribution was analysed by semi-quantitative RT-PCR in various normal tissues from whole brain, liver, heart, kidney, lung, trachea (Clontech), spleen, colon, stomach, testes, ovary (Ambion) and in paired tumour/normal stomach tissues as described in the original paper **I**. Briefly, cDNA was synthesised from 4 μg of total RNA by random hexamer priming using First-Strand cDNA Synthesis Kit (MBI Fermentas); 1/15 of synthesised cDNA was amplified in a total reaction volume of 20 μl containing 200 μM of dNTPs, 2 U of Taq polymerase (Fermentas, Lithuania) and 10 pmole of each primer (for amplification of TACC1-A: forward 5'-AGGAGGAGGATTCGCAAGC-3' and reverse 5'-TTGTTCCGAGGACTGCCGAG-3', TACC1-F: forward 5'-CTTTGACGAATCCATGGATCC-3' and reverse 5'-AATTTCACTTGTTCAGTAGTC-3', GAPDH (used as reference gene): forward 5'-GTCATCCCT GAGCTAGACGG-3' and reverse 5'-

GGGTCTTACTCCTTGGAGGC-3'). Optimal cycle numbers for each transcript (i.e. 35 cycles for TACC1-A, 31 cycle for TACC1-F and 25 cycles for GAPDH) were defined as described in the original paper **I**. For co-amplification of TACC1-A and F, TACC1-F forward primer and reverse primer 5'-TGGTAGACACAGGAACATTGG-3' were used.

To analyze the exon composition of TACC1 mRNA 5' variants expressed in gastric cancer tissues 5'-RLM-RACE (RNA ligase-mediated rapid amplification of cDNA ends) analyses were performed using a FirstChoise™ RLM-RACE kit (Ambion) according to manufacturer's protocol. Briefly, 10 μg of gastric cancer total RNA was dephosphorilated and ligated to RNA adapters after removal of cap structure from full-length mRNAs. A random primed reverse transcription and nested PCR with adapter-specific forward primers and TACC1-specific reverse primers were performed, obtained PCR products were cloned using InsT/AcloneTM PCR Product Cloning Kit (MBI Fermentas) and sequenced.

2.2.2. CONSTRUCTION OF PHAGE-DISPLAYED CDNA EXPRESSION LIBRARIES

Construction of λ and T7 phage-displayed TAA mini-libraries

Two phage surface display vectors – λKM8 (kindly provided by Dr. Olga Minenkova, Kenton Labs[20]) and T7Select 10-3b (Novagen) – were used for cloning antigenic regions of 15 tumour associated antigens (TAAs) or antigen families as described in the original paper **IV**. Briefly, either full–length ORFs, known antigenic regions or the antigenic regions predicted using an algorithm developed by Welling *et al*[432] (the antigens and the regions chosen for cloning are listed in the Table 7) were amplified by RT-PCR using High Fidelity PCR enzyme mix (Fermentas) using testis or melanoma-derived cDNAs as a template. The primer sequences (listed in Appendix 1) contained restriction sites enabling directional cloning of the PCR products into the vectors. For the amplification of genes from gene families, degenerated primers were designed to amplify representative set of antigens.

λKM8 and T7Select 10-3b vectors were digested with NotI/SpeI and NotI/SalI restriction enzymes (Fermentas), respectively (Figure 18), dephosphorylated and purified. The PCR fragments were digested with the corresponding restriction enzymes and purified. Five nanograms of each digested PCR product was ligated into λKM8 or T7 Select 10-3b vector (100 ng), and 1/5 of each ligation mixture was subjected to *in vitro* packaging resulting in mini-libraries of size 1-5 x 10^5 pfu and 0.5-5 x 10^4 pfu, respectively. The obtained TAA mini-libraries were once amplified in BB4 (for λ phage) or IPTG-induced BLT5615 (for T7 phage) *E. coli* cells and the aliquots were stored at +4°C or as glycerol stocks at -80°C.

Construction of T7 phage-displayed cDNA expression libraries

One testis, five melanoma, one prostate cancer and two gastric cancer T7-displayed cDNA expression libraries were constructed using T7Select 10-3b vector and OrientExpress cDNA library construction system (Novagen) according to the manufacturer's instructions. Briefly, to construct a testis library (designated T7-T1), 150 μg of testis total RNA (Ambion) was subjected to mRNA isolation by Dynabeads mRNA purification kit (Invitrogen) following manufacturer's instructions. Four micrograms of the mRNA was converted to cDNA using HindIII random primers (5'-TTNNNNNN-3') that was followed by directional EcoRI and HindIII linker ligation to cDNAs, digestion with the corresponding restriction enzymes, ligation into pre-digested T7Select 10-3b vector arms and packaging *in vitro* by using T7Select Packaging extract (Novagen).

Five melanoma libraries (designated T7-Mel-T2, T7-Mel-T4, T7-Mel-525, T7-Mel-445 and T7-Mel-1816) were constructed from a single tumour specimen-derived mRNAs (0.5-1 μg each) (Table 4), two gastric cancer libraries (designated T7-GaCaP5 and T7-GaCaP6) were constructed from two mRNA pools (of total amount 4 μg each) derived from five and six gastric cancer tumour tissue specimens, respectively (Table 4), and a prostate cancer library (designated T7-OPCT) was constructed from pooled mRNAs (of total amount 4 μg) derived from three prostate cancer cell lines OPCT 1, 2 and 3. Briefly, tumour tissues were homogenised in liquid nitrogen and then used for total RNA isolation with TRI reagent (Sigma) that was subsequently used for mRNA isolation with Dynabeads mRNA Purification kit (Invitrogen). The mRNA was subjected to cDNA synthesis and T7Select 10-3b vector-based library construction as described above.

All the libraries were amplified once in IPTG-induced BLT5615 *E. coli* cells, the lysates were centrifuged to remove cell debris, and the phage-containing supernatants were stored in aliquots at +4 °C or as glycerol stocks at -80 ^{o}C.

2.2.3. ENRICHMENT OF T7 PHAGE-DISPLAYED LIBRARIES

The biopanning procedure

The procedure was used for the routine enrichment of serum-reactive clones from testis, melanoma and gastric cancer cDNA expression libraries prior to immunoscreening. To negatively select for IgG-encoding phage clones, 100 μl of protein G-coated magnetic beads (Pierce) were washed with 2 x 1 ml of ImmunoPure (G) IgG binding buffer (BB, Pierce), blocked with 5 % non-fat dried milk in TBS, 0.05 % Tween-20 for 30 min at room

temperature (RT); ~10^{11} pfu from an amplified library were mixed with >1 volume of BB, added to the beads and incubated for 2 h at RT under agitation (this step was omitted in the case of T7-T1 library enrichment). Next, beads were discarded, 2 µl of patient's serum was added to the library and incubated overnight at +4 °C under agitation. All sera were preabsorbed with CNBr-Sepharose 4B-coupled BLT5615 *E. coli* and T7 phage lysates prior to biopanning. Two hundred microliters of pre-blocked Protein G-coated beads were resuspended in the phage-serum solution and incubated for 2 h at RT under agitation. The beads were washed 5 x 1 ml of BB, 4 x 10 min with 1 ml of TBS, 0.05 % Tween-20, 3 x 1 ml of BB and resuspended in phage extraction buffer (PEB, 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 6 mM $MgSO_4$). The bound phages were titrated, amplified and stored at +4 °C until subjected to immunoscreening.

To compare the antigen repertoires after the first and second cycle of biopanning, ~5 x 10^{10} pfu from the T7-T1 library were subjected to the biopanning procedure as described above. Approximately 8 x 10^3 pfu from the biopan that was subjected to immunoscreening while the rest was amplified and subjected to the second round of biopanning according to the same protocol, titrated and ~8 x 10^3 pfu were immunoscreened. The serum-reactive clones were isolated, subcloned to monoclonality and their insert sequences determined as described thereafter.

Differential enrichment of the T7-OPCT library

For differential selection of prostate cancer-associated antigens (a schematic overview can be seen in Figure 25), six pools of sera collected from prostate cancer patients undergoing two independent DC-based immunotherapy trials (i.e. Pr-ONY and Pr-NRH) were assembled:

(i) four pools of prostate cancer patients' sera (designated as Pr-PreV-R-P6, Pr-PV-R-P6, Pr-PreV-NR-P6 and Pr-PV-NR-P6) representing two groups of serum samples – i.e. pre-vaccination (PreV) and post-vaccination (PV) – were further subdivided according to patients' response to treatment (i.e. responders (R) and non-responders (NR)). Each of the four pools included six serum samples;

(ii) two pools of prostate cancer patients' post-vaccination sera, designated as ONY-PV-R-P7 and ONY-PV-NR-P3, representing seven serum samples derived from patients responding to vaccination and three serum samples derived from patients not responding to vaccination, respectively.

Approximately 10^{11} pfu of amplified library was mixed with >1 volume of BB and 2 µl of each serum comprising the serum pool A (Figure 25) was added to the beads and incubated overnight at +4 °C under agitation. Two hundred microliters of protein G-coated magnetic beads (Pierce) were added to the phage-antibody mixture and incubated in RT for 2 h with agitation. The beads with antibody-phage complexes were washed The beads were washed 5 x 1 ml of BB, 4 x 10 min with 1 ml of 0.05 % Tween-20, 3 x 1 ml of BB and resuspended in PEB, titrated and subjected to immunoscreening while the unbound phage solution was further used for the selection of serum pool B-reactive clones following the same procedure. All serum specimens were preabsorbed with the phage and *E. coli* lysates prior to selection.

2.2.4. IMMUNOSCREENING OF THE PHAGE DISPLAY LIBRARIES

Immunoscreening of T7 and λ phage-displayed TAA mini-libraries

The procedure has been described in detail in the original paper **IV**. Briefly, for T7-displayed TAA mini-libraries, ~10^3 pfu from each library was spotted on gridded LB/carbenicillin agar plates pre-coated with LB top agarose containing BLT5615 cells and grown for ~2 h at 37 °C until plaques reached ~1 mm in diameter. For screening of λ-displayed TAA mini-libraries, BB4 cells together with NZY top agarose were plated on NZY agar plates, infected with the same amount of phages and the plates were incubated at 37 °C for 6-8 h. Further, in either case plates were overlaid with Protan nitrocellulose (NC) filters (Schleicher & Schuell) and incubated for 1 h at 37 °C. The filters were blocked for 1 h in 5% non-fat dried milk in TBS, 0.05 % Tween-20 and then incubated overnight at RT with 1:200 diluted patients' serum that has been preabsorbed with *E. coli*-phage lysate. The serum-reactive clones were detected by incubating the filters with AP-conjugated anti-human IgG Fcγ–specific secondary antibody (Pierce) and NBT/BCIP (Fermentas). Ten serum-reactive clones in each mini-library per each serum were isolated and subjected to secondary screenings by using a plaque assay to obtain monoclonal phage stocks; briefly, the phage stocks were diluted to 3-5 pfu/µl, spotted on gridded LB/carbenicillin agar plates pre-coated with LB top agarose containing IPTG-induced BLT5615 cells and grown for ~2 h at 37 °C until plaques reached ~1 mm in diameter. Further procedure was done according to the protocol described above. Separate positive plaques were chosen and isolated from the plate, resuspended in 100 µl of PEB and stored in +4 °C until further analyses.

Immunoscreening of T7-displyed libraries

For screening of T7 phage-displayed libraries, BLT5615 cells were grown in LB supplemented with 1×M9 salts, 0.4 % glucose, 1 mM $MgSO_4$ and carbenicillin (50 µg/ml) to OD_{600}=0.5 and then induced with IPTG for 30 min. Approximately 8 x 10^3 pfu from enriched library premixed with molten LB top agarose containing the 1/10 volume of the IPTG-induced cells, 1 mM IPTG and carbenicillin (50 µg/ml) was plated per 150 mm Ø LB/carbenicillin agar plate. Plates were incubated at 37 °C for ~2 h until visible plaques appeared and then

overlaid with Protan nitrocellulose (NC) filters (Schleicher & Schuell) and incubated for 1 h at 37 °C. Further immunoscreening procedure was done as described in the original paper **IV**. All available serum-reactive clones were isolated and subjected to secondary screenings and subcloned to monoclonality.

2.2.5. ANTIGEN IDENTIFICATION AND DEVELOPMENT OF TUMOUR ANTIGEN DATABASE

The monoclonal serum-reactive phages were directly subjected to PCR analyses to amplify inserted cDNA sequences by using primers flanking the insert (T7-Up2: 5'-CTTCGCCCAGAAGCTGCA-3', T7-Down: 5'-AACCCCTCAAGACCCGTTTA-3', λKM8-Up: 5'-CAATCTGTGTGGGCACTCG-3', λKM8-Down: 5'-CGGCTGGTAATGGGTAAAGG-3'). One microliter of phage solution was used as a template for 35-cycle amplification following conditions 45 s at 94 ^{o}C, 30 s at 50 ^{o}C and 45 s at 72 ^{o}C. PCR products were purified and directly sequenced using ABI Prism BigDye Terminator v3.1 cycle sequencing kit and 3130 Genetic Analyser (Applied Biosystems). DNA sequences were analysed using BLAST tool at www.ncbi.nlm.nih.gov, Translate tool at www.expasy.org and compared against sequences available at Cancer Immunome Database[13].
The clone insert sequences were deposited into a Tumour Antigen database that was developed on the basis of MySQL. All the available information about the identified antigens including translated protein sequences, official gene name, chromosomal localisation, brief description of gene function and putative structural motifs of the protein as well as the patient-related clinical information was deposited in the database as well. Additional tools were developed to allow automatic generation of EST profiles for each record, local nucleotide and protein BLAST search as well as search by keywords. The database provides links to the Cancer Immunome Database, Entrez Gene and CGAP expression databases.

2.2.6. WESTERN BLOT ANALYSES OF THE IDENTIFIED ANTIGENS

Determination of antigen copy number variation

Recombinant antigen copy number per T7 phage particle was determined as described in the original paper **IV**. Briefly, selected phage clones were amplified in *E. coli* BLT5615 cells, precipitated from the lysates by NaCl/PEG, titrated and ~10^9 pfu of each phage were subjected to Western blot analysis with 1:5000 diluted rabbit HRP-conjugated anti-T7 tag antibody (ABcam) recognising the N terminus of T7 coat protein 10B. To ascertain that unincorporated recombinant proteins are not co-precipitated with the phage particles, NaCl/PEG precipitated phage solution containing ~10^{10} pfu was filtrated through 100,000 NMWL filter units (Millipore) and the flow-through fraction was run in parallel with the phage samples as a negative control. For quantity calibration a standard curve was constructed from five serial three-fold dilutions of one phage clone. The images were analysed using GelWorks software (Ultra-Violet Products).

Confirmation of recombinant antigen serum reactivity

cDNA inserts of four serum-reactive clones were amplified by PCR and cloned into a prokaryotic GST expression vector pGEX-4T-1, purified using the Microspin GST Purification Module (Amersham Biosciences) according to the manufacturer's instructions in order to obtain either the natural products of these genes or the out-of-frame peptides displayed by the serum-reactive clones. GST protein was also expressed and purified for the negative control. Purified proteins (500 ng) were subjected to Western blot analysis by using either 1:20,000 diluted HRP-conjugated goat anti-GST antibody (Amersham Biosciences) or 1:200 diluted patient's sera followed by incubation with 1:10,000 diluted HRP-conjugated goat anti-human IgG antibody (Sigma) as described in the original paper **IV**.

2.2.7. ANTIGEN MICROARRAYS

Development of T7 phage-displayed antigen microarrays

The development of phage microarrays and profiling of autoantibody responses in cancerous and non-cancerous sera was done according to standard operating procedures (SOPs) that were developed by Pawel Zayakin. Briefly, the development of SOPs covered the evaluation of different variables, including high-titre phage stock preparation method (e.g. plate lysates vs liquid lysates), phage growing conditions (e.g. growing volumes, agitation, *E. coli*-phage ratios), and formats (e.g. separate tube vs 96-well plate formats) as well as optimisation of phage printing conditions. Next, microarray slide processing procedure was elaborated that included elucidation of optimal serum preabsorbtion technique, appropriate antibody dilutions and washing conditions.
To generate the phage microarray, a panel of 730 phage clones encoding different antigens and 30 non-recombinant phage clones was assembled in 96-well plates. BLT5615 *E. coli* cells were grown from fresh cultures until OD_{600} reached 0.8, induced by IPTG for 30 min, aliquoted in 96 deep-well plates (Whatman) (500 µl per well) and then infected with 5 µl of low-titre monoclonal phage stocks, grown at 37 ^{o}C, 220 rpm until complete cell lysis. Then 6.4% glycerol was added and plates were centrifuged to remove cell debris and random phage clones were subjected to PCR-based quality control. The obtained high-titre phage stocks were either stored at -80 ^{o}C or directly subjected to the second round of amplification using 1 µl of the high-titre phage

stock. Then 6.4% glycerol and 0.01% sodium azide was added, lysates were clarified by centrifugation and directly used for the production of the antigen microarray. The phages were spotted in duplicates onto two-pad nitrocellulose-coated FAST slides (Whatman) using QArray^{mini} compact arrayer with 150-micron solid pins (Genetix). Printed microarray slides were stored at +4 ^{o}C until further processing but no longer than one week.

Autoantibody profiling by using antigen microarrays

The microarrays were blocked in 5 % non-fat dried milk in TBS, 0.05 % Tween-20 for 1 h at RT, incubated with preabsorbed 1:200 diluted patients' sera for 2 h at RT under agitation and then washed 4 x 15 min in TBS, 0.5% Tween-20. Next, slides were incubated with 1:10,000 diluted anti-T7 tail fiber antibody (Novagen) for 1 h at RT, washed as described above, and then incubated with Cy5-labelled goat anti-human IgG antibody (1:1500) and Cy3-labelled goat anti-mouse IgG antibody (1:3000) (both antibodies with minimal cross-reactivity against IgGs of other species, Jackson ImmunoResearch) for 1 h at RT, then washed again, rinsed with distilled water and dried by centrifugation for 1 min at 1000 rpm. The microarrays were read using AQuire two-laser confocal scanner (Genetix) and the images were analysed using Genetix QScan software. For each spot the mean Cy5 and Cy3 signals were background-subtracted, averaged between replicates, and the Cy5/Cy3 ratios were calculated for each antigen and normalised by that of non-recombinant phages. Spots with Cy3 signals lower than 50% of the mean Cy3 signal intensity across the array were excluded from further analysis. A cut-off value for defining cancer-specific antigens was set as >4 standard deviations (SD) above the average Cy5/Cy3 ratios in healthy donors.

Development of T7 and λ phage-displayed antigen microarrays

High-titre phage stocks were prepared for nine T7 and λ phage clones encoding the same TAAs isolated by immunoscreening from TAA mini-libraries as well as four non-recombinant phage clones according to SOPs that were developed by Pawel Zayakin. Briefly, BLT5615 and BB4 *E. coli* cells were grown from fresh cultures until OD_{600} reached 0.8, and then infected with T7 and λ phage stocks, respectively, and grown until complete lysis. Phage lysates were prepared for printing as described above and arrayed in five replicates onto nitrocellulose-coated 16-pad FAST slides (Whatman). The microarrays were processed following conditions described above; corresponding slides were incubated with 1:10,000 diluted anti-T7 tail fiber (Novagen) or 1:1500 diluted anti-gpV (λ tail protein) mouse monoclonal antibody (kindly provided by Dr. Mauruzio Cianfriglia) to normalise the serum-derived signal against the number of phage particles per spot. A cut-off value for defining serum-reactive antigens was set as >3 standard deviations (SD) above the mean ratio for the 20 non-recombinant phage spots.

3. RESULTS AND DISCUSSION

The original publications **I-IV,** which are included at the end of the dissertation partially form the basis of the current study and are ordered according to the description of results in the current section. The review article (ref. **V**) that has been published in *Current Cancer Therapy Reviews* overviews the current success of the use of autoantibodies as biomarkers in cancer patients. It summarises the recent proteomic approaches (other than conventional SEREX) that have been successfully applied for the identification of novel TAAs and autoantibody profiling, discusses molecular basis of autoantibody production in cancer patients and their putative role in immune responses against tumours, and give an insight into clinical value of autoantibody signatures in the early detection of cancer as well as monitoring the response to different treatment modalities. Thus, the contents of this paper are in part reflected in the Literature Review section and will be discussed in the current section as well.
In this dissertation, the main emphasis will be laid on the results obtained from the application of conventional SEREX and phage display-based SEREX approaches to different types of cancer, and on the evaluation of these approaches for their suitability in the assessment of cancer patients' autoantibody responses in large-scale.

3.1. APPLICATION OF THE CONVENTIONAL SEREX APPROACH

The conventional SEREX approach makes use of cancer patient's tumour tissues and autologous serum antibodies as it is shown in Figure 14. This approach allows to systematically search for genes whose products had elicited high-titre IgG antibody production in cancer patients. Importantly, this approach is applicable to nearly all tumour types and is less technically demanding than T cell epitope cloning (see section 1.2.3.) – there is no need for the establishment of tumour cell lines and pre-characterised CTL clones since operatively removed tumour tissue specimen is used instead as a source of mRNA messages that cover all spectrum of actively transcribed genes. This also allows to escape *in vitro* artefacts that are induced by culturing tumour cells.

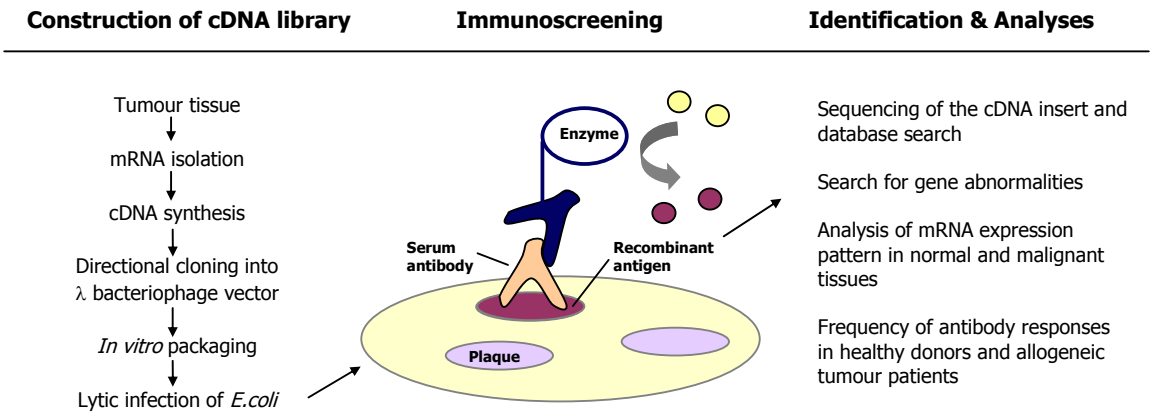


Figure 14. The principle of the conventional SEREX approach. The approach is based on the construction of tumour-derived cDNA expression libraries in the bacteriophage λ ZAPII vector under the inducible *lacZ* promoter. Bacteria are infected with the recombinant bacteriophages and grown on a Petri dish. After lytic plaques appear, the protein cargo is transferred onto a nitrocellulose membrane that is then incubated with 1:100-1:1000 diluted cancer patient's serum, and the serum-reactive spots are detected by secondary antibody-enzyme conjugate. The corresponding plaque is then isolated from the plate and immunoscreened repeatedly to monoclonality and subjected to sequence, mRNA expression and serological analyses[400].

When antigens are selected and identified, they can further be characterised for their reactivity against allogeneic sera derived from healthy individuals and tumour patients to select cancer-related antigens. Besides, the expression analyses of identified antigens in tumour and normal tissues are often integrated in SEREX studies. These and other analyses are used to address different questions. For

example, is the humoural response to the antigen cancer-restricted? Why is the protein immunogenic? Does the antigen play a role in tumourigenesis, if any, and what is this role, etc.[6,433]?

3.1.1. SEREX ANALYSIS OF GASTRIC CANCER ANTIGENS (REF. I)

Identification of serum-reactive clones

In order to search for tumour antigens eliciting autoantibody responses in gastric cancer patients, the conventional SEREX approach was applied to moderately differentiated, ulcerated gastric adenocarcinoma (Table 4). Immunoscreening of ~8×10^5 clones from gastric cancer cDNA expression library with autologous serum resulted in the identification of 11 serum-reactive clones. In addition, ~3×10^5 clones from the library were immunoscreened with allogeneic serum from a patient suffering from poorly differentiated infiltrative gastric adenocarcinoma, and this resulted in the isolation of nine serum-positive clones.

Sequence analyses of full-length inserts (ranging from 0.5-2.5 kb) of the identified serum-reactive clones and GenBank database homology search revealed that they represent 14 distinct genes (listed in Table 5). From these, only two (i.e. centrosomal protein CEP290 and uveal autoantigen UACA) were eliciting antibody responses in both gastric cancer patients. Both of the antigens have been previously identified by SEREX approach in several types of cancer, however autoantibody responses to these antigens revealed not to be cancer-specific (Table 6) indicating that they may represent cancer-independent autoantigens. Amongst the identified antigens, there are four proteins with yet unknown function. Others represent functionally diverse proteins, majority of which are intracellular proteins and this is consistent with other SEREX studies[6]; besides, they include genes with known or putative involvement in carcinogenesis (see Table 5 for brief characterisation). No mutations were found by the clone insert sequence analyses; nevertheless, sequence analysis of the clone Ga55 insert revealed that it includes an alternative 36 bp exon and represents previously unknown *TACC1* splice variant, designated as TACC1-F.

The vector system exploited in the conventional SEREX protocol ensures that the recombinant proteins are expressed in soluble form as C-terminal fusions to β-galactosidase. Analyses of the insert sequences revealed that eight from the 14 antigens (i.e. 57%) are translated in correct reading frame in relation to β-galactosidase while others are translated as frame-3 peptides or represent 5'UTRs of the genes (Table 5). With regard to the latter, it is possible that the natural proteins are translated from internal ribosome translation sites (IRES) in *E. coli*, however, it can not be excluded that antibodies recognise the out-of-frame peptides synthesised as fusion proteins with β-galactosidase, which either represent mimotopes or tumour-specific antigens derived from cancer-associated frame-shifting mutations. However, all of the identified antigens were subjected to further preliminary characterisation in order to select most attractive tumour antigen candidates that could potentially represent novel immunotherapy targets.

SEREX antigens fall into different human tumour antigen categories. It has been generally assumed that a cancer-associated expression pattern – i.e. expression restricted to cancers and (i) germ cells (i.e. CT antigens), (ii) the cell type from which the cancer originated (differentiation antigens), (iii) foetal tissues (oncofoetal antigens) or (iv) neurons (onconeural antigens) as well as protein overexpression in cancer cells may underlie the immunogenicity of SEREX-defined antigens. Besides, variety of molecular alterations including mutations, translocations and splicing defects have been pointed to elicit B cell responses, although only in few cases such alterations have been found and clearly demonstrated to induce B cell responses[6,414,434,435]. Apart from these mechanisms, it could also be possible that other reasons may lead to self protein immunogenicity, for example altered post-translational modifications[332-334], misfolding[436], aberrant subcellular localisation resulting for instance from virus infection[335] or exposure on the surface of apoptotic cells as it was demonstrated for β-actin in medullary breast cancer[181]; however, no experimental data is available to anticipate the prevalence of such mechanisms with regard to SEREX-defined autoantigens.

To gain an insight whether the identified antigens fall into these categories, the identified antigens underwent preliminary characterisation that involved (i) serological analyses to distinguish antigens with cancer-specific sero-reactivity, (ii) analysis for their mRNA tissue distribution to search for differentiation and CT antigens and (iii) comparison of the mRNA expression levels in paired cancerous and normal tissues to identify potential overexpressed antigens.

Table 5. Genes identified by applying the conventional SEREX to gastric cancer

Clone ID	Gene symbol	NCBI Reference Sequence	Cancer-specific serology	SEREX database	Reading frame	Product, characterisation
Genes isolated by autologous screening						
Ga19*	*NARG1*	NM_057175.3	Yes	1 (Glioma)	Correct	NMDA receptor regulated 1. Orthologous to mouse *tbdn-1* acetyltransferase which is involved in regulation of angiogenesis.
Ga27	*GRN*	NM_002087.2	Yes	-	Correct	Granulin. Epithelial cell growth factor. Upregulated in brain tumours and highly tumourigenic cell lines.
Ga28/ Zg3	*CEP290*	NM_025114.3	No	5 (OvCa, PrCa, LuCa, Lymphoma)	Correct	Centrosomal protein 290kDa. Function unknown, 13 putative coiled-coil domains. The presence of antibodies against this protein is associated with several forms of cancer.
Ga34	*EMCN*	NM_016242.2	No	-	Correct	Endomucin. A mucin-like sialoglyco-protein that interferes with the assembly of focal adhesion complexes and inhibits interaction between cells and the extracellular matrix.
Ga44	*RARS*	NM_002887.3	Yes	-	Frame 3	Arginyl-tRNA synthetase. Belongs to the class-I aminoacyl-tRNA synthetase family.
Ga50*	*PPHLN1*	NM_016488.5	No	-	5'UTR	Periphilin 1. May play a role in epithelial differentiation and contribute to epidermal integrity and barrier formation.
Ga52/ Zg15	*UACA*	NM_001008224.1	No	8 (Glioma, BrCa, CoCa, LuCa, EsoCa)	5'UTR	Uveal autoantigen with coiled-coil domains and ankyrin repeats. A novel candidate for eye muscle autoantigens in thyroid-associated ophthalmopathy.
Ga55*	*TACC1*	NM_001122824.1	Yes	2 (GaCa, PrCa)	Correct	Transforming acidic coiled-coil containing protein 1. Microtubule stabilising protein, multifunctional gene expression regulator during interphase.
Ga71	*FXR1*	NM_005087.2	Yes	-	Frame 3	Fragile X mental retardation-related protein 1. A RNA binding protein. Overexpressed in squamous cell carcinomas. Known autoantigen.
Genes isolated by allogeneic screening						
Zg2	*ZNF609*	NM_015042.1	No	-	Correct	Zinc finger protein 609. Function unknown.
Zg4*	*NUCB2*	NM_005013.2	Yes	-	Correct	Ca^{2+} binding protein. Multifunctional protein involved in calcium homeostasis as well as TNF signalling regulation, and appetite regulation in brain.
Zg5	*FAM63B*	NM_001040450.1	No	1 (CoCa)	ND	Family with sequence similarity 63, member B. Function unknown.
Zg10	*SWAP-70*	NM_015055.2	No	16 (CoCa, BrCa, ReCa, LuCa, Mel, HL)	Frame 3	Switch-associated protein 70. Involved in Ig heavy chain class switching. Expressed predominantly in B lymphocytes.
Zg14*	*RRP7A*	NM_015703.3	Yes	-	Correct	Ribosomal RNA processing 7 homolog A (S. cerevisiae). Function unknown.

* These cDNA sequences have been deposited in the GenBank (accession numbers: AY039238-AY039242 and AF450266);
Abbreviations: Ca – cancer, Br – breast, Ov – ovarian, Co – colon, Ga – gastric, Eso – esophageal, Pr – prostate, Re – renal cell, Lu – lung, Mel – melanoma, HL – Hodgkin's lymphoma, UTR – untranslated region, ND – not determined.

Serological characterisation of the identified antigens

All of the identified antigens were searched for the overlap with records in the SEREX database[13]. This search revealed that six from the 14 identified antigens are represented by one or more clones that have been cloned from various cancers in previous SEREX studies (Table 5); from these, only one gene, *TACC1*, had been so far identified by immunoscreening of a gastric cancer library.

Among the rest of the antigens there are three (i.e. ZNF609, RARS and FXR1) that have been either directly or indirectly linked with humoural responses. Although antibody responses against ZNF609 have yet not been reported, other zinc finger proteins that represent DNA binding protein family are frequently identified in SEREX studies[13]. Similarly, the Arg-tRNA synthetase encoded by *RARS* was identified by SEREX for the first time in addition to other particular aminoacyl-tRNA synthetases, nucleotide sequences of which are already deposited in the SEREX database[13]. Besides, autoantibodies against aminoacyl-tRNA synthetases are found in a subgroup of patients with the idiopathic myopathies, polymyositis and dermatomyositis[437]. Another of the identified genes, *FXR1*, encodes known autoantigen in scleroderma patients[438]. Antibody production against the rest of the antigens (i.e. GRN, EMCN, PPHLN1, NUCB2, and RRP7A) had not been previously demonstrated.

Summarising the data obtained from other original SEREX studies, they have resulted in the identification of different numbers of serologically active proteins in an individual tumour starting from a single antigen by applying SEREX to haematological malignancies[439] and ending up to as many as 40 and 43 different antigens in studies analysing poorly differentiated gastric adenocarcinoma[10] and melanoma[440], respectively, indicating the broad heterogeneity of tumour-induced B cell responses in patients. Overall, these studies have shown that tumour-induced autoantibody responses are detected in nearly all cancer patients; however, only on average 20-25% (ranging from 7-50%) of the detected antibody responses are found to be restricted to cancer patients and are not found in cancer-free individuals.

In order to determine the specificity of the defined autoantibody responses in our study, we applied a plaque assay and assessed the frequency of the humoural responses against the serum-reactive clones in panels of sera from gastric, colon, breast and prostate cancer patients as well as healthy donors. Antibody responses against seven of the 14 antigens (i.e. 50%) showed similar reactivities with sera from cancer patients and healthy donors, while the others revealed to be cancer-specific (Tables 5 and 6). From these, three antigens (GRN, FXR1 and NUCB2) showed gastric cancer-restricted specificity including one (i.e. GRN) that reacted exclusively with the autologous patient's sera. Such antigens with autoantibody responses restricted to cancer patients should be considered as valuable target candidates for inclusion into sero-diagnostic or prognostic test panels.

Table 6. Reactivity of SEREX-defined antigens with sera from healthy donors and cancer patients.

Antigen clone ID	Gene symbol	Healthy donor	Gastric cancer*	Colon cancer	Breast cancer	Prostate cancer
Zg14	*RRP7A*	0/25	1/22	0/23	1/11	0/3
Ga19	*NARG1*	0/28	1/22	1/23	4/11	1/3
Ga71	*FXR1*	0/36	2/54	0/13	0/11	0/3
Zg4	*NUCB2*	0/36	3/56	0/23	0/11	0/3
Ga44	*RARS*	0/39	3/54	0/23	1/11	0/3
Ga27	*GRN*	0/39	1/54	0/23	0/11	0/3
Ga55	*TACC1*	0/39	1/54	0/23	1/11	0/3
Ga50	*PPHLN1*	1/39	3/54	0/23	2/11	0/3
Zg2	*ZNF609*	1/25	2/22	0/23	0/11	0/3
Ga34	*EMCN*	2/28	7/54	2/23	0/11	0/3
Zg10	*SWAP-70*	3/25	4/22	2/23	2/11	0/3
Ga28/Zg3	*CEP290*	4/28	3/22	1/23	3/11	2/3
Zg5	*FAM63B*	9/25	4/22	8/23	2/11	1/3
Ga52/Zg15	*UACA*	27/39	25/54	18/23	5/11	3/3

* Includes the reactivity with sera used for immunoscreening of the cDNA expression library.

Antigen mRNA expression analyses

To search for CT and differentiation antigens (which are clinically the most attractive targets; see section 1.2.3.) among the identified genes, tissue distribution of their mRNA expression levels was assessed in brain, liver, heart, trachea, lung, kidney, spleen, colon, stomach, testis, ovary and peripheral blood lymphocytes. None of the genes revealed to encode for a novel CT antigen. Three of the genes (*NUCB2*, *UACA* and *NARG1*) showed differential mRNA distribution pattern, however, none of them encoded for a differentiation antigen. Interestingly, the TAAC1-F transcript variant encoded by clone Ga55 was strongly expressed in brain and gastric cancer tissues, but not in normal stomach and other normal tissues analysed while the transcript variant lacking the alternative exon was detected universally in all tissues tested (Figure 1 in the original paper **I**) indicating that alternative splicing regulation may be altered in tumours. The other genes showed ubiquitous mRNA expression levels.

Antigens overexpressed in tumours are frequently identified in SEREX studies and this is considered to be one of the most frequent reasons for non-mutated self antigen immunogenicity, therefore we compared differences in the antigen mRNA expression levels in paired gastric tumour and adjacent relatively normal tissue specimens derived from 20 gastric cancer patients by semi-quantitative RT-PCR analysis. The mRNA levels of target genes were normalised to those of *GAPDH* and *ACTB*. These analyses revealed that amongst the identified genes, in three cases mRNA levels were upregulated in the analysed gastric tumour specimens in comparison to matched normal tissues – 2.0-6.5 fold increase in mRNA level of *NARG1* in three out of 20, 2.3-7.4 fold increase of *UACA* in three of 20 (see Figure 2 in the original paper **I**), and 2.3 and 2.9 fold increase of *GRN* in two of nine gastric cancer specimens were observed. No correlation was observed when the relative overexpression was compared to the serological data of the corresponding patients, indicating that the overexpression most likely did not serve as a trigger for autoantibody production against these proteins. Surprisingly, mRNA levels of *NUCB2* revealed to be 2-11 fold lower in 10 of 20 gastric cancer specimens indicating this protein as putative tumour suppressor. Noteworthy, the expression of the alternatively spliced *TACC1* transcript variant F was detected in 3 of 20 cancer tissue specimens, but not in the adjacent tissues. In the remaining cases, mRNA levels of these genes were similar in cancer and non-cancerous tissues

3.1.2. MOLECULAR CHARACTERISATION OF NUCB2 AND TACC1

Basing on the data obtained from antigen characterisation, two of the TAAs identified in the conventional SEREX study – NUCB2 and TACC1 – were selected as most attractive tumour antigen candidates and were subjected to more detailed molecular characterisation in order to assess possible reasons for their immunogenicity.

NUCB2 is an antigen with tumour-specific posttranslational modification (Ref. II)

NUCB2 is an EF-hand Ca2+ binding protein that is implicated in various physiological processes like calcium homeostasis, hypothalamic regulation of feeding and TNF receptor shedding[441-443]. In our study we identified NUCB2 as gastric cancer antigen and collective sequence and mRNA expression analyses revealed that it does not belong to any of the major human tumour antigen categories (Table 2); however, antibody responses against NUCB2 were restricted exclusively to gastric cancer patients (in 3 out of 56, Table 6). In order to assess the molecular mechanisms underlying NUCB2 immunogenicity in gastric cancer patients, the antigen was further in-depth analysed.

In support to our previous results, a 2-95 fold downregulation of *NUCB2* mRNA in 28 out of 36 stomach tumour specimens when comparing to adjacent normal tissues was demonstrated by quantitative RT-PCR (Figure 2, B in the original paper **II**); in 16 of the tissue pairs this was confirmed also at protein level by Western blot (Figure 4, A in **II**). Apart form downregulation, Western blot analyses revealed that NUCB2 is posttranslationally modified in gastric mucosa and cancerous tissues (Figure 15), and that the PTMs include phosphorylation as well as yet unidentified PTM (other than O- or N-glycosylation), which was detected in nine out of 16 gastric tumour tissue specimens including that derived from the patient whose serum was used for immunoscreening, but not in the matched normal stomach tissues. The PTM of NUCB2 was specific for malignant cells since it was demonstrated in AGS gastric cancer cell line. Although no conclusive evidence was gained to prove the association of the PTM with the immunogenicity of NUCB2, such mechanism could account for the generation of humoural immune responses against a significant proportion of tumour-derived

proteins – PTM aberrations in cancer tissues are common event[286]. Although SEREX does not support direct identification of proteins carrying aberrant PTMs (since they are expressed in bacterial system), they may lead to conformational changes of a protein resulting in altered display of protein regions usually hidden from B cells, which thus might represent novel B cell epitopes.

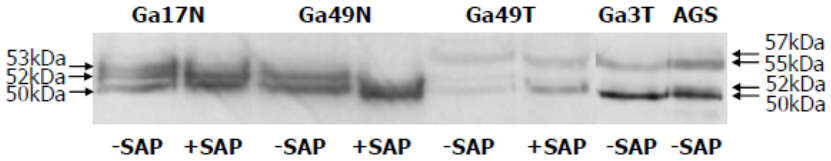


Figure 15. Western blot analysis of NUCB2 posttranslational modifications. Western blot analysis of normal stomach mucosa and gastric cancer specimens revealed that in addition to the 50 kDa band, which represents mature NUCB2 protein, there are two to four closely spaced bands that migrate in the range between 50 and 57 kDa. The treatment of protein extracts with shrimp alkaline phosphatase (SAP) revealed that 53 and 52 kDa bands in normal tissues and 52 and 57 kDa bands in tumour tissue represented phosphorylated forms of NUCB2. The 55 and 57 kDa bands were present only in the tumour tissues, including Ga3T – tumour tissue from the patient with anti-NUCB2 autoantibodies, and AGS gastric cancer cells.

Splice variant antigen TACC1 (Ref. III)

Another serum-reactive protein identified by immunoscreening of the gastric cDNA expression library is TACC1 – a centrosome and microtubule-associated protein, which is essential for mitotic spindle function[444]. Sequence analysis of the clone Ga55 insert revealed that it includes an alternative 36bp exon (i.e. exon 4a, Figure 16, A) and represents previously unknown TACC1 splice variant, designated as TACC1-F (previously published TACC1 transcript variant was designated as TACC1-A[444]). Besides, antibody responses against Ga55 were detected exclusively in cancer patients (Table 6).

In order to analyse the transcript variant distribution, RT-PCR analyses were applied to various normal (i.e. brain, liver, heart, kidney, lung, trachea, spleen, colon, stomach, testis, ovary, PBLs) and paired tumour/normal stomach tissues. Results showed that the isoforms are expressed differentially – TACC1-A transcripts were ubiquitous (although in different levels), but TACC1-F expression at significant levels were detected only in brain (Figure 2 in the original paper **III**). While significant differences in TACC1-A mRNA expression between cancerous and adjacent normal tissues were not observed, TACC1-F transcripts were abundant in tumours (in six out of 10 specimens) and almost undetectable (Figure 16, B) in all normal stomach tissues indicating that regulation of TACC1 mRNA splicing is altered in gastric tumours. Importantly, four more transcript variants of TACC1 gene were identified by RLM-RACE analyses in this study, including TACC1-D, another splice variant that contains exon 4a, expression of which was detected exclusively in gastric cancer, but not any of the normal tissues analysed (Figure 2 in the original paper **III**).

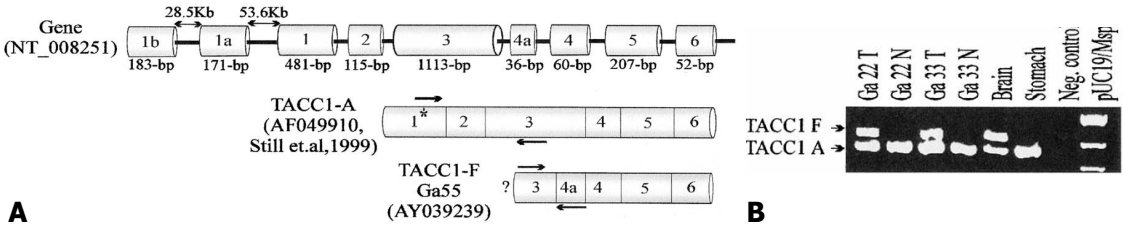


Figure 16. A | Schematic representation of exon composition in the 5' region of gene *TACC1* and two of the transcript variants. The SEREX-defined clone Ga55 encodes a novel splice variant of *TACC1* (i.e. TACC1-F), which encompasses alternative 36 bp exon 4a that disrupts a nuclear localisation signal. **B |** Co-amplification of TACC1 transcript variants A and F (primers for expression analysis are indicated by arrows in **A**).

It has been shown that TACC1 overexpression in drosophila and HeLa cells leads to protein aggregate formation[445,446]. We proposed that inappropriate expression of TACC1 isoforms in gastric cancer cells may lead to dysfunction of TACC1 and possibly the formation of protein aggregates, which might have served as a trigger for autoantibody production. Alternatively, TACC1-F might represent novel onconeural antigen. Cancer-restricted expression of TACC1 isoforms F and D indicates that these isoforms may represent genetic marker for gastric cancer.

As demonstrated by this study, the conventional SEREX approach serves as valuable tool for novel TAA identification. Taken together, the SEREX analyses of gastric cancer resulted in the identification of 14 serum-reactive clones, eight from which encoded for novel TAAs. Against seven of the identified antigen clones autoantibody responses were detected exclusively in cancer patients' sera thus suggesting their relevance as sero-diagnostic markers. Amongst the identified antigens, no viral, mutated, CT, differentiation or overexpressed antigens were found; however, detailed characterisation of two of the TAAs revealed that TACC1-F is encoded by a novel splice variant of *TACC1* and might belong to onconeural antigen category, and that NUCB2 might have elicited humoural responses due to cancer-specific posttranslational modifications.
The potential of this approach has been demonstrated by other studies, for example, the application of SEREX approach to oesophageal cancer has lead to the identification of a novel CT antigen NY-ESO-1, which is amongst the most immunogenic human tumour antigens identified to date[284] – not only antibody responses, but also integrated CD4+ and CD8+ T cell responses have been demonstrated against this antigen in various tumour types[6,219,447], and this antigen has been involved in multiple cancer immunotherapy trials[285]. It has to be stressed that high-titre IgG switched antibody targets are searched by SEREX, which generally can not be generated without cognate T cell help – thus, the SEREX antigen repertoire is considered to represent CD4+ T cell antigen repertoire as well.
However, like every approach, SEREX technology has its own inherent biases, advantages and disadvantages[433]. With regard to biases in antigen repertoire, recombinant proteins are expressed within a bacterial system, which does not support the expression of posttranslationally modified protein isoforms found in humans. Thus, significant proportion of tumour antigens that are represented, for example, by cell surface glycans will be missed as well as point to the fact that SEREX approach is limited in its ability to identify antigens that could serve as targets for antibody-based treatments. Nevertheless, the release of periplasmic proteins that are involved in protein folding during phage-induced lysis of bacteria allows at least partial folding of recombinant proteins thus enabling the identification of conformational epitopes apart from linear epitopes[433,448]. The attempt to overcome this limitation was made by the development of RAYS approach, or recombinant antigen expression on yeast surface – the use of this eukaryotic expression system is aimed to enable more natural protein folding and posttranslational modifications[15]. Next, although SEREX allows searching for tumour-related IgG targets systemically, it is biased towards the identification of high abundance messages and thus low abundance mRNAs encoding relevant antigens might be missed. Besides, only those gene products will be selected that are actively transcribed in the tumour tissue at the time of surgery. In order to enrich the transcripts encoding for potential B cell targets, scientists have used testis-derived mRNA for library construction since testis has a highly representative transcriptome due to the extensive DNA hypomethylation[413,414]. Complementarily, such an approach enables specific search for CT antigens recognised by cancer patient sera.
Although the conventional SEREX approach is less technically demanding than T cell epitope cloning, it has been proven to be extremely time and labour-consuming method. Multiple technical peculiarities characterise the approach: (i) there is a need to deplete for serum antibodies that react with antigens expressed by *E. coli* and bacteriophage prior to immunoscreening in order to avoid troublesome background signals; (ii) a pre-screening step with anti-IgG secondary antibody alone is necessary to exclude false positive signals generated by cloned IgG transcripts, since tumour tissues are often infiltrated by plasma cells, which are the source of IgG transcripts in libraries; the screening of tumour cell lines rather than tumour tissue have been used to circumvent this problem (this option also exclude the use of mRNAs derived from normal stromal cells that surround tumours)[402-404]; (iii) tens of rounds of immunoscreening have to be performed in order to isolate representative set of serum-reactive clones (usually 0-1 positive signals are found per 8×10^3 phage clones), and often limited availability of cancer patient sera makes this procedure particularly time-consuming. Apart form above listed, the conventional SEREX approach is not suitable for simultaneous monitoring of

autoantibody profiles in multiple cancer patients, which is dictated by the used cloning system – i.e. phage clones can not be used directly for the generation of antigen microarrays.

3.2. EVALUATION OF λ AND T7 PHAGE DISPLAY SYSTEMS FOR THE ASSESSMENT OF AUTOANTIBODY RESPONSES (REF. IV)

In order to make the SEREX technique more time and labour-effective, several laboratories worldwide have tried to combine phage display with the SEREX approach, where the recombinant cDNAs are expressed as fusion proteins with one of the phage coat proteins. Thus physical link between the displayed protein and the encoding cDNA is generated, making the phage target selectable[16,18-20,416,449,450]. This enables the enrichment of a cDNA expression library for serum-reactive clones prior to immunoscreening, which is achieved via biopanning (Figure 22). Moreover, the recombinant phage particles can be directly printed onto glass slides to produce antigen microarrays that enables the monitoring of antibody responses against hundreds to thousands of antigens simultaneously using only few microliters of serum[426,451-453].

To date, four phage surface display vector systems have been used for the identification of serologically active tumour antigens: (i) filamentous phage M13 surface protein **pVI-based display vector system** has been applied to search for serum-reactive protein identification in breast cancer[449] (the capsid of M13 phage is composed of major coat protein pVIII (~2700 copies) and minor proteins pIII, pVI, VII and IX, each represented in 5 copies[454]); (ii) **The pJuFo vector system** represents another M13 phage-based vector system, and it has been used for the interrogation of humoural responses in prostate cancer patients[16]; in this system, the protein surface display is enabled by strong interaction between two leucine zipper polypeptides Jun and Fos – Jun domain is expressed as a fusion protein with pIII whereas the leucine zipper domain of Fos is co-expressed as an N-terminal fusion peptide to a recombinant protein[455]; (iii) **λKM vector series** – Minenkova and colleagues have applied the λ phage display-based SEREX to define tumour antigens in breast cancer – the cDNA expression libraries were constructed to allow recombinant protein fusion to either C- or N-terminus of the head protein D (gpD) by exploiting either λKM4[19] and λKM8 or λKM10 vectors[20], respectively, and (iv) **T7Select 10-3b vector system** that has been designed to display recombinant proteins as fusion proteins with 10B capsid protein; to date, this system has been applied to various types of cancer[18,416,450].

In the current study, we choose to analyse surface display systems that are based on lytic phages T7 and λ, and this was done for several reasons. First, the assembly of M13 phage takes place in the periplasm of *E. coli* and the repertoire of displayed fusion proteins thus is strongly biased to those, which can be exported through the bacterial inner membrane and still retain their correct folding in the oxidising environment of the periplasmic space. In contrast, capsids of lytic phages, such as λ and T7 phages, are assembled in the cytoplasm therefore the repertoires of cDNA libraries do not have the biological constraints similar to M13 phage. Apart from this, the choice of the selected display vector systems was dictated by the availability of the vector DNAs.

The T7Select 10-3b vector (purchased from Novagen) and λKM8 vector (kindly provided by Dr. Olga Minenkova from Kenton Labs) were chosen for the study. In λKM8 vector, cDNAs are expressed as N-terminal fusions of the phage capsid protein gpD, from which they are separated by a flexible GS linker (composed of tandem Gly-Ser repeats) to facilitate λ head assembly while in T7 phage they are fused to the C-terminus of the coat protein 10B (Figure 18). Thus, one of the major differences between these vector systems is that cloned cDNA fragments containing stop codons can not be displayed on the surface of λ phage while can be on T7.

Initially, these vector systems were evaluated for their ability to display a range of known, clinically relevant tumour antigens by cloning the members of 15 TAA families into the selected display vectors. Then, they were compared with regard to their suitability for the generation of phage-displayed antigen microarray and monitoring of autoantibody responses by using the microarray. These analyses were done in order to choose the most suitable phage display expression system for further study, which was aimed for the identification of a comprehensive list of serum-reactive proteins in melanoma, prostate cancer and gastric cancer.

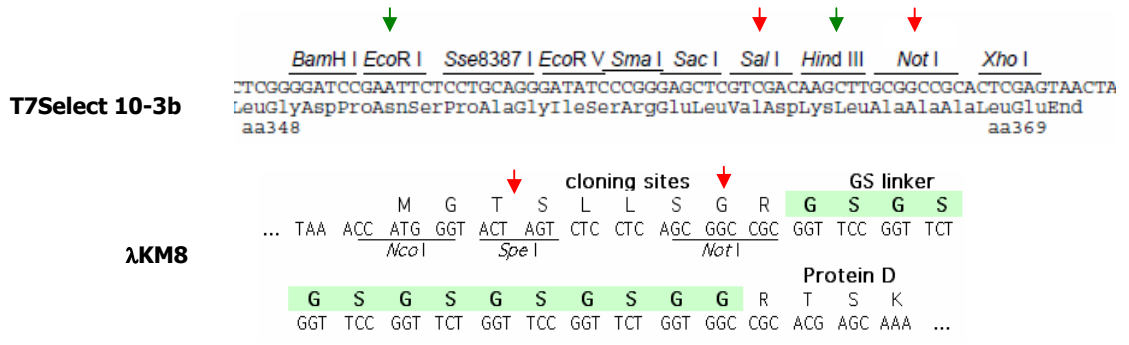


Figure 18. The cloning sites of the phage surface display vectors used in the current study. In the T7Select 10-3b vector system cDNAs are expressed as C-terminal fusions of the coat protein 10B while in the λKM8 vector system – as N-terminal fusions of the surface protein gpD, from which they are separated with flexible GS linker. Red arrows designate the cloning sites used for TAA mini-library construction while green arrows – those for testis and tumour-derived cDNA expression library construction (see the following sections 3.3. and 3.4.).

3.2.1. TUMOUR-ASSOCIATED ANTIGEN DISPLAY ON λ AND T7 PHAGE SURFACES

Construction and immunoscreening of TAA mini-libraries

In order to compare the T7Select 10-3b and λKM8 phage display vector systems for their ability to display a range of clinically relevant tumour antigens, 12 cancer-testis (CT) antigen family members and three other tumour antigens (Table 7) were amplified by RT-PCR and directionally cloned into the vectors. For genes with long ORFs the potential linear B cell epitopes were predicted and chosen for cloning using an algorithm developed by Welling *et al*[432] while for shorter transcripts complete ORFs were amplified. In the case of tyrosinase, the immunodominant region that has been shown to react with sera from melanoma patients, but not with sera from healthy donors[456] was chosen. For the cloning of multiple genes from antigen families, such as MAGEA and SSX, degenerated primers were designed and used for the amplification.

Cloning of the respective RT-PCR products into λKM8 and T7Select 10-3b vectors resulted in TAA mini-libraries in size of 1-5 $\times 10^5$ pfu and 0.5-5$\times 10^4$ pfu, respectively. Five random clones from each mini-library were selected and sequenced in order to assess the repertoire of the cloned antigens (shown in Table 7), as well as to verify the insertion of cDNAs in the correct reading frame relatively to the phage coat proteins.

After one round of amplification, both of the TAA mini-library sets were immunoscreened with sera from 21 melanoma patient in order to select serum-reactive clones and to compare the serum-reactive antigen repertoires. From the 21 serum specimens eight were collected from melanoma patients in a time period of 2-6 months after the Mel-TYR vaccination trial (see Appendix 4 for listing). Approximately 2 $\times 10^3$ pfu from each of the λ and T7 phage-displayed TAA mini-libraries were used for immunoscreening. One to 10 serum-reactive phage clones were purified from each TAA mini-library and identified by sequencing their cDNA inserts. In total, 224 and 103 serum-reactive clones, which represented 36 and 18 individual antigens, were isolated from T7 and λ-displayed TAA mini-libraries, respectively. Eighteen out of the 21 melanoma patients' sera recognised at least one of the TAAs cloned.

Table 7. Antigens comprising TAA mini-libraries.

Antigen family	Number of mRNAs*	Regions cloned (nt positions; NCBI RefSeq No)	Different genes found by sequencing five random clones	
			T7Select 10-3b	λKM8
CTAG	2	86-400, 338-628; NM_001327	CTAG1B, CTAG2	CTAG1B, CTAG2
MAGEA	10	209-618, 590-1137; NM_005362	MAGEA1, 2, 3, 4, 9	MAGEA1, 3, 4, 6
SSX	10	58-401, 58-617; NM_005636	SSX2	SSX1, 2
BAGE	1	195-330; NM_001187	BAGE	BAGE
GAGE	8	84-410; NM_001472	GAGE2, 7, 8	GAGE1, 8
MAGEB	4	98-516; NM_002364	MAGEB2,4	MAGEB2
MAGEC	2	931-1453; NM_016249	MAGEC1, 2	MAGEC1, 2
SPANX	6	57-347; NM_022661	SPANXA2, SPANXE	SPANXD
LDHC	1	245-785; NM_017448	LDHC	LDHC
CT45	4	246-798; NM_001017417	CT45-1	CT45-1
THEG	1	778-1176; NM_016585	THEG	THEG
CTAGE	6	130-1070, 311-1070, 1052-1616; 1589-2354; NM_203354; 11-235; NM_022663	CTAGE1, 3, 5, 5Δex7	CTAGE1, 5
MTA1	1	1394-1772; NM_004689	MTA1	MTA1
TYR	1	494-710; NM_000372	TYR	TYR
MLANA	1	54-408; NM_005511	MLANA	MLANA

* Number of known members of the antigen family that theoretically could be amplified with the selected primers.

Comparison of serum-reactivities against TAAs displayed on λ and T7 phages

The results of the T7 and λ TAA mini-library immunoscreening are summarised in Table 8. Taken together, relatively good concordance in the recognition of antigens displayed on λ and T7 phages was observed – the reactivity against antigens from CTAG, MAGEA, MAGEC, SSX and GAGE families was detected in both expression systems whereas no reactivity against BAGE, MAGEB, SPANX, LDHC, CT45, THEG, MTA1, TYR and MLANA was observed in any of them.

However, regarding the sensitivity of the display systems, T7Select 10-3b showed better performance in terms of both, signal intensities and the number of positive sera reactive with members from CTAG, MAGEA, MAGEC and GAGE antigen families, while both systems showed equal performance in detection of reactivity against and SSX2 (Table 8). For example, eight melanoma patients' sera reacted with CTAG1B and seven with CTAG2 when these antigens were displayed on T7 phage, while only four of them reacted with CTAG1B and three with CTAG2 when expressed by λKM8. In immunoscreening, positive signal intensities for the serum specimens that did not recognise the corresponding λ phage displayed antigens were low (not shown), thus indicating that these discrepancies might have been raised due to less effective display of these antigens by λ phage in comparison to that on T7 page.

Only exception of the observed differences in sensitivity between both system was CTAGE5 – antibody responses against this antigen were detected when its N-terminal fragment was displayed on the surface of λ phage (recognised by two sera), but not T7 phage (Table 8). At the same time, four sera reacted with T7 phage clones expressing 56 aa polypeptide (sharing six amino acids with the λ-displayed protein) derived from CTAGE pseudogene that was followed by a stop codon. CTAGE5 N-terminal fragment was amongst the longest recombinant peptides cloned, and it has been suggested that recombinant protein display density on the T7 phage is variable and depends on the size of the hybrid coat protein[457]. This suggested that the detection of serum antibodies against CTAGE5 might have been hampered by very low display density of the recombinant protein on T7 phage (noteworthy, the display density of CTAGE5 N-terminal fragment on T7 phage was demonstrated to be the lowest amongst the cloned antigens, for which the display density was assessed by Western blot analyses, Figure 20).

Table 8. Antigens identified by immunoscreening of λ and T7 phage-displayed TAA mini-libraries and individual sera recognizing the respective antigens.

TAA mini-library	Antigen	Reactive sera*	
		T7 Select 10-3B	**λKM8**
CTAG	CTAG1B (NY-ESO-1)	MA001643, MA000703, MA000161, MA000445, MA00SK, MA00AM, MA00550, MA000513	MA001643, MA000703, MA000161, MA000445
	CTAG2 (LAGE-1)	MA001643, MA000703, MA000161, MA000445, MA00SK, MA00AM, MA00550	MA001643, MA000703, MA000445
	CTAG1B-ORF2	UKRV-Mel31 MA000703	- -
MAGEA	MAGEA1	MA000513	MA000513
	MAGEA2	MA000513	MA000513
	MAGEA3	MA000513	MA000513
	MAGEA12	MA000513	-
	MAGEA2 antisense	MA00WF	-
MAGEC	MAGEC1	MA000703	MA000703
	MAGEC2	MA000703	-
SSX	SSX2	MA000951	MA000951
CTAGE	CTAGE5		MA000273, MA000525
	CTAGE pseudogene	MA000525, MA000273, MA001816, MA001111	-
	CTAGE1 antisense	MA001404	-
GAGE	GAGE3-7 subgroup	MA000445, MA001111, MA001404	MA000445
	GAGE1, 8	MA000445	MA000445
	GAGE2-ORF2	MA00GG	-
	GAGE7-ORF2	MA001643	-

* Sera are ranked by the signal intensities in immunoscreening.

Further, from the melanoma sera analysed, five reacted with T7 phage clones expressing CTAG1B, GAGE2 and GAGE7 ORF-2 peptides as well as MAGEA2 and CTAGE1 antisense peptides (Table 8). All these cDNAs contain stop codons when translated as fusion proteins with the coat protein 10B in the T7Select system, and such peptides per definition can not be displayed by using λKM8 vector system. Amongst the antigens identified by T7 mini-library immunoscreening, there were multiple different CTAG1B (commonly known as NY-ESO-1) variants, which were encoded by cDNA inserts containing various frame-shifting and in-frame deletions (alignment can be seen Figure 19, C). At lesser extent, the same was observed for the closely related CT antigen CTAG2 (i.e. LAGE-1). These variants most likely were generated due to RT-PCR errors, since the cDNAs encoding N and C-terminal fragments of CTAG antigens contained multiple self-complementary regions that flanked the borders of the deleted regions. It is worth of notion that CTAG1B-ORF2 and CTAG2-ORF2 (known as CAMEL) peptides are well known CD4+ and CD8+ T cell antigens, whose immunogenicity is supposed to be associated with the translation of the genes in alternative ORF in cancer (including melanoma) cells[308,458]. However, humoural responses specifically recognising such peptides have not been demonstrated to date[13,294].

Therefore, in order to clarify whether melanoma patient-derived autoantibodies react with the common CTAG1B peptide or specifically recognise out-of-frame peptides, the CTAG1B phage clone variants were tested with the 21 melanoma patients' sera by plaque assay. Results showed that serum antibodies are capable of distinguishing between CTAG1B and CTAG1B-ORF2 peptides. In the example of MA001643 serum, autoantibodies seem to recognise the common peptide of the antigen N-terminal fragment with the single exception there being clones CT35 and CT177 (positions 2B and 2C in Figure 19, B), which encode only for first ten amino acids of CTAG1B followed by frame 2 peptide (for alignment see Figure 19, C). However, the clone's CT31-expressed peptide, first 16 amino acids of which corresponds to CTAG1B protein is recognised by the serum antibodies allowing to suggest that the B cell epitope is localised within the region of the first 16 amino acids. Another example is the UKRV-Mel31 serum, which exclusively recognises one of CTAG1B-ORF2 peptide

variants represented by clones CT31 and CT35, but not other CTAG1B clones. To our knowledge, these results for the first time demonstrate that antibody responses may have been generated specifically against ORF2 peptide of CTAG1B.

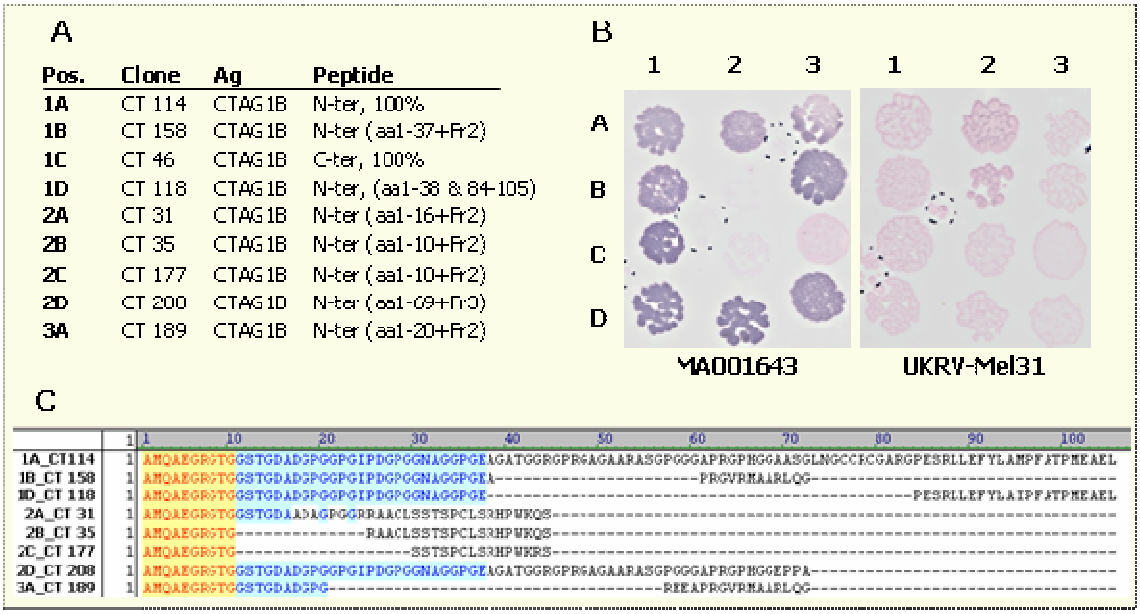
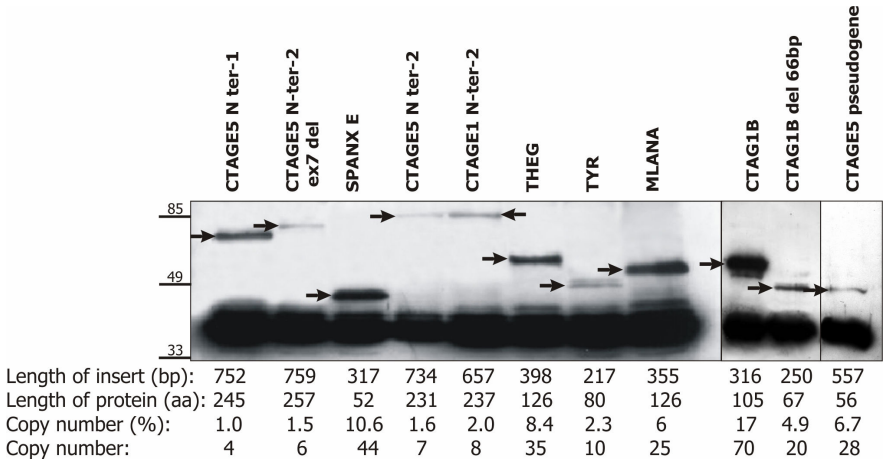


Figure 19. Differential serum reactivities against CT antigen CTAG1B and CTAG2 variants displayed on T7 phage. A | A partial list of serum-reactive CTAG clones used for the macro-array analysis. **B** | The serum reactivities against the CTAG clones (only the corresponding fragment of the macro-array is shown); dotted rounds mark T7 phage without an insert. **C** | Alignment of the selected CTAG1B N-terminal peptide variants displayed on T7 phage.

Recombinant antigen display density on T7 and λ phages

The display density of recombinant proteins and its regulation is different in these two vector systems. The capsid shell of T7 phage is composed of 415 copies of surface protein encoded by the gene *10*. The capsid normally contains two forms of protein, 10A (344 aa) and 10B (397 aa). Although 10B normally makes up ~10%, functional capsids may be composed entirely of either of the two forms. In the T7Select 10-3b system, the expression of 10B-recombinant protein hybrids is ensured by the phage genome, which has been shown to result in a display of 5-15 copies of recombinant protein per phage (that makes 1.2-3.6% of the total surface protein content), while the rest of the wild-type 10A capsid protein is provided by a IPTG-inducible plasmid in the complementing host BLT5615[450]. The capsid of λ phage contains 415 copies of gpE and 405-420 copies of gpD, to which N-terminus recombinant proteins are fused – the latter form protruding trimeric structures that are essential for the capsid stability[459]. In the λKM8 system, wild-type copies of protein D are provided by a copy of the respective gene in the genome of the phage and the capsid was shown to be composed of ~50% recombinant proteins at least in the case of scFv antibody display[460] indicating that the gpD-based λ display vector system has inherently higher display capacity in comparison to the T7Select 10-3b system.

Unfortunately, it was not possible to compare the display density for the antigens of interest between the two vector systems directly due to unavailability of an antibody recognising gpD protein. Nevertheless, we tried to determine the display density range for the recombinant TAAs of different size per chimeric T7 phage capsid. Serum-negative antigens were included in the analyses as well in order to verify that the lack of reactivity against the TAAs is not due to the failure to display the respective antigens. Selected T7 phage clones encoding 11 different antigens (listing can be seen in Figure 20) were amplified and phage particles were precipitated from bacterial lysates. Purified phages were subjected to Western blot analysis and probed with an antibody generated against the N-terminus of 10A/B, which thus recognises both the fusion protein and the non-recombinant coat protein.



Length of insert (bp):	752	759	317	734	657	398	217	355	316	250	557
Length of protein (aa):	245	257	52	231	237	126	80	126	105	67	56
Copy number (%):	1.0	1.5	10.6	1.6	2.0	8.4	2.3	6	17	4.9	6.7
Copy number:	4	6	44	7	8	35	10	25	70	20	28

Figure 20. Copy number of TAA fusion proteins per T7 phage particle determined by Western blot using antibody against N-terminus of the coat protein 10A/10B. The lower band represents wild-type protein 10A, arrows indicate the fusion proteins of the respective sizes. Quantification of wild-type/fusion protein ratio was done by construction of a standard curve from a five 3-fold dilutions of one phage on separate gels (not shown) and the intensities of bands were calculated using GelWorks software.

Results are represented in Figure 20, which shows that (i) all the analysed phage clones express fusion proteins of corresponding lengths thus indicating that the lack of serum-reactivities against at least for the four antigens (i.e. SPANXE, THEG, TYR and MLANA) in immunoscreening represent true negative calls, (ii) the display density of the recombinant proteins per phage vary markedly and is the highest in phages expressing N-terminus of CTAG1B (~17% that represent ~70 copies per phage) and lowest in phages expressing N-terminus of CTAGE5 (~1% representing ~4 copies per phage) clearly showing that, although the display density of T7 phage indeed is variable, it does not correlate directly with the size of the displayed proteins and thus might be dependent upon other recombinant antigen-related factors, which most likely include biochemical properties, hydrophilicity and hydrophobicity as well as folding characteristics.

To our knowledge, so far there is one study addressing the display capacity comparison of λ and T7 phages. In a model experiment, two cDNAs of different lengths were cloned into a gpD-based λ display vector and the T7Select 10-3b vector and tested in a plaque assay, in which λ display system performed with much higher sensitivity. Authors showed by Western blot analysis that the display density of the λ phage is high (representing ~90% gpD copies) irrespective of the protein size while T7 phage display capacity was generally low and dependent upon recombinant protein size[457]. These observations are in line with our data regarding serum-reactivity against CTAGE5, but conflict with regard to the rest of the serum-positive antigens, which showed higher sensitivity when displayed by T7 phage. Thus it was reasoned that the lower sensitivity demonstrated by λKM8 display system in our study could be explained by the recombinant protein location in the N-terminus of gpD, which could hamper the spatial accessibility of the antigen by serum antibodies (in contrast to those expressed in the C-terminus of 10B on the T7 phage). In fact, the analysis of the gpD crystal structure demonstrated that the N-termini up to Ser 15 are disordered and are located near the three-fold axis of gpD trimer on the side that binds to the capsid surface and hence at least partially may be hidden under the gpD trimer[459].

3.2.2. EVALUATION OF THE T7 AND λ PHAGE DISPLY SYSTEM SUITABILITY FOR THE PRODUCTION OF ANTIGEN MICROARRAYS

The use of phage surface display-based expression systems, in contrast to that exploited in the conventional SEREX, enables the direct production of recombinant phage microarrays that could be further used for high-throughput survey of autoantibody signatures in cancer patients. At the time point, when the study was started, several attempts to generate and use the T7 phage-based microarrays for the TAA discovery and/or the assessment of autoantibody responses in cancer patients had been made[425,453], but the suitability of λ phage for this purpose has not been assessed.

In order to compare the suitability of the T7Select 10-3b ad λKM8 display systems for the production of antigen microarrays, nine T7 and λ phage serum-reactive clones encoding the same TAAs and four non-recombinant phages were amplified and spotted in five replicates onto nitrocellulose-coated glass slides. The microarrays were tested with the seven melanoma serum samples (see Table 9 for listing), which were used for the immunoscreening of the TAA mini-libraries.

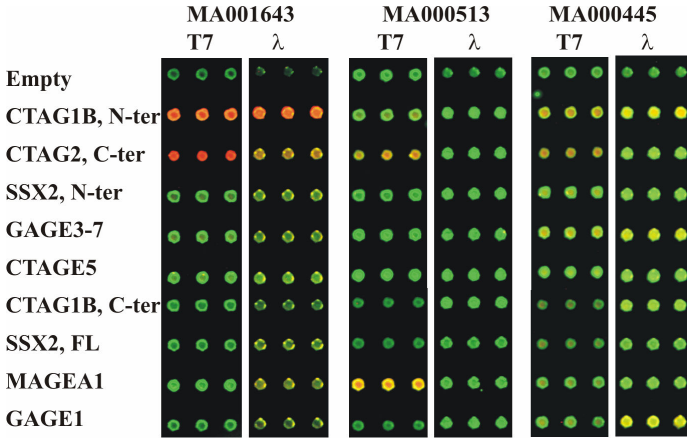


Figure 21. Comparison of serum reactivities against the T7 and λ phage-displayed antigens on microarrays. High-titre phage clone stocks were directly spotted on FAST slides in quintuplicate, probed with preabsorbed sera from seven melanoma patients (detected with Cy5-labelled secondary antibody). In order to quantify the amount of phages in each spot, microarrays were afterwards probed with monoclonal antibodies against tail protein of T7 or λ phage (detected with Cy3-labelled secondary antibody). Only partial array images are shown.

The variation between replicates was less than 10% for both display systems. Similarly to immunoscreening data, the T7Select system showed higher sensitivity for the detection of autoantibodies against CTAG1B and MAGEA1 while both systems detected anti-SSX2 and GAGE3-7 antibodies with comparable signal-to-noise ratios, but anti-CTAGE5 antibodies were detected only in λKM8 system (yet, only in one out of the two CTAGE-positive serum samples) (Table 9). In the majority of cases the antigens, which were found to be serum-positive by the immunoscreening of TAA mini-libraries, were defined as positive using the selected cut-off value (>3 SDs above the mean Cy5/Cy3 ratios of 20 non-recombinant phage spots) in microarray screening.

Nevertheless, some inconsistencies when comparing microarray data to plaque immunoscreening were found as well. Although T7 phage clones expressing GAGE1 and C-terminal fragment of CTAG1B were identified by plaque immunoscreening using MA000445 and MA001643 sera, respectively, the signal intensities for these clones probed by the respective sera did not reach the defined cut-off value. Similarly, λ phage clones expressing CTAGE5 and C-terminal fragments of CTAG antigens revealed to be sero-negative when probed with the sera that were used to isolate these clones from TAA mini-libraries. Lowering the cut-off value would result in the detection of false positive signals due to the variability in the background signal intensities of serum non-reactive clones. At the same time, MA000951 serum was defined as CTAG1B-positive by microarray screening while no such reactivity

was detected by plaque assay. Hence the microarray screening and plaque immunoscreening have comparable, but not identical sensitivities for the detection of autoantibodies.

Table 9. Detection of autoantibodies against T7 and λ phage-displayed TAAs using microarrays.

Serum	MA001643		MA000513		MA000445		MA000951		MA000161		MA000273		MA000525	
Antigen | Phage	T7	λ	T7	λ	T7	λ	T7	λ	T7	λ	T7	λ	T7	λ
CTAG1B, N-ter	**37.9**	**17.5**	**28.8**	**3.9**	**8.2**	**5.1**	**2.1**	1.4	**39.3**	**2.8**	0.9	1.5	1.9	0.9
CTAG2, C-ter	**58.8**	3.2	**64.4**	1.5	**9.8**	2.1	1.3	1.0	**43.2**	1.6	1.4	1.6	1.4	0.9
SSX2, N-ter	2.3	1.2	2.9	1.0	2.5	1.7	**3.5**	**1.9**	3.5	1.1	1.1	1.6	1.6	0.9
GAGE3-7	2.2	1.2	3.0	1.0	**5.2**	**4.8**	1.5	1.4	3.4	1.4	1.2	1.6	1.4	1.0
CTAGE5	2.0	1.2	2.5	2.1	1.7	1.8	1.2	1.1	3.3	1.4	0.9	**2.5**	1.3	1.2
CTAG1B, C-ter	4.6	**2.7**	3.0	0.9	2.3	2.8	1.6	1.2	1.1	1.6	0.9	1.6	1.5	0.9
SSX2, FL	2.3	1.0	2.8	0.6	2.4	1.2	**4.3**	**2.1**	1.8	0.8	1.0	1.4	1.2	0.9
MAGEA1	2.3	1.2	**119.7**	**2.7**	1.7	1.7	1.6	1.4	1.6	1.3	1.2	1.7	1.4	1.0
GAGE1	2.2	1.3	2.8	1.3	3.0	**5.7**	1.6	1.2	2.9	1.7	0.8	1.5	1.5	1.1

Cy5/Cy3 ratios for each antigen were normalised by that of non-recombinant phages, and the cut-off value for serum-positive clones was set as >3 SDs above the average of all the spots (marked with bold).

In principle, both phage surface display systems revealed to be equally suitable for the generation of recombinant phage microarrays and monitoring of autoantibody responses in cancer patients – it was possible to obtain high-titre phage stocks without any additional purification or concentration steps, and the phage capsids remained sufficiently stable. In most of the cases, the recombinant antigens retained their sero-reactivity after phage amplification thus allowing to suggest that there is no generation of phage revertants that acquire growth advantages over original clones.

Summarising all of the obtained data regarding the comparison of the two phage surface display systems, each revealed to have their own advantages and disadvantages. With regard to the choice of phage display system that could be successfully applicable for further study, it was decided to give the preference to T7Select system, and this was done for several reasons. First, although λKM8 display system has been shown to ensure stable and high fusion protein representation in chimeric capsids irrespective of recombinant protein size[457], this study demonstrated that it is less sensitive for antibody-mediated detection, plausibly due to recombinant protein localisation in the N-terminus of coat protein gpD that makes it less accessible for antibodies. Second, the use of λKM8 system for further study was not feasible due to the lack of commercially available anti-λ tail antibody that is a prerequisite for microarray-based analyses. And finally, T7 phage in comparison to λ has more favourable biological properties – it is chemically stable, very easy to grow and it replicates more rapidly than bacteriophage λ (and M13)[450].

The considerable copy number variation of fusion proteins (i.e. 4-70 copies per phage particle) demonstrated by the T7Select 10-3b system in this study, however, is a disadvantage of this system, and suggests that the signal strength in immunoscreening and microarray analyses may depend not only to antibody titre, but also on the copy number of recombinant protein per phage particle. As well, this confers a risk of missing positive signals due to a very low display of a recombinant protein as it was shown in the case of CTAGE5. A solution of this problem would be to clone cDNAs of limited length to avoid the expression of long fusion proteins, which has been shown by this study and others[457] to have lower representation in chimeric capsids. Apart from that, an appropriate solution to this problem would be the construction of a T7Select-based vector that would allow monitoring the copy number of fusion proteins per phage particle.

One of the advantages of the λKM8 display system is that it allows the expression only of in-frame antigens while the T7Select 10-3b system permits the expression of out-of-frame peptides as well; thus the use of λKM8 would be more appropriate for studies aimed for the identification novel cancer immunotherapy targets. In this study, several TAAs (i.e. MAGEA2, CTAGE1, GAGE2 and 7) expressed in alternative reading frames were identified by the T7Select system. Whether or not these antigens, similarly to CTAG antigens, are translated in alternative ORFs in cancers remains to be determined. In this scenario they may reveal to be novel tumour-specific antigens; however, it is more plausible that they represent mimotopes recognised by cross-reactive antibodies that, however, could potentially represent valid biomarkers.

3.3. ELABORATION OF T7 PHAGE DISPLAY-BASED SEREX APPROACH (REF. IV)

The following part of the study was focussed to the elaboration of an optimal protocol that could be further applied for the identification of a comprehensive set of serum-reactive proteins (i.e. immunome) in melanoma, prostate and gastric cancer, as well as to the characterisation of the antigen repertoire that can be identified using the T7Select phage display system.
Testis-derived RNA was used for the construction of a T7 phage-displayed cDNA expression library (designated T7-T1), which resulted in a size of 8 x 10^6 pfu. Testis was selected due to the characteristics shared between germ cell and neoplastic cell transcriptomes. The selection of serum-reactive clones was based on the enrichment of the library via biopanning by using protein G-coated magnetic beads, which was followed by the library immunoscreening with sera from nine melanoma patients and serum-reactive clone identification.

3.3.1. THE OPTIMISATION OF BIOPANNING PROCEDURE

The surface display of recombinant proteins ensured by the T7Select system enables the selection of serum-reactive clones via biopanning (the principle of biopanning is shown in Figure 22). By using this advantage, antigen selection is quickened up enormously when comparing to the conventional SEREX protocol. Moreover, it also allows to perform a negative selection – for example, depletion of IgG-encoding phage clones prior to immunoscreening or antigens recognised by sera from healthy individuals. However, the biopanning procedure holds multiple technical and principal variables, the uses of which may vary significantly in different references and this prompted us for the elaboration of biopanning procedure that would ensure most efficient enrichment of T7-displayed cDNA expression libraries.
In order to elaborate an optimal biopanning procedure, various conditions and variables were tested and compared; besides, the optimal number of biopanning cycles was assessed through comparing the level of enrichment for serum-reactive clones and complexity of antigen repertoires after one and two rounds of biopanning.

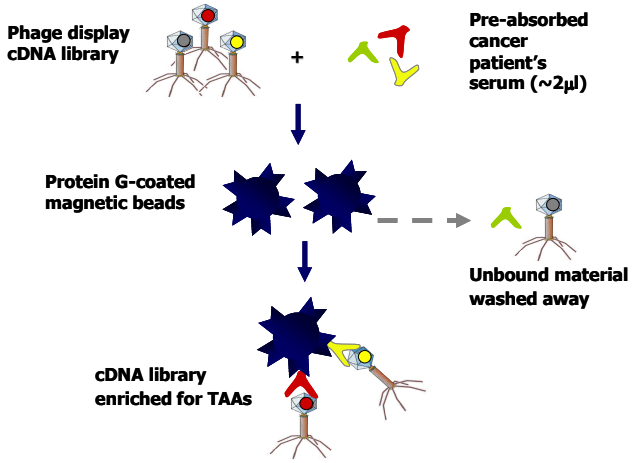


Figure 22. The principle of biopanning. First, a phage display cDNA library is incubated with a pre-absorbed cancer patient's serum. Phage-antibody complexes are then selected by magnetic beads coated with protein G, which binds antibody constant regions. After several rounds of washes, it results in a library enriched for serum-reactive clones that may be directly subjected to the immunoscreening procedure. When enriching tumour-derived libraries, they are pre-incubated with protein G-coated beads to remove IgG-encoding phage clones prior to adding serum (not shown).

Choosing the optimal biopanning conditions

The biopanning procedure optimisation was done on the basis of the MagnaBind™ Protein G magnetic beads and ImmunoPure Binding/Elution buffer system (Pierce). Empirical determination of the optimal biopanning conditions involved testing of multiple variables, and the differences in the efficiency of biopanning were judged from the number of positive clones detected by immunoscreening of 8×10^3 pfu from the obtained biopans:

(i) *serum and phage concentrations* – up to two microliters of serum and 0.5-1 x 10^{11} pfu of a library was determined as optimal for binding to 200 µl of the magnetic beads;

(ii) *conditioning of the beads* – beads blocked in 5 % marvel in TBS, 0.05 % Tween-20 vs beads in ImmunoPure Binding Buffer (BB) were tested; ~3 times higher enrichment with serum-positive clones was obtained, when blocked beads were used for the selection indicating blocking step as crucial for removal of serum non-reactive phage background;

(iii) *incubation buffer composition* – antibody-phage complex formation and the binding efficacy of such complexes to the magnetic beads was compared when using BB vs TBS, 0.05 % marvel (which would be the optimal choice for phage viability); as demonstrated by the recovered phage titres (i.e. 3 x 10^5 and 4.5 x 10^4 from initial 5 x 10^{10} pfu, respectively), incubation in BB revealed to be more suitable for antibody binding to the beads;

(iv) *incubation conditions* – it was estimated that incubation steps of ~2-3 h at RT are sufficient for antibody-antigen and bead-antibody complex formation, however, prolonged incubation periods at 4 ^{o}C overnight were estimated to be slightly more efficient;

(v) *washing conditions* – washing of beads with bound antibody-phage complexes in buffers with different composition (i.e. BB vs BB and TBS, 0.05 % Tween-20) and washing cycle numbers were evaluated; it was estimated that (1) the use of detergent-containing washing buffer contributes to increased serum-positive/non-reactive phage ratios, and (2) 10 cycles of washing are necessary and sufficient to remove non-bound phage background;

(vi) *incubation order* – the efficacy of protein G-serum antibody-phage particle complex formation was compared between two different procedures: (1) preabsorbed serum was incubated with ~5x10^{10} pfu of T7-T1 library that was followed by an incubation period prior to adding protein G beads (as shown in Figure 22), and (2) serum antibodies were first bound to beads and after an incubation period the library was added. The use of these two protocols resulted in the ~5 x 10^5 pfu of biopans in both cases; however, immunoscreening clearly showed that that better enrichment is achieved by using the first approach (serum-positive/negative phage ratios were approximately six times higher);

(vii) *elution* – tittering of enriched libraries after elution by using ImmunoPure IgG Elution buffer resulted in dramatic drop of the recovered phage titre in comparison to non-eluted biopans, which most likely was due to high acidity of the elution buffer (i.e. pH 2.8) affecting the phage viability; alternatively, the beads with bound phage were resuspended in phage extraction buffer and used directly for immunoscreening, which revealed to be suitable alternative to the elution.

At the time point when the study was started, the T7Select vector had been exploited in three studies aimed for the serological tumour antigen identification in breast cancer[18,425] and non-small cell lung cancer[416]. In these and following studies authors have used differential biopanning procedure, in which healthy donor serum-reactive phage clones are first depleted from the libraries that further is followed by the positive selection with cancer patient-derived sera. Nevertheless, we have chosen to avoid such an approach. The rationale for this was based on the overall goal of the study, which was aimed to generate TAA microarray comprising as broad as possible antigen repertoire that could be applicable for the identification of autoantibody biomarkers for early detection of cancer, cancer diagnosis, and response to therapy. Since several studies have shown that autoantibody responses against transformed cell-derived proteins are induced early in tumour progression or even in pre-malignant lesions[7,8], the use of such negative selection could potentially result in loss of important antigen set, against which age matched healthy donors with pre-malignant conditions or undetected early-stage tumours could have been generated humoural responses. Besides, the autoantigens recognised by healthy donor serum-derived antibodies may reveal potential negative predictors of cancer[461].

Choosing the optimal number of biopanning cycles

Commonly, four-to-five biopanning cycles are performed in order to reach significant enrichment of a target of interest from a phage display library[416,425,453]. The enriched library resulting from one biopanning cycle is amplified prior to next one, and this allowed to suspect that due to the variable antibody titres in sera, antibody affinities to antigens and viability of different phage clones such an approach may lead to the overrepresentation of particular antigen-encoding phage clones and underrepresentation of others.

In order to assess the level of redundancy increase and thus select the optimal number of biopanning cycles for further analyses, the repertoires of antigens selected after the first and the second round of biopanning using two different serum samples were assessed and compared. Approximately 5×10^{10} pfu from T7-T1 library were used for biopanning with MA002079 and LGP-Mel 150 sera that resulted in the recovery of ~5×10^5 pfu in both cases; ~8×10^3 pfu from the enriched libraries were subjected to immunoscreening, which resulted in the detection of ~80 and 60 serum-reactive clones, respectively. The remaining phages were once amplified and subjected to the second round of biopanning. Immunoscreening of ~8×10^3 pfu from the second biopan with the respective serum resulted in the detection of >130 positive clones thus clearly showing that higher enrichment with serum-reactive clones is achieved (Figure 23).

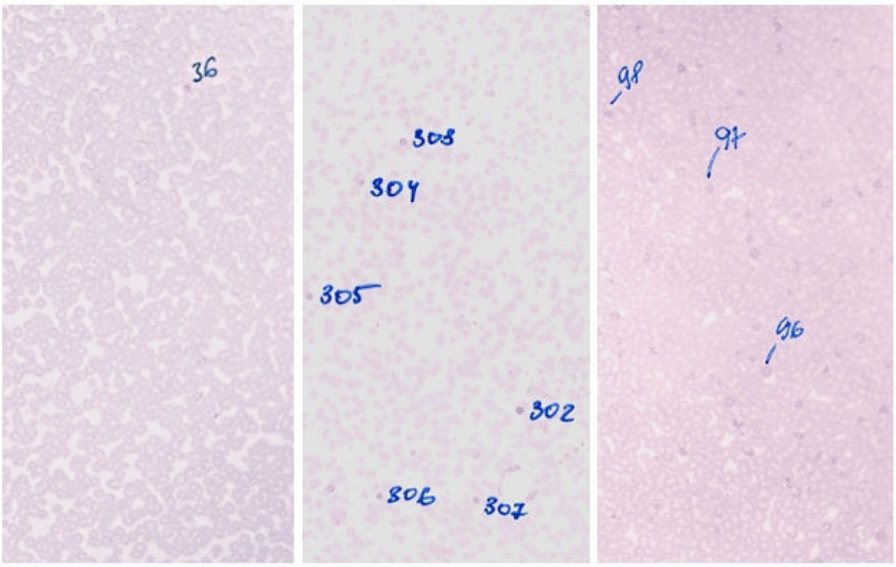


Figure 23. The comparison of primary immunoscreening outcomes before and after library enrichment. A | Without enrichment (~0-1 positive signals per 8 x 10^3 pfu). **B** | After the first biopanning cycle (~60-80 positive signals per 8 x 10^3 pfu). **C** | After the second biopanning cycle (>130 positive signals per 8 x 10^3 pfu).

A proportion of randomly selected serum-reactive phage clones were isolated, immunoscreened to monoclonality and sequenced to compare the antigen repertoires in the first and the second biopan. A considerable increase of redundancy in the antigen repertoire was observed – the representation of different antigens among clones detected in the first and the second biopan fell from 73 to 10% and 92 to 50% when screened with MA002079 (see Appendix 2 for the complete representation of identified antigens) and LGP-Mel 150 sera, respectively, which is consistent with the observations seen in other studies[416]. Therefore, in order to balance enrichment versus desired diversity of serum-reactive clones, it was decided to use a single round of biopanning throughout the study.

3.3.2. SELECTION AND CHARATERISATION OF SERUM-REACTIVE CLONES FROM T7 PHAGE-DISPLAYED TESTIS CDNA LIBRARY

Identification of serum-reactive clones

In order to assess and explore the serum-reactive protein repertoire that can be selected from T7-displayed cDNA expression libraries, four serum samples from individual melanoma patients (i.e. MA002079, MA00GG, LGP-Mel 150 and LGP-Mel 143) and a serum pool from five melanoma patients (Mel-Pool-5, see Appendix 4) were used to select antigens from the T7-T1 library by applying the elaborated biopanning procedure. Next, ~10^4 pfu from each biopan were immunoscreened with the respective sera and, taken together, this resulted in the detection of 436 serum-reactive phage clones (i.e. ~40-150 reactive clones per serum), which were isolated and purified via secondary immunoscreening. The cDNA inserts from monoclonal phage lysates were amplified by PCR and sequenced.

Sequence analyses showed that these 436 clones represent 243 different antigens; however, they also revealed that only in 25 cases (~10%) cDNAs were translated in their natural reading frames (see Table 10 for listing and brief characterisation). Six of these antigens have been previously identified by the conventional SEREX in different tumour types (but not melanoma) and 11 represent protein families whose other members have been detected by SEREX[13]. Three serum-reactive clones encoded for autoantigens, which are known to induce autoantibody production in autoimmune disorders (LMOD1, AKAP12) or infertility (SPAG8). Noteworthy, mRNA expression of SPAG8 was detected in one out of ten melanoma tissue samples and testis (and only trace levels in other normal tissues, results not shown) indicating that it may represent novel cancer-testis gene. One of the clones contained a hybrid cDNA generated by in-frame fusion of *ATP2C1* and *ANKRD24* genes; however no evidence for the expression of this fusion mRNAs in the testis tissue was found, suggesting that most likely it represents a cloning artefact. No immune responses against the remaining four in-frame antigens (i.e. SENP1, TEF, SLU7 and DKFZP566E164) have been reported before thus demonstrating that the repertoire of antigens identified by T7 phage display-based SEREX approach overlaps with that of the conventional SEREX, and at the same time this approach allows the detection of novel antigens.

Amongst the rest of the 219 serum-reactive clones there were 12, which represented novel splice variants or uncharacterised genes (with at least two ESTs confirming that these sequences are transcribed) with unknown protein sequences, while the remaining clones expressed short, most likely unnatural peptides encoded by inserted fragments of intergenic DNA (n=52), mtDNA (n=9), rRNA (n=3) and 5'or 3' UTRs or cDNAs cloned out-of-frame relatively to the coat protein 10B (n=143). With regard to the latter, it can not be excluded that some of these 143 clones, much like CTAG1B-ORF2 peptide, indeed represent cancer-specific antigens that could be generated by frame-shifting mutations, defects in pre-mRNA splicing or aberrations in translational controls in cancer cells. However, it is more feasible that mimotopes of other immunogenic proteins are encoded by these clones, particularly those 52 clones that contain intergenic regions with no evidence of expression.

Confirmation of serum reactivity against selected antigens by Western blot

In order to confirm that serum antibodies recognise the displayed recombinant antigens, two in-frame antigens (SPAG8 and LRRC50) and two out-of-frame peptides (encoded by *GOLGA5* and *LAMC1*) were expressed as GST fusion proteins and analysed by Western blot by LGP-Mel 150 serum, which was used for the selection of these antigen clones. Noteworthy, GOLGA5 (or golgin-84) belongs to golgin protein family, members of which are known autoantigens[462] while LAMC1 is known to be overexpressed in breast cancer[463], therefore corresponding in-frame proteins of these antigens were expressed and analysed as well. Besides, purified GST alone was used as a negative control.

The results confirmed serum-reactivity against the two in-frame antigens SPAG8 and LRRC50 as well as out-of frame peptides LAMC1 and GOLGA5. No reactivity was detected against in-frame LAMC1 and GST proteins while weak serum-positive signal was demonstrated against GOLGA5 protein. Although LGP-Mel 150 serum contained also low-titre antibodies against GOLGA5 in its natural reading frame (Figure 24, A), the results clearly demonstrated that serum antibodies had selected for the out-of frame GOLGA5 peptide and that there is no translation of these products in alternative reading frames in *E. coli* due to, for example, frame-shifting mutations in T7 genome.

Table 10. In-frame antigens identified by screening T7-T1 library with sera from melanoma patients.

Gene symbol	Protein	Serum	Position (aa) on Ref Seq	SEREX DB ID*
CCDC84	Coiled-coil domain containing 84	MA002079	186-280, NP_940891.1	-
HDLBP	High density lipoprotein binding protein (vigilin)	MA002079	1161-1268, NP_005327.1	309, GaCa
KIF27	Kinesin family member 27	MA002079	715-940 & 715-1128[#], NP_060046.1	-
YBX1	Y box binding protein 1	MA002079	313-324, NP_004550.2	-
MASK-BP3	MASK-4E-BP3 alternate reading frame gene	MA002079	1482-1540, NP_065741.3	1082, ReCa
LIG1	DNA ligase I	Mel-Pool-5	1-141, NP_000225	-
LMOD1	Leiomodin 1	Mel-Pool-5, MA00GG, LGP-Mel 150[‡]	463-507, NP_036266.2	2374, FS
COPS4	COP9 signalosome subunit 4	Mel-Pool-5	15-136, NP_057213.2	-
R3HDM2	R3H domain-containing protein 2	Mel-Pool-5	285-513, R3HD2_HUMAN	1233, BrCa
ANXA11	Annexin A11	Mel-Pool-5	1-71, NP_665876.1	81, LuCa
DKFZP566E164	Hypothetical protein LOC25858	Mel-Pool-5	3-76, CAB55995.2	-
SENP1	Sentrin/SUMO-specific protease 1	LGP-Mel 143	355-618, NP_055369.1	-
KIF1B	Kinesin family member 1B isoform b	LGP-Mel 143	316-458, NP_055889.2	-
ATP2C1/ ANKRD24	Calcium-transporting ATPase 2C2 /ankyrin repeat domain 24	LGP-Mel 143, LGP-Mel 150[‡]	775-785, NP_055676.2 / 614-650, NP_597732.1	-
SPAG8	Sperm associated antigen 8	LGP-Mel 143, LGP-Mel 150[‡]	9-99, NP_758516.1	-
AKAP12	A-kinase anchor protein 12	LGP-Mel 143, LGP-Mel 150	1465-1622 or 1382-1529[#], NP_005091.2	-
CCDC92	Coiled-coil domain-containing protein 92 (Limkain beta-2)	LGP-Mel 150	75-200, NP_079416.1	-
ATP5G2	ATP synthase, H+ transporting, mitochondrial F0 complex	LGP-Mel 150	56-107, NP_005167.2	-
LRRC50	Leucine rich repeat containing 50	LGP-Mel 150	626-725, NP_848547.3	-
SLU7	Step II splicing factor	LGP-Mel 150	110-228, NP_006416.3	-
Similar to: LRRC37A	Similar to: leucine rich repeat containing 37A	MA00GG	803-870, NP_055649.3	-
HNRPH1/RPS2	Hybrid of heterogeneous nuclear ribonucleoprotein H1 & ribosomal protein S2	LGP-Mel 143	383-416, NP_062543.1 / 268-278, NP_002943.2	182 (RPS2), CTCL, PrCa
TEF	Thyrotrophic embryonic factor (novel splice variant)	MA00GG	1-52 & 162-215, NP_003207.1	-

* Identification number of an antigen in the Cancer Immunome Database[13];
[#] Two partially overlapping clones encoding the same antigen were isolated;
[‡] The same phage clone was isolated with multiple sera.
Abbreviations: Ca – cancer, Ga – gastric, Re – renal cell, FS – fibrosarcoma, Br – breast, Lu – lung, CTCL – cutaneous T cell lymphoma, Pr – prostate.

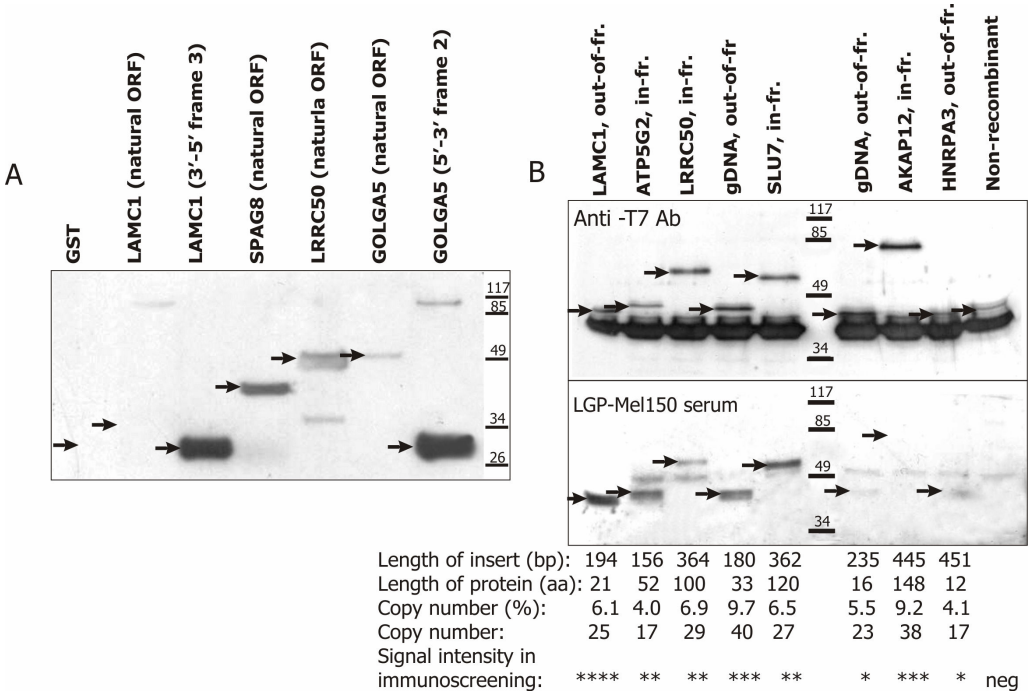


Figure 24. Western blot analyses of serum reactivity against selected antigens. A | In-frame and out-of-frame peptides encoded by the selected serum-reactive phages were expressed as GST C-terminal fusion proteins and tested for the reactivity with LGP-Mel 150 serum. In parallel, the fusion proteins were tested with anti-GST antibody to confirm the expression of the fusion protein and to determine its size (not shown). The arrows indicate the location of GST fusion proteins. **B** | Approximately 10^9 pfu from the selected phage clone lysates were separated by SDS/PAGE and tested with anti-10B antibody to determine the copy number of recombinant proteins per phage particle (quantification was done as described in Figure 20) with patient's serum that was used for the immunoselection.

Noteworthy, similar percentage (i.e. 6-16%) of in-frame antigens in comparison to that determined by this study has been found in other studies aimed to select TAAs from T7 phage-displayed cDNA libraries[18,428,452]. Predominance of out-of-frame peptides, although at lesser extent, also have been detected by using pJuFo phage display system[16] and λ phage surface display system[19]. When comparing the conventional and phage display-based SEREX approaches, the application of the former also results in the selection of significant proportion of serum-reactive clones (i.e. ~35-45%) that contain cDNAs fused to *β*-galactosidase gene in non-natural reading frame (as demonstrated by results from our studies and personal communication to Dr. G. Li, Nottingham and Dr. S. Eichmuller, Heidelberg). Although it is possible that the natural products of these genes could be produced by means of putative alternative ribosomal binding sites in the cloned cDNA sequences, to our knowledge it has never been experimentally confirmed. Thus, it can be assumed that a considerable fraction of these antigens also represents mimotopes.

Determination of selected antigen display density on T7 phage

Next, the differences in the copy number variation between in-frame and out-of-frame antigens displayed on the immunoselected phage clones were compared. The relatively high proportion of out-of-frame peptides amongst the clones identified in the T7-displayed library immunoscreening allowed to hypothesise that this could be associated with higher display density of these peptides than natural ORFs due to their shorter size (the size of the out-of-frame peptides ranged from two to 56 aa, on average 21 aa, while in-frame proteins ranged from 10 to 414 aa, on average 120 aa) that could result in the detection of low-affinity antibodies due to a higher valence of the epitope carrier. In order to test this hypothesis, eight high-titre serum-reactive phage clones encoding four in-frame and four out-of-

frame antigens as well as empty T7 phage were probed in Western blot with the serum LPG-Mel 150, which was used for the antigen selection, and anti-10B antibody as described above (see section 3.2.1.).
As shown in Figure 24, B, the copy number variation of recombinant protein per phage particle ranged from 17 to 40 (4-10%); however, no correlation between the copy number and (1) the size of the fusion protein (in support to our previous results), (2) the size of the insert and (3) signal intensity in Western blot and plaque immunoscreening with patient's serum was observed. The serum antibody signals were detected against all fusion proteins except AKAP12, suggesting that the anti-AKAP12 antibodies recognise a discontinuous epitope while all the other antigens display linear epitopes.
Collectively, these data suggest that the increased detection of serum-reactivity against out-of-frame peptides is an intrinsic feature of the T7Select 10-3b phage display system that is not associated with the variations in the copy number of recombinant proteins per phage particle. It is feasible, that phage clones expressing shorter peptides may become overrepresented during library amplification – a possible explanation of this could be that structural or biochemical properties of longer in-frame antigens may have obstructive impact on the phage capsid assembly in contrast to shorter mimotopes sequences. Apart from this, variations in the accessibility of the C-terminus of the coat protein 10B also may play a role in this phenomenon.

Taken together, the usage of phage surface display-based approach for the selection of autoantibody targets provides a time and labour-effective alternative to the conventional SEREX approach. However, the antigen repertoire that could be selected by applying the T7Select 10-3b phage display system is strongly biased to the identification of mimotopes and this can be seen, on the one hand, as disadvantage of this system since in addition to mimicking tumour cell-derived antigens, the out-of-frame peptides may resemble protein as well as non-protein antigens of various infectious agents, thus negatively influencing the probability of finding cancer-associated biomarkers among mimotopes in comparison to in-frame antigens.
However, a study by Lin and co-workers has showed that in a panel of 130 selected phage clones recognised by autoantibody biomarkers of head and neck cancer that were identified through systematic T7-displayed antigen microarray analyses only eight clones (i.e. 6%) represented in-frame antigens suggesting high potential of mimotopes as cancer biomarkers[428]. Another study of autoantibody signatures in prostate cancer identified 22 T7 phage-displayed antigen detector group, from which only 5 clones (comprising 23%) encoded for in-frame antigens[453]. It is known that recombinantly expressed peptides rich in aromatic amino acids mimic carbohydrate structures thus making possible to identify autoantibody biomarkers that recognise carbohydrate antigens *in vivo*[464], which represent a significant subset of tumour-specific antigens[286]. Apart from that, short linear peptides are known to mimic discontinuous epitopes, which are suggested to represent a majority of all B cell epitopes[464]. Thus, the expression of recombinant peptides capable to mimic both carbohydrate and conformational epitopes, expression of which is limited in the selected expression system, should be considered as an advantage since the antibodies recognising such mimotopes might represent valuable biomarkers. Considering all this, it was decided in further study to exploit all the identified individual serum-reactive clones (i.e. in-frame as well as out-of-frame) for the generation of antigen microarray.

3.4. LARGE-SCALE ASSESSMENT OF AUTOANTIBODY REPERTOIRES IN CANCER PATIENTS

Next part of the study was aimed for the antigen discovery, which was followed by the generation of a phage-displayed antigen microarray – the T7Select 10-3b display system was exploited for the identification of a comprehensive set of antigens associated with melanoma as well as prostate and gastric cancer, and the identified antigen phage clones were subjected to the generation of an antigen microarray applicable for high-throughput monitoring of autoantibody responses.

3.4.1. LARGE-SCALE IDENTIFICATION OF TUMOUR-ASSOCIATED ANTIGENS

Identification of tumour-associated antigens was done according to the elaborated procedure, which included (i) negative selection of IgG encoding phage clones from tumour tissue-derived T7 phage-displayed libraries, (ii) library enrichment via a single round of biopanning (Figure 22) with an exception of prostate antigen selection that was done according to a differential biopanning procedure (Figure 25), (iii) immunoscreening of the enriched libraries with autologous and/or pooled allogeneic sera from cancer patients suffering from the particular cancer type (all sera were preabsorbed against the proteins expressed by the vector system prior to analyses), (iv) serum-positive clone purification through secondary immunoscreening and (v) antigen identification by sequencing of serum-positive phage clone inserts.

All the clone-related information including clone insert nucleotide sequences, amino acid sequences of the translated peptides, the official gene name, chromosomal localisation, summary about the function of the gene and structural motifs of the protein and clinical information about the patients whose tissue material and sera were used for antigen selection, antigen overlap with Cancer Immunome database records etc. was deposited in the Tumour Antigen database (an example can be seen in Appendixes 3A and 3B). In addition, another function was embedded into the database that allows to automatically perform BLAST search against human EST database (http://www.ncbi.nlm.nih.gov) and classify the retrieved EST sequences as cancer/normal/brain/testis or cell line by searching the respective keywords in their description, which thus gives the preliminary view of tissue distribution of the antigens.

The decision of sequencing all serum-reactive clone inserts throughout the study was made since it gives an advantage of assembling a pre-characterised antigen panel for the generation of an antigen microarray that would be particularly rich in unique clones without any excessive overrepresentation of frequently selected antigens. Such a microarray would be of increased value for further applications. In contrast, vast majority of the studies surveying of autoantibody responses by means of T7 phage-displayed antigen microarray have followed a strategy of choosing random clones[426,428,452,453,465] or random serum-reactive clones[425] from enriched libraries after three to five rounds of differential biopanning that inevitably leads to reduced representation of the antigen repertoire.

Identification of antigens recognised by melanoma patients' sera

In total 243 different melanoma antigens were identified previously via T7-T1 library immunoscreening with sera from nine melanoma patients (see section 3.3.2 and Table 11). In addition, five primary melanoma (Table 4) tissue-derived cDNA expression libraries were constructed by using the T7Select 10-3b vector system (the size of each library is given in Table 11) that was followed by the selection of serum-reactive clones by using autologous sera. Besides, the libraries were enriched and immunoscreened with a serum pool (i.e. Mel-PV-Pool-5) that was composed from serum specimens collected from five melanoma patients at time period of 2-6 months after the Mel-TYR immunotherapy trial (which involved tyrosinase peptide, IL-2 and IFN-α) in order to discover potential antigens, to which antibody responses could have been induced by the vaccine. (Table 11).

Table 11. The cDNA libraries and sera used for melanoma antigen discovery.

cDNA library	Size of primary library (pfu)	Sera used for positive selection*	Serum-reactive clones	Different antigens	In-frame antigens
T7-Mel-T2	4 x 10^5	LGP-Mel-T2	30	26	-
T7-Mel-T4	3 x 10^5	LGP-Mel-T4	16	12	1
T7-Mel-1816	0.6 x 10^5	MA001816	30	19	1
T7-Mel-445	8 x 10^5	MA000445	85	44	3
		Mel-PV-Pool-5	47	20	-
T7-Mel-525	9 x 10^5	MA000525	48	38	3
		Mel-PV-Pool-5	42	35	3
Pooled T7-Mel-T2, T4, 1816		Mel-PV Pool-5	35	32	-
Subtotal melanoma libraries:		**10**	**333**	**226**	**11 (10)‡**
T7-TAA mini-libraries#	0.5-5 x 10^4	8 post-vaccination & 13 pre-vaccination melanoma	224	36	17
T7-T1	8 x 10^6	9 melanoma	436	243	25
Total:		**26**	**993**	**505**	**52**

* See Appendix 4 for detailed list of the melanoma sera used for melanoma antigen discovery;
# See section 3.2.1. for detailed representation of TAA mini-library immunoscreening data;
‡ The number of different antigens given in brackets.

Overall, 333 serum-reactive clones encoding 226 different antigens were isolated by immunoscreening of ~4 x 10^4 pfu from each of the melanoma library biopans. Sequencing analyses revealed that among the identified clones there are only 10 that contains cDNAs of known genes that were fused in-frame to the T7 phage coat protein 10B ensuring the display of natural antibody ligands (see Table 12). From these, only for an eukaryotic translation elongation factor EEF1A1 and OSBPL10 corresponding records can be found in the SEREX database (Table 12); noteworthy, autoantibody responses against EEF1A1 have been reported in 66% of patients with Felty syndrome, a complex autoimmune condition[466]. GAGE antigens that belong to CT antigen category as well have been found to elicit antibody responses in cancer patients[467] and the phage clones encoding for GAGE antigens were selected by MA000445 serum through both, TAA mini-library and the T7-Mel-445 library immunoscreening (Tables 8 and 12).
Amongst the rest of the antigens no other proteins have been reported to elicit autoantibody responses, however, four from the remaining antigens (i.e. CBX3, SFRS18, SMARCC1 and ZCCHC9) belong to protein families, other members of which have been identified by SEREX, while some of the others are shown to be associated with carcinogenesis – RFWD2 is a negative regulator of p53, which is overexpressed in breast and ovarian adenocarcinomas[468]; SMARCC1 expression has been linked with prognostic value – it is upregulated in prostate cancer and positively correlates with tumour recurrence and dedifferentiation[469] while, on contrary, colorectal cancer patients with tumours displaying high levels of SMARCC1 proteins had a significantly better overall survival rates[470].

Summarising the TAA mini-library, T7-T1 and five melanoma cDNA library immunoscreening data, melanoma antigen repertoires were interrogated by using serum specimens from 26 patients, and this resulted in the identification of 505 different serum-reactive clones; 196 of these were selected by the eight post-vaccination serum specimens. Approximately 10% of the melanoma-associated antigen phage clones encoded for proteins expressed in their natural reading frames.

Table 12. In-frame antigens identified by melanoma library immunoscreening.

Gene symbol	Protein	Serum	Position (aa) on NCBI RefSeq	SEREX DB ID*
RFWD2	Ring finger and WD repeat domain 2; Critical negative regulator of p53	LGP-Mel-T4	431-468, NP_071902	-
CBX3	Chromobox homolog 3; a component of heterochromatin	MA001816	10-44, NP_009207	-
GAGE[#]	G antigen; known CT antigen family member	MA000445	4-40 & 5-70[‡], NP_001459.2	-
GALNT1	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 1/ 28S ribosomal RNA; initiate mucin-type O-linked glycosylation	MA000445	518-559, NP_065207.2	-
SFRS18	Splicing factor, arginine/serine-rich 18	MA000525	100-167, NP_116259.2	-
SMARCC1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 1; a transcription factor	MA000525	674-799, NP_003065.3	-
EEF1A1	Eukaryotic translation elongation factor 1 alpha 1	MA000525	242-306, NP_001393.1	406, Mel; 2672, CTCL; 2553, CoCa
OSBPL10	Oxysterol binding protein-like 10, an intracellular lipid receptor	Mel-PV-Pool-5	748-764, NP_060254.2	2659, CoCa
KLRAQ1	KLRAQ motif containing 1	Mel-PV-Pool-5	19-117, NP_001129102.1	-
ZCCHC9	Zinc finger, CCHC domain containing 9	Mel-PV-Pool-5	208-271, NP_115656.1	-

* Identification number of an antigen in the Cancer Immunome Database[13];
[#] Particular member from the GAGE antigen family can not be determined;
[‡] Two partially overlapping clones encoding the same antigen were isolated.
Abbreviations: Ca – cancer, Mel – melanoma, CTCL – cutaneous T cell lymphoma, Co – colon.

Identification of prostate cancer antigens

Induction of heterogeneous autoantibody responses after immunotherapy has been demonstrated by several studies[408,471,472]. Since one of the future applications of the antigen microarray is the discovery of autoantibody signatures that could predict the cancer patients' response to immunotherapy, there was a need to assess the antigen repertoire, antibody responses against which are generated by defined vaccination strategy. To accomplish this, prostate cancer patient serum samples that have been obtained prior and after two independent DC-based immunotherapy trials (i.e. Pr-ONY and Pr-NRH) were used for the selection of prostate cancer-associated antigens. A T7 phage-displayed cDNA expression library T7-OPCT of size 8 x 10^5 pfu was constructed from mRNA specimens derived from three prostate cancer cell lines (i.e. OPCT-1, OPCT-2 and OPCT-3). The enrichment of the T7-OPCT library was done in different manner in comparison to the above described – i.e. (i) no negative selection for IgG encoding phage clones were done since cell lines were used for the construction of the library and (ii) the library was subjected to differential biopanning procedure in order to enrich for (1) antigens eliciting antibody responses in prostate cancer patients after vaccination as well as (2) antigens that have induced B cell responses in cancer patients responding to vaccination.
First, 12 prostate cancer patients enrolled in the Pr-NRH vaccination trial were selected for their autoantibody repertoire analyses. From these patients, six were responding to the treatment while the other six were non-responders (patients were classified according to T cell response). Serum specimens from these 12 patients that had been collected prior and three months after the vaccination were preabsorbed with *E. coli* and T7 phage lysates and used for the generation of four 6-serum pools

– i.e. pre-vaccination and post-vaccination pools from responders (Pr-PreV-R-P6 and Pr-PV-R-P6) as well as pre-vaccination and post-vaccination pools from non-responders (Pr-PreV-NR-P6 and Pr-PV-NR-P6). To assess the autoantibody responses induced by the immunotherapy in responders and non-responders, T7-OPCT library was differentially enriched (schematic overview of the biopanning procedure is given in Figure 25) and immunoscreened with the corresponding serum pools. Apart from the T7-OPCT library immunoscreening, these serum pools were used for immunoscreening of the T7-TAA mini-libraries as described above (see section 3.2.1.) in order to assess the antibody responses against these antigens in prostate cancer patients as well as to get an insight of potential vaccine-induced changes in the detected autoantibody responses against these TAAs.

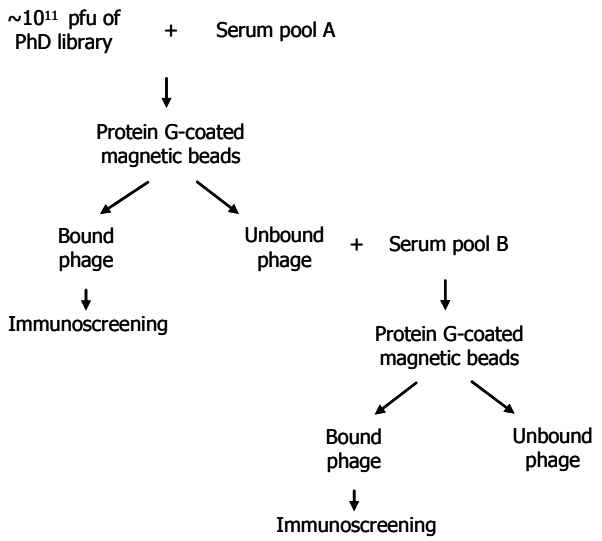


Figure 25. The schematic overview of the differential biopanning procedure. The T7-OPCT library was subjected to the differential biopanning procedure aimed (i) to enrich the library for clones reactive with post-vaccination sera (serum pool A there is represented by pools Pr-PreV-R-P6 or Pr-PreV-NR-P6 and serum pool B – Pr-PV-R-P6 or Pr-PV-NR-P6, respectively) and (ii) to enrich the library for clones reactive with responder serum antibodies (serum pool A there represents ONY-PV-NR-P3 and serum pool B – ONY-PV-R-P7). PhD – phage display.

Next, 10 prostate cancer patients enrolled in the Pr-ONY vaccination trial were selected for their autoantibody repertoire analyses. Serum specimens collected 51 weeks after vaccination from three patients not responding as well as sera from seven patients responding to vaccination were combined to generate serum pools ONY-PV-NR-P3 and ONY-PV-R-P7, respectively. The patients were classified according to PSA responses. The two serum pools were used for differential enrichment of antigen-encoding phage clones in the libraries T7-OPCT and T7-T1, which were pooled prior to selection (Figure 25). The biopans were immunoscreened with the corresponding serum pools to identify the selected antigen repertoires.

Taking together, a serum-reactive protein repertoire was selected and identified by interrogating autoantibody repertoires from 34 serum specimens that had been obtained from 22 prostate cancer patients undergoing the two DC-based immunotherapy trials (Table 13).

In total, 362 serum-reactive clones were isolated by immunoscreening of the T7-displayed cDNA libraries. Among them, 80 and 90 different gene products were identified in T7-OPCT and T7-OPCT+T7-T1 library analyses, respectively, while the immunoscreening of TAA mini-libraries resulted in the identification of nine different gene products. From the identified antigens 52 different antigen clones were selected by pre-vaccination sera while the analysis of autoantibody repertoire in patients after vaccination resulted in the identification of 148 different antigen clones. From the all identified antigen repertoire 113 were selected by antibodies from patients that did not respond to the

immunotherapy while 87 were defined by serum antibodies from responders. Clone insert sequencing analyses also revealed that among the 179 different clones 13 contains cDNAs of known genes that were fused in-frame to the T7 phage coat protein 10B (see Table 14 for the listing and brief characterisation).

Table 13. The cDNA libraries and sera used for antigen discovery in prostate cancer.

cDNA library	Sera used for positive selection	Serum-reactive clones	Different antigens*	In-frame antigens
T7-OPCT	Pr-PreV-R-P6	18	17	-
	Pr-PV-R-P6	42	28	1
	Pr-PreV-NR-P6	48	26	1
	Pr-PV-NR-P6	48	32	4
T7-TAA mini-libraries	Pr-PreV-R-P6	2	2	-
	Pr-PV-R-P6	10	3	1
	Pr-PreV-NR-P6	9	7	3
	Pr-PV-NR-P6	10	7	2
Pooled T7-OPCT &T7-T1	ONY-PV-R-P7	80	37	6
	ONY-PV-NR-P3	95	41	1
Total:	**34 (22 patients)**	**362**	**200 (179)** [#]	**19 (14)** [#]

* Number of different antigens identified in each biopan is given, some of which were selected by more that one serum pool.
[#] Number of different antigens is given in brackets.
Abbreviations: Pr – prostate, PreV – pre-vaccination, PV – post-vaccination, R – responders, NR – non-responders.

Three of these antigens (i.e. CTAG1B, CTAG2 and CTAGE1) were selected by TAA mini-library immunoscreening. Antibody responses against CTAG antigens ranging between 8-19% of prostate cancer patients have been reported previously[16,415,473]. However, the expression and humoural responses against CTAGE antigens, which have been identified in cutaneous T-cell lymphoma, have never been reported in prostate cancer patients so far. The translation of the cDNA clone most likely representing a CTAGE pseudogene leads to the display of a protein that shows 96% similarity to CTAGE1 antigen (see amino acid positions in Table 14). Although the clone results in a coterminous stop codon, the detected autoantibody responses against the 53 aa long peptide (total length of the recombinant peptide is 56 aa) most likely represent the antigen-induced calls. Regarding the potential vaccination-induced autoantibody responses against the identified TAAs, the immunoscreening results indicate that humoural responses against CTAGE are induced in the responder pool (Table 14), however, detailed analyses of individual patient pre- and post-vaccination serum would be needed to confirm this observation.
Amongst the other 10 in-frame antigens there are three proteins (i.e. PDAP1, MYH11 and RPS2) that have been identified by conventional SEREX studies (Table 14), from these only RPS2 had been identified in prostate cancer. Importantly, 17 individual clones representing two different myosin heavy chain protein fragments encoded by *MYH11* mRNA were isolated by ONY-PV-R-P7 serum pool indicating that autoantibody formation against this gene product might be induced by the vaccination. Interestingly, localisation of functionally related protein β-actin on the surface of apoptotic tumour cells has been shown to trigger autoantibody production in medullary carcinoma of the breast[181]. Four more antigens, RNF126, FAM20C, ZBTB48 and HNRPH1, belong to protein families, other members of which have been detected by SEREX[13]. Noteworthy, HNRPH1 belongs to heterogeneous nuclear ribonucleoprotein family, members of which are well-known autoantigens known to induce autoantibody production in autoimmune disorders[474]. Another antigen NAMPT represents a potential malignant astrocytoma/glioblastoma serum marker for poorer prognosis of the disease outcome[475], while the autoantibody responses against the rest of the antigens have not been reported so far.

Table 14. In-frame antigens selected with prostate cancer patients' sera.

Gene symbol	Protein	Serum	Position (aa) on NCBI RefSeq	SEREX DB ID*
CTAG1B	Cancer/testis antigen 1B	Pr-PreV-NR-P6 Pr-PV-NR-P6	1-104, NP_001318.1	1546, OvCa, 57, EsoCa
CTAG2	Cancer/testis antigen 2	Pr-PreV-NR-P6 Pr-PV-NR-P6	1-104, NP_758965.1	1160, BrCa
Similar to CTAGE[#]	Cutaneous T-cell lymphoma-associated antigen	Pr-PreV-NR-P6 Pr-PV-NR-P6 Pr-PV-R-P6	299-352, NP_758441.2	2021, Lymphoma
NAMPT	Nicotinamide phosphoribosyl-transferase, pre-B-cell colony enhancing factor 1	Pr-PreV-NR-P6 Pr-PV-NR-P6	450-510, NP_005737.1	-
JARID2/ RNF126	Hybrid of Jumonji, AT rich interactive domain 2 & ring finger protein 126	Pr-PV-NR-P6	847-857, NP_004964.2 /208-298, NP_919442.1	-
ZBTB48	Zinc finger and BTB domain containing 48	Pr-PV-NR-P6	298-311, NP_005332.1	-
FAM20C	Family with sequence similarity 20, member	Pr-PV-NR-P6	1-58, NP_064608.2	-
CFDP1	Craniofacial development protein 1	Pr-PV-R-P6	77-137, NP_006315.1	-
POGK/ gDNA	Hybrid of Pogo transposable element with KRAB domain & gDNA	ONY-PV-R-P7	108-160, NP_060012.3	-
VARS	Valyl-tRNA synthetase	ONY-PV-R-P7	1008-1077, NP_006286.1	-
PDAP1	PDGFA associated protein 1	ONY-PV-R-P7	152-181, NP_055706.1	2352, FS
MYH11	Myosin, heavy chain 11, smooth muscle	ONY-PV-R-P7	1584-1679, 1817-1873, NP_074035.1	100, Teratoma
FN1	Fibronectin 1	ONY-PV-R-P7	523-566, NP_997640.1	-
HNRPH1/ RPS2	Hybrid of heterogeneous nuclear ribonucleoprotein H1 & ribosomal protein S2 (out-of-frame)	ONY-PV-NR-P3	383-416, NP_062543.1/ 268-278, NP_002943.2	182 (for RPS2), PrCa

* Identification number of an antigen in the Cancer Immunome Database[13];
[#] At protein level shows 96% identity to CTAGE1, possibly encoded by a CTAGE1 pseudogene.
Abbreviations: Pr – prostate, PV – post-vaccination, PreV – pre-vaccination, R – responders, NR – non-responders, Ca – cancer, Ov – ovary, Eso – oesophageal, Br – breast, FS – fibrosarcoma.

Identification of gastric cancer and gastritis-associated antigens

Two T7-displayed gastric cancer cDNA expression libraries (designated as T7-GaCaP5 and T7-GaCaP6) were constructed from stomach tumour specimens derived from five and six cancer patients, respectively (Table 4). These libraries were immunoscreened with the autologous serum pools GaCa-P5 and GaCa-P6. An additional set of serum-reactive clones was immunoselected from the T7-GaCaP6 library by using an allogeneic gastric cancer patient-derived serum pool GaCa-P6-II. In parallel, gastric cancer-associated antigens were selected from pooled T7-GaCaP5 and T7-T1 libraries by using two 5-serum pools from patients suffering from intestinal type gastric adenocarcinoma (designated as GaCa-Int-P5) and diffuse type gastric adenocarcinoma (GaCa-Dif-P5). See Table 15 for overview on libraries and sera used for the identification of gastric cancer-associated antigens.
Next, in order to assess the autoantibody responses induced in premalignant conditions of gastric cancer and early steps of gastric carcinogenesis, the libraries were subjected to the selection of antigens by various gastritis-associated serum pools. A pool of sera derived from five patients suffering from atrophic gastritis (designated Gs-Pool5) was used for immunoscreening of T7-GaCaP5 library while two pools comprising serum samples from patients with *H. pylori* positive gastritis (HP^+-

Gs-P5) and *H. pylori* negative gastritis (HP^--Gs-P5) were used for the selection of antigens in pooled T7-GaCaP5 and T7-T1 libraries (Table 15).

Table 15. The cDNA libraries and sera used for antigen discovery in gastric cancer.

cDNA library	Size of primary library (pfu)	Sera used for positive selection*	Serum-reactive clones	Different antigens	In-frame antigens
T7-GaCaP5	3.9×10^6	GaCa-P5	45	16	1
		Gs-Pool5	41	24	2
T7-GaCaP6	8×10^6	GaCa-P6	150	78	8
		GaCa-P6-II	58	41	1
Pooled T7-GaCaP5 &T7-T1		GaCa-Int-P5	88	45	2
		GaCa-Dif-P5	82	53	4
		HP^+-Gs-P5	93	50	4
		HP^--Gs-P5	71	49	-
Total:		**42**	**628**	**356**	**22**

* The serum pools contained no overlapping serum samples;
Abbreviations: HP – *H. pylori*, Ca – cancer, Ga – gastric, Gs – gastritis, Int – intestinal, Dif – diffuse.

After a negative selection step for IgG encoding phage clones the libraries were enriched by the corresponding serum pools by applying the routine biopanning procedure that was followed by the biopan immunoscreening. Taking together, a serum-reactive protein repertoire was selected by interrogating autoantibody repertoires from 27 individual gastric cancer and 15 gastritis patients that resulted in the identification of 233 and 123 different antigen clones, respectively.

Among the antigen clones identified by serum antibody repertoire from gastric cancer patients, 16 encoded for proteins translated in-frame regarding surface protein 10B. From these, two gene products have been identified by previous studies by applying conventional SEREX to other tumour types (Table 16). Among the rest of the antigens there were six (i.e. RPS19, BAT2D1, CCDC11, RNF14, ATP2A2, IDH1) that belong to protein families, other members from which have been demonstrated by SEREX to elicit humoural responses in cancer patients[13].

Although other gene products have not been yet demonstrated to elicit disease-associated humoural responses, several of them have been linked to cancerogenesis. Allograft inflammatory factor 1 (AIF-1) has been proposed to have a role in pathogenesis, proliferation and involution of hemangioma[476]; besides it has been implicated in promotion of breast tumour growth via activating NF-kappaB signalling, which consequently leads to in the in promotion of tumour growth resulting from up-regulated expression of cyclin D1[477]. Another of the identified antigens RNF14 is involved in enhancing cell cycle progression and cell proliferation via induction of cyclin D1 in colon cancer[478]. Germline alterations of ATP2A2 have been associated with predisposition to lung and colon cancer[479] while RPN2 gene has been associated with drug docetaxel resistance in breast cancer[480]. The other antigens have not been associated with carcinogenesis neither they have been reported to elicit humoural immunity.

Regarding the gastritis-associated antigen identification, six out of 123 isolated antigen clones encoded for in-frame antigens, which are listed and briefly overviewed in Table 17. Serum antibody responses against two of the identified antigens PTBP1 and SC65 have been demonstrated by conventional SEREX studies (Table 17). Importantly, autoantibody production against synaptonemal complex protein SC65 has been demonstrated in cases of interstitial cystitis[481]. Another of the antigens, eIF3i, although have not been identified to elicit humoural responses in cancer patients, belongs to a family of eukaryotic translation initiation factors, which has been represented in SEREX-defined antigen repertoire[13], and it has been shown that carcinoma-associated eIF3i overexpression is linked to facilitated mTOR-dependent growth transformation[482].

Table 16. In-frame antigens selected with gastric cancer patients' sera.

Gene symbol	Protein	Serum	Position (aa) on NCBI RefSeq	SEREX DB ID*
RPS19	Ribosomal protein S19	GaCa-P5	113-145, NP_001013.1	-
C1GALT1	Core 1 synthase, glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase, 1	GaCa-Int-P5	156-168, NP_064541.1	-
BAT2D1/ CCDC11	Hybrid of HBxAg transactivated protein 2 & coiled-coil domain containing 11 (both in-frame)	GaCa-Int-P5	459-518, NP_055987 / 300-386, NP_659457.2	-
RNF14	Ring finger protein 14	GaCa-Dif-P5	461-474, NP_004281.1	-
C16orf82	Chromosome 16 open reading frame 82, hypothetical protein	GaCa-Dif-P5	1-154, NP_001139017.1	-
Hypothetical gene LOC284861	Hypothetical protein	GaCa-Dif-P5	59-182, XP_932893.2	-
ESCO1	Establishment of cohesion 1 homolog 1	GaCa-Dif-P5	24-60, NP_443143.2	-
AIF1	Homo sapiens allograft inflammatory factor 1	GaCa-P6	76-93, NP_116573.1	-
HSBP1	Homo sapiens heat shock factor binding protein 1	GaCa-P6	64-76, NP_001528.1	-
ATP2A2	ATPase, Ca^{2+} transporting, cardiac muscle, slow twitch 2	GaCa-P6	216-301, NP_001672.1	-
RPN2	Ribophorin II	GaCa-P6	333-368, NP_001129243.1	-
HP1BP3	Heterochromatin protein 1, binding protein 3	GaCa-P6	47-116, NP_057371.2	-
RRBP1	Ribosome binding protein 1 homolog 180 kDa	GaCa-P6	72-119, CAD38684.1	2086, ND, 2128, Mel
BFAR	Bifunctional apoptosis regulator	GaCa-P6	89-118, NP_057645.1	-
IDH1	Isocitrate dehydrogenase 1 (NADP+), soluble isoform	GaCa-P6	151-194, NP_005887.2	-
ZNF282	Zinc finger protein 282	GaCa-P6-II	262-402, NP_003566.1	653,ThyCa; 1065, ReCa; 2381, Sar

* Identification number of an antigen in the Cancer Immunome Database[13];
Abbreviations: HP – *H. pylori*, Ga – gastric, Ca – cancer, Int – intestinal, Dif – diffuse ND – not defined, Mel – melanoma, Thy – thyroid, Re – renal cell, Sar – sarcoma.

Although induction of humoural responses against the rest of the antigens has not been demonstrated, all of them have been implicated in gastric carcinogenesis – amphiregulin is an autocrine growth factor for various malignant cells including gastric cancer[483], hepatocarcinoma[484], and breast cancer[485] and it is upregulated in *H. pylori*-induced carcinogenesis in mice[486], SPARC is a protein overexpressed in gastric adenocarcinomas[487], while ATG2B has been shown to harbour frame shifting mutations in gastric and colorectal cancers with microsatellite instability[488]. Although preliminary, these results indicate that the survey of humoural responses of gastritis patients may reveal potential autoantibody biomarkers for early detection of gastric cancer. However, assessment of humoural responses of representative set of patients against all 123 identified gastritis-associated antigen clones will allow to define such autoantibody signatures.

Table 17. The identified gastritis-associated in-frame antigens.

Gene symbol	Protein	Serum	Position (aa) on NCBI RefSeq	SEREX DB ID*
SC65	Synaptonemal complex protein SC65	Gs-Pool5	383-437, NP_006446.1	2334, malignant fibrous histiocytoma
SPARC	Secreted protein, acidic, cysteine-rich (osteonectin)	Gs-Pool5	13-80, NP_003109.1	-
PTBP1	Polypyrimidine tract binding protein 1 (a hnRNP)	HP^+-Gs-P5	318-352, NP_114368.1	2663, CoCa
EIF3I	Eukaryotic translation initiation factor 3, subunit I	HP^+-Gs-P5	177-219, NP_003748.1	-
ATG2B	ATG2 autophagy related 2 homolog B (S. cerevisiae)	HP^+-Gs-P5	1008-1037, NP_060506.5	-
AREG	Amphiregulin (schwannoma-derived growth factor)	HP^+-Gs-P5	140-193, NP_001648.1	-

* Identification number of an antigen in the Cancer Immunome Database[13].
Abbreviations: HP – *H. pylori*, Gs – gastritis, Co – colon, Ca – cancer.

It has to be mentioned that an independent study aimed for the meta-analyses and in-depth molecular characterisation of all of the selected in-frame antigens identified in melanoma, prostate and gastric cancer as well as gastritis is currently underway to reveal novel potential immunotherapy targets as well as to define the underlying reasons for the antigen immunogenicity.

3.4.2. PROFILING OF AUTOANTIBODY RESPONSES BY PHAGE-DISPLAYED ANTIGEN MICROARRAY

In the conventional SEREX studies novel tumour-associated antigen discovery is usually followed by their immunoscreening with small panels of sera from normal individuals and cancer patients in order to select antigens with cancer-specific immuno-reactivity. Although this approach, named *petit serology*, is useful to choose antigens worth of further analyses, it is laborious and has several limitations – it requires large amounts of sera and only small numbers of specimens can be tested simultaneously[6]. Modifications of this plaque assay-based technique have been introduced – SMARTA (serological mini-arrays of recombinant tumour antigens) that exploits a 96-well format for serological mini-arrays[489], and SADA (serum antibody detection array) that involves hundreds of antigen phage clones immobilised on a tiny membrane surface[490]. Both of the techniques are suitable alternatives when the goal is to screen larger number of SEREX-defined antigens against a limited number of serum samples.
As an alternative to *petit serology*, sero-epidemiology of SEREX-defined antigens has been assessed in a quantitative manner by application of an approach called *grand serology* that involves ELISA tests with recombinantly expressed antigens, which enables the profiling of autoantibody responses in larger scale[6,491]. However, although this approach does not require the serum preabsorbtion and is more compliant regarding serum amounts necessary for the analyses, the recombinant expression and purification of tens or hundreds of TAAs is not a trivial and cost-effective task.
The application of these approaches for surveying of humoural responses in cancer patients has demonstrated that the autoantibody responses against each individual antigen is generally low, rarely exceeding 10-15%[489,491,492], indicating that no single antibody may serve as good biomarker while the combination of individual TAAs results in enhanced cancer detection[490,492].This clearly points to the need of expanding the list of antigens with cancer-specific humoural responses as well as the elaboration of approaches that enable high-throughput profiling of autoantibody responses in cancer patients.
A tumour antigen microarray generated by printing crude phage lysates onto nitrocellulose-coated slides obviously serves as a time, labour and cost-efficient alternative that can be used for

simultaneous monitoring of autoantibody responses in multiple serum samples against hundreds of serum-reactive clones. Nonetheless, the conventional SEREX approach does not enable the direct utilisation of the isolated antigen phage clones since recombinant antigens are produced in soluble form by the used vector system. In contrast, the phage display principle ensures physical incorporation of recombinant proteins into the phage surface and this attractive feature permits the development of microarrays directly by omitting extra antigen expression and purification steps.

Development of the T7 phage-displayed antigen microarray prototype

The final task of this work was to develop the identified antigen microarray that would be suitable for high-throughput survey of autoantibody responses in cancer patients. The first prototype of T7 phage-displayed antigen microarray has been generated on the basis of the antigens identified in this study – a panel of sero-reactive phage clones comprising 730 phage clones encoding for the 505 melanoma and 179 prostate cancer antigens, and 46 antigens selected from T7 TAA mini-libraries through clone sequence-based screening (not listed) as well as 30 non-recombinant phage clones were included in the microarray.

The development of standard operating procedures (SOPs) that were used for the production and processing of phage-displayed antigen microarrays was done in an independent study by Pawel Zayakin and will not be discussed herein in detail. It covered the evaluation of different variables, including high-titre phage stock preparation method (e.g. plate lysates vs liquid lysates with or without subsequent precipitation, standardisation of phage titres), phage growing conditions (e.g. growing volumes, agitation, *E. coli*-phage ratios), and formats (e.g. separate tube vs 96-well plate formats) as well as the choice of slide surface chemistry and optimisation of phage printing parameters. The elaboration of the microarray slide processing procedure included elucidation of an optimal serum preabsorbtion technique, appropriate antibody dilutions and incubation periods as well as washing conditions.

Shortly, all the individual phage clones were obtained in high-titre through their amplification in IPTG-induced liquid BLT5615 *E. coli* culture in 96-deepwell plates. After the bacterial culture lysis, the high-titre phage stocks were subjected to PCR-based quality control to monitor possible phage cross-contaminations. The obtained high-titre phage stocks then were used for repeated amplification cycle to standardise phage titres; the lysates were clarified by centrifugation and supernatants were directly subjected for printing in duplicates onto nitrocellulose-coated two-pad FAST slides.

Profiling of autoantibody responses in cancer patients and healthy donors

T7 phage-displayed antigen microarrays were used for autoantibody profiling in melanoma and prostate cancer patients as well as healthy individuals in order search for autoantibody signatures capable of distinguishing between the cancer patients and cancer-free individuals. The microarray was tested with sera from 55 prostate cancer and 121 melanoma patients, and 136 healthy individuals. Each array was probed with 1:200 diluted preabsorbed serum and antibody recognising T7 phage tail protein (for normalisation of serum-derived signal against the number of phage particles in each spot) that were detected with Cy5-labelled anti-human IgG and Cy3-labelled anti-mouse secondary antibodies, respectively. For each spot mean Cy5 and Cy3 signals were background-subtracted, averaged between replicates, Cy5/Cy3 ratios were calculated for each antigen and normalised by that of non-recombinant phages. The average variation between replicates typically was <10%, which is generally acceptable variation in immunoassays.

The analysis of the microarray revealed the existence of differences in the autoantibody profiles between healthy individuals and cancer patients; an example of microarray images demonstrating such differences in sera from melanoma patient and healthy donor is shown in Figure 26. All the obtained microarray data were subjected to the preliminary analysis in order to determine the antigens that are predominantly recognised by the sera from cancer patients. The average Cy5/Cy3 ratios in healthy donors (HD) was calculated for each antigen, and a threshold of four standard deviations (SD) above the average Cy5/Cy3 ratios in HD was set to define antigens preferentially reacting with cancer patients' sera (i.e. the antigens with equal serum reactivities from HD and cancer patients would be defined a serum-negative using this threshold).

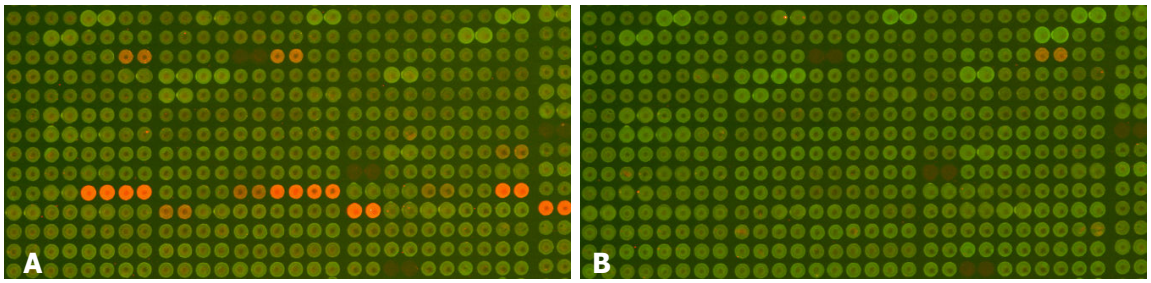


Figure 26. A comparison of serum profiles from single melanoma patient (A) and healthy donor (B). Partial microarray images (composite Cy3 (phage) and Cy5 (serum) signals) showing autoantibody profiles in serum from a melanoma patient and a healthy donor.

Data obtained from these preliminary analyses suggested that 245 and 182 antigens are preferentially recognised by sera form patients with prostate cancer and melanoma, respectively, and not with healthy donors' sera. The top ten markers in melanoma and prostate cancer are listed in the Tables 18 and 19, respectively. Among the best markers in both studies there are known CT antigens CTAG1B (NY-ESO1) and CTAG2 (LAGE-1), as well as previously unknown antigens like SLU7 and LRRC50 in melanoma and EEF1A1 in prostate cancer, and a number of out-of-frame peptides, most of which more likely represent mimotopes of cancer-associated antigens. The detection of out-of-frame peptides amongst the best markers points to their value for autoantibody biomarker discovery.

Table 18. Top ten antigens specifically recognised by sera from melanoma patients.

Clone	Gene	Reading frame	HD (%) n=77	Mel I (%) n=22	Mel II (%) n=23	Mel III (%) n=23*	Mel IV (%) n=40*	Mel total* (%) n=121	p-value[#]
CT38	CTAG1B, N-ter	In-frame	0.0	13.6	8.7	17.4	27.5	19.8	0.0007
CT159	CTAG2, N-ter	In-frame	0.0	13.6	8.7	17.4	30.0	18.2	0.0015
CT43	CTAG2, C-ter	In-frame	0.0	13.6	8.7	13.0	25.0	16.5	0.0030
CT291	HADH	- strand	0.0	13.6	0.0	4.3	22.5	14.9	0.0062
CT290	SPEN	- strand	0.0	13.6	0.0	4.3	17.5	12.4	0.0173
CT104	CTAG1B, C-ter	In-frame	0.0	9.1	8.7	13.0	25.0	12.4	0.0173
39	SLU7	In-frame	0.0	9.1	13.0	8.7	10.0	9.1	0.0633
62	RP11-485O17	gDNA	0.0	4.5	8.7	8.7	10.0	7.4	0.1166
19	LRRC50	In-frame	0.0	0.0	4.3	13.0	7.5	7.4	0.1166
126	FAM122A	Frame 2	0.0	4.5	4.3	0.0	2.5	5.8	0.2087

* Includes samples form various melanoma stages and patients undergoing immunotherapy;
[#] p-value calculated by applying χ^2 test to compare antibody frequencies in HD and melanoma patients.

Table 19. Top ten antigens specifically recognised by sera from prostate cancer patients.

Clone	Gene	Reading frame	Healthy donors (%) (n=59)*	Prostate cancer (%) (n=55)	p-value[#]
7	EEF1A1	In-frame	0.0	16.4	0.0149
10	PLCG2	Frame 3	0.0	16.4	0.0149
CT208	CTAG1B, N-ter	In-frame	0.0	14.5	0.0264
334	TUBG1	- strand	0.0	14.5	0.0264
CT159	CTAG2, N-ter	In-frame	0.0	12.7	0.0460
CT43	CTAG2, C-ter	In-frame	0.0	12.7	0.0460
CT104	CTAG1B, C-ter	In-frame	0.0	10.9	0.0788
CT291	HADH	- strand	0.0	10.9	0.0788
371	RP11-677O4	gDNA	0.0	10.9	0.0788
CT290	SPEN	- strand	0.0	9.1	0.1322

* Sera from age and sex-matched healthy donors were used in the study;
[#] p-value calculated by applying χ^2 test to compare antibody frequencies in HD and prostate cancer patients.

Amongst the human tumour antigens, CT antigens are considered to be one of the most promising targets for cancer immunotherapy[490]; besides, autoantibodies against these antigens have been shown to be valid biomarkers for cancer detection as well as monitoring the course of tumour treatment and prediction of relapse[496,497]. The obtained serological data regarding CT antigens are summarised in Table 20. In line with results of other studies, the highest frequency of antibody responses were observed against CTAG antigens followed by MAGEA and GAGE[491,498]. Increasing frequency of autoantibody responses against CTAG antigens was observed in stage III and IV melanoma patients; however, ~14% of patients with stage I melanoma had high-titre anti-CTAG antibodies thus indicating that such responses may be used for early detection of cancer.

Table 20. Reactivity against CT antigens (%).

	CTAG1B, N-ter	CTAG1B, C-ter	CTAG1B-ORF-2	CTAG2, N-ter	CTAG2, C-ter	MAGEA	SSX2	GAGE	MAGEB	MAGEC	CTAGE	CTAGE pseudo	THEG	SPANX
Mel I (n=22)	13.6	9.1	-	13.6	13.6	-	-	-	-	-	4.5	-	4.5	-
Mel II (n=23)	8.7	8.7	-	8.7	8.7	4.3	-	-	-	-	-	4.3	-	-
Mel III (n=23*)	17.4	13.0	8.6	17.4	13.0	21.7	-	13.0	-	4.3	-	-	-	4.3
Mel IV (n=40*)	27.5	25.0	-	30.0	25.0	2.5	2.5	5.0	-	-	-	2.5	-	-
PrCa (n=55)	12.7	10.9	-	10.9	10.9	10.9	5.5	7.3	3.6	9.1	9.1	3.6	1.8	1.8
HD (n=136)	-	-	-	-	-	-	-	1.3	-	-	-	2.6	-	-

* Including melanoma patients undergoing immunotherapy.

According to the obtained data, 91% and 70% of all analysed prostate cancer and melanoma patients' sera, respectively, showed reactivity against at least one of the cancer-specific antigens indicating that on the basis of the autoantibody repertoire analyses it is possible to develop sero-diagnostic test with significantly higher specificity and sensitivity than any other of the currently used biomarker – for example, the PSA test reaches ~85-90% sensitivity and only ~40% specificity[5,493,494]. Besides, it was estimated that stage I melanoma can be detected with 64% sensitivity, stage II – with 55%, stage III – with 76% and stage IV – with 77% sensitivity thus in principle demonstrating that autoantibodies are produced in early-stage cancer patients.

However, the 30% of the melanoma and 9% prostate cancer sera analysed did not react with any of the cancer-specific antigens represented on the microarray, indicating that either the selected approach for the microarray data analyses (i.e. the set threshold) did not allow the detection of the low titre cancer-specific antibody responses or humoural responses in these patients are poorly induced. To gain an insight whether these patients indeed lack the tumour-induced antibody responses, three such melanoma (Appendix 5) and two prostate cancer patient's sera were individually subjected to the selection of antigens from T7 phage display libraries (pooled T7-T1, T7-Mel-525 and T7-Mel-445, and pooled T7-T1 and T7-OPCT, respectively). Taking together, immunoscreening of 2-3 x 10^4 pfu from each biopan resulted in the identification of 6-13 different serum-reactive clones, in total serum-reactivity against 26 and 24 antigens were found in the prostate and melanoma patients, from these three in-frame antigens were identified by melanoma sera – i.e. HNRNPH3, NUP153 and NUP43, all belonging to the protein families, other members of which have been recorded in the SEREX database and are known to be associated with autoimmune conditions[474,495], indicating that these antibody responses most likely are not cancer-associated. Whether the detected humoural responses against the other serum-reactive phage clones are cancer-restricted remains to be determined.

It has to be stressed that the signals in these preliminary analyses were classified simply as positive or negative according to the set threshold; nevertheless, the obtained data are quantitative and currently we are working on the development of mathematical methods that could be used for the data analysis, and this is not a trivial task as most of the tools for microarray data analysis are developed for the analysis of DNA arrays and aimed for the detection of small differences consistent in all the samples while we are simultaneously searching for responses against several hundreds of different targets. However, these preliminary results have demonstrated the proof of principle that autoantibodies can be successfully used as cancer biomarkers, yet the current test did not reach sufficiently high sensitivity. To improve this, the next version of phage-displayed antigen microarray is currently being generated

on the basis of all the antigens identified in this study; besides, phage clones expressing known and putative in-frame TAAs were included in the phage panel used for the microarray generation (not listed) that presumably could increase the sensitivity of the yet to be determined cancer-specific autoantibody biomarker set.

The antigen repertoire of the next version of the microarray

The development of the final version of the phage-displayed antigen microarray is underway. The serum-reactive antigen repertoire of this microarray include all serum-reactive phage clones identified by this study: (i) 529 antigens that have been selected by sera from 29 melanoma patients, (ii) 179 antigens that have been selected by 34 serum specimens from 22 prostate cancer patients, (iii) 233 antigens that have been selected by 27 gastric cancer patients' sera as well as (iv) 123 antigens that have been identified by immunoscreening of gastric cancer-derived library by sera from 15 gastritis patients. Among the printed serum-reactive antigen clones there were 92 that encoded for in-frame antigens while the overwhelming majority of the rest displayed unnatural peptides generated from inserted fragments of intergenic DNA, mtDNA, rRNA, 5'or 3' UTRs or cDNAs cloned out-of-frame relatively to the T7 phage coat protein 10B. Apart from such clones, there was a proportion of serum-reactive clones (n=33) that encoded for hitherto uncharacterised gene products. In total, the antigen microarray will comprise ~1300 recombinant phage clones as well as 40 randomly distributed non-recombinant T7 phage clones.

To our knowledge, this is so far the largest antigen microarray generated by means of the T7 phage display system if considering the number of different serum-reactive antigen phage clones represented. In parallel to our study, several studies have been conducted in order to search for autoantibody signatures specific for cancer patients by means of T7-displayed protein microarrays. Most of them performed the antigen discovery on microarrays thus omitting immunoscreening step. Fernandez-Madrid *et al*. analysed humoural responses in breast cancer by probing of a microarray comprising 938 random serum-reactive clones (selected after 4-10 rounds of positive selection), which resulted in the identification of 12 phage predictor group (possessing 77% sensitivity and 88% specificity). This group included Annexin XI-A, a diagnostic biomarker distinguishing between invasive ductal carcinoma from ductal carcinoma *in situ*[425]. Zhong and colleagues succeeded in development of an antigen microarray comprising 4000 phage clones that were randomly selected from non-small cell lung cancer-derived libraries after four rounds of differential biopanning. From these, only 212 phage clones revealed to be serum-reactive and included a set of five antigens, humoural responses against which were able to diagnose this malignancy with 89% accuracy[426,427]. Another study by Tainsky lab explored ovarian cancer – differentially selected 480 T7 phage-displayed antigens were serologically analysed on a macro-array, which resulted in the identification of 62 individual serum-reactive clones that were further analysed in large-scale on a microarray; artificial neuronal network (ANN) analyses of the microarray data identified a group of diagnostic markers containing six mimotopes (reaching 32% sensitivity and 94% specificity)[452,499]. Lin *et al.* identified 130 marker set (having 80% sensitivity and 90% specificity) by analysing humoural responses in head and neck cancer patients by using an antigen microarray comprising 5133 phage clones randomly chosen after 3-5 rounds of differential biopanning[428], while in a recent study by Chen and colleagues an antigen microarray comprising 2304 phage clones selected from lung adenocarcinoma-derived cDNA library after five rounds of differential biopanning was used for the definition of an autoantibody profile specific to this malignancy – autoantibodies against 22 peptides were capable of discriminating between cancer and control group with 85% sensitivity and 86% specificity[465]. The same experimental design was applied to search for autoantibody biomarkers in prostate cancer – serological probing of 2304 element microarray resulted in the identification of 22 antigen detector group possessing 88% specificity and 82% sensitivity[453]. The T7 phage display-based discovery of autoantibody biomarkers has not been applied to melanoma and gastric cancer so far.

Taking together, these studies clearly demonstrate that the combination of individual autoantibody biomarkers enables more precise and sensitive detection of cancer. Considering the complexity of the microarray generated by the selected approach and the preliminary data obtained from analysing the first prototype of the antigen microarray, it could be expected that its application may result in the identification of autoantibody signatures possessing even higher specificity and sensitivity.

Forthcoming applications of the antigen microarray

Three types of cancer have been searched for proteins eliciting B cell responses in the current study – melanoma, prostate cancer and gastric cancer. Early diagnosis of gastric cancer is crucial problem in Eastern Europe, including Latvia – this region is amongst those with the highest gastric cancer incidence, and the high mortality of this cancer in Latvia (accounting for ~10% of cancer-related deaths) is mostly due its late diagnosis, strongly indicating a need for novel biomarkers of early detection of this malignancy. Melanoma, although not the most frequent one, is the deadliest form of skin cancer[120]. It differs from the majority of solid tumour types with its high metastatic rates, low survival of patients with the metastatic disease and resistance to conventional treatment[121]. Although the diagnosis of skin-born melanomas may seem to be feasible task, the role of early melanoma diagnosis can not be overestimated – when melanoma is diagnosed and treated early, the chances for long-term survival are significantly higher. Besides, most of the current cancer immunotherapy trials are focused on fighting advanced stage melanomas[348], and there is increased need for biomarkers that could contribute to monitoring of vaccine-induced immune responses as well as predict whether the patient will benefit from the specific treatment or not. Prostate cancer, in its turn, is actual problem worldwide – it is most prevalent type of cancer among men, and its incidence and mortality, although has experienced slight decrease in the last years, is still high[136]; dramatic increase in prostate cancer incidence and mortality has been experienced also in Latvia (Figure 4). The widely applied PSA testing fails to identify a small but significant proportion of aggressive tumours, and only about one third of men with positive PSA test results have a positive biopsy[5,493]. Not only better diagnostic, but also prognostic non-invasive biomarkers are needed for prostate cancer to predict the natural course of disease, which would allow to choose more appropriate treatment for each patient[493]. Apart from that, the treatment of hormone-refractory prostate cancer is challenging task, and several emerging therapies for the treatment of this disease are currently under investigation, including immunotherapy[500,501], pointing to the need of novel biomarkers to assess the probability that a patient will benefit from a particular treatment.

It has been shown by several studies that antibodies against proteins expressed by transformed cells are generated early in cancerogenesis or even in pre-malignant lesions[7,236,461]. However, the role of such antibody responses in anti-tumour immune response and their clinical significance is still uncertain and controversial. On the one hand, the cancer immunoediting concept suggests that the cancer immunosurveillance mechanisms ensure the elimination of significant proportion of early (premalignant) tumours while minority of them progresses into bulk tumours due to the immunoselection of less immunogenic cell variants (section 1.2.2.)[230]. This implies that signatures of anti-tumour immune responses might be found in individuals who will never develop clinically detectable disease. For example, Spisek *et al*. have demonstrated that patients with monoclonal gammopathy, a premalignant condition of myeloma, frequently develop spontaneous protective humoural and cellular immune responses to SOX2, a putative cancer stem cell antigen. Although this antigen is expressed in myeloma, spontaneous immunity to SOX2 was not detected in any myeloma patient[461]. On the other hand, studies by Blankenstein *et al.* have demonstrated that premalignant occurrence of antigen-specific antibodies correlates with CTL unresponsiveness and enhanced tumourigenesis[7,236]. Collectively, these and other studies indicate that antibodies may reveal to be positive or negative predictors of proceeding from pre-malignant to malignant state of cancerogenesis depending on what kind of initial immune response – host protective or tumour-promoting – is generated. However, it is generally not known against what antigens the humoural immune response is directed in either of these cases; as well it is not known whether the antigen repertoires are different or overlapping.

Therefore, in order to identify potential autoantibody **biomarkers for early detection of cancer**, the repertoire of selected antigens represented on the microarray will be systematically evaluated by assessing their reactivity with panels of sera from patients with different stage tumours as well as premalignant lesions (e.g. atrophic gastritis), and age and sex-matched healthy donors. Besides, sera from patients suffering from various autoimmune disorders will be included in the analyses in order to define the autoantibody repertoire overlap between autoimmune conditions and cancer.

The fact that autoantibodies can be exploited for early detection of cancer has been in principle demonstrated by several studies. For example, Zhong *et al*. applied T7 phage-displayed antigen

microarray consisting of 212 serum-reactive phage clones to analyse autoantibody profiles in early stage lung cancer, which resulted in the identification of five markers that could discriminate between stage I lung cancer and healthy control sera with 91.3% sensitivity and 91.3% specificity. Moreover, on the basis of this marker set it was possible to correctly classify 32 of 40 cancer sera from blood samples drawn one to five years before the diagnosis[427]. In another study by Chapman *et al*. a serological test was developed on the basis of seven known TAA panel, which were able to detect invasive breast cancer with 64% sensitivity and ductal carcinoma *in situ* with 45% sensitivity while the specificity of the panel was 85% in both cases[502].

It is not entirely clear what immunological processes are reflected by SEREX-defined autoantibodies and what biological roles they have. An autoantibody profile is often considered as a reflection of an individual's immunological experience with minor effector role in anti-tumour immune responses. However, since the induction of high-titre IgG responses is generally dependent upon CD4+ T cell help, the repertoire of SEREX-defined antigens reflect the repertoire of activated CD4+ T cells. In some cases integrated CD4+, CD8+ and B cell responses have been demonstrated against SEREX-defined antigens including but not restricted to NY-ESO-1[314], MAGE-A3[503] and melanocyte differentiation antigen RAB38/NY-MEL-1[504,505]. In such cases the presence of autoantibodies may signify for CTL responses against the particular antigen in the patient. Nevertheless, this is unlikely to be the case for the majority of SEREX-defined antigens – considering all the accumulated data from SEREX studies, it has become increasingly evident that an overwhelming proportion of antigens identified by SEREX are non-altered self proteins (i.e. wild-type autoantigens) that are expressed in different levels in normal tissues and are not known to be aetiologically implicated in autoimmune conditions[6].

Hiroshi Shiku and colleagues have performed a series of studies in mice to address the role of SEREX-defined autoantibodies in the anti-tumour immune response. Collectively they have demonstrated that: (i) co-immunisation of mice with a strong CTL epitope and an unrelated SEREX-defined wild-type antigen remarkably enhances CD8+ T cell response against the CTL epitope and heightens resistance to pulmonary metastasis, which is mediated by CD4+ helper T cells[506]; (ii) immunisation with SEREX-defined wild-type antigen alone, in turn, resulted in immunosuppression mediated by CD4+CD25+ regulatory T cells[507] letting to propose that the SEREX-defined antigens are recognised by Tregs and may induce T cells with suppressive activity[508]; (iii) IFN-γ produced by CD8+ T cells in co-immunisation experiments had a crucial role in shifting the balance from the generation and/or activation of CD4+CD25+ regulatory T cells to augmented CTL activity[509]. Collectively these studies suggest that the SEREX-defined autoantibody repertoire can be considered as the refection of both, T helper and regulatory T cells; however, it remained to be determined what mechanism underlay the concordant repertoire of self-reactive B cells and CD4+CD25+ regulatory T cells.

Irrespective of what kind of immunological processes – CTL or Treg-mediated – the defined autoantibody profiles may reflect, they might reveal valid biomarkers that could be exploited for the prediction of the outcomes of immunotherapy thus enabling more individualised treatment strategies. Although so far, at least to our knowledge, the humoural responses induced by treatment have not been surveyed by means of established high-throughput autoantibody profiling techniques, the analyses of antibody responses against individual antigens or sets of TAAs have in principle demonstrated that immunotherapy and standard treatment induced B cell responses in cancer patients. For example, Ehlken with colleagues showed that very individualised serological responses are induced by vaccination of melanoma patients with cytokine gene-modified autologous tumour cells[408]. Twenty-seven TAAs were identified by SEREX using pool of five post-vaccination sera, and against 18 of them antibody responses were found to be induced by the vaccination. Moreover, authors showed a putative correlation between the induction of humoural responses and increased lytic activity of CTLs in several patients; however, the relevance of such autoantibodies as predictive markers for protective response or as indicative markers for CTL activity could not be assessed, most likely due to very individualised humoural responses in each patient. Next, in a large survey of 200 melanoma patients treated with high-dose adjuvant IFN-α2b the induction of common autoantibodies was seen in 26% of the cases, which showed statistically significant correlation with the prolonged overall and relapse-free survival in melanoma patients[471]. In line with this study, the correlation of a DC vaccine-associated elevation of serum anti-nuclear antibody to better clinical responses has been shown[510].

Besides, autoantibody induction as a result of standard treatment has been demonstrated – a study by Nesslinger *et al.* showed that in 14-29% of prostate cancer patients cancer-specific autoantibody responses are elicited in four to nine months after radiation and hormone therapy; the treatment-induced autoantibodies recognised a diverse repertoire of TAAs and in most cases persisted for at least 30 months[409].

Thus, encouraged by these findings, in forthcoming experiments we intend to systematically analyse the repertoire of the T7 phage-displayed antigens on microarray, which comprises antigens that have been selected by post-vaccination sera by serum samples obtained from the patients enrolled in the Mel-TYR, Pr-ONY and Pr-NRH immunotherapy trials. The definition of autoantibody profiles in pre-vaccination and post-vaccination sera and the correlation of potential post-vaccination specific autoantibody signatures in these patients with the obtained clinical and immunological data could lead to the identification of specific **prognostic markers of the patients' response to the immunotherapy**, which could be further used for improved management of individual patient's treatment. Apart from biomarker discovery, the data obtained from such analyses may help in more comprehensive understanding of the nature of induced immune responses and clinical responses of the patients to the vaccination. In this regard, it would also be interesting to assess the type of generated autoantibody responses in cancer patients – differential probing of the microarray with secondary antibodies specific to a particular immunoglobulin G isotype would be potentially helpful for the understanding of the role of autoantibodies since type 1 humoural responses (i.e. IgG1 and IgG3 subtype antibodies) are associated with acute inflammatory states, which are linked to protective anti-tumour immunity[219,374].

CONCLUSIONS

The results of the dissertation can be summarised as follows:

1. The application of the conventional SEREX approach to gastric adenocarcinoma resulted in the identification of 14 serum-reactive clones, eight from which encoded for novel tumour-associated antigens. Autoantibody responses against a half of the identified antigens were detected exclusively in cancer patients' sera thus suggesting their relevance as sero-diagnostic or predictive markers.
2. Detailed molecular characterisation of two of the identified tumour-associated antigens – TACC1 and NUCB2 – revealed that the expression of cancer-associated alternative splice isoforms and altered, cancer-specific posttranslational modifications, respectively, may underlay their immunogenicity in cancer patients.
3. Characterisation of two different lytic phage surface display vector systems, the λKM8 and T7Select 10-3b, demonstrated that:
   (i) both vector systems were equally capable to display a range of known antigens from 15 TAA families;
   (ii) the T7-based system is more sensitive regarding the detection of serum antibodies against the majority of displayed TAAs, which most likely is due to better spatial accessibility of the antigens when displayed on T7 phage;
   (iii) the λKM8 system, although less sensitive for antibody-mediated detection, is more appropriate for the identification of novel immunotherapy target genes while the T7Select 10-3b system, in contrast to λKM8, allows the expression of out-of-frame peptides that might represent valid autoantibody biomarkers or novel tumour-specific antigens;
   (iv) the biological properties of both phage display systems allow their exploitation for the production of phage-displayed antigen microarrays and microarray-based serum autoantibody profiling.
4. The phage-displayed antigen microarray analyses demonstrated that microarray-based screening and plaque immunoscreening have comparable, yet not identical sensitivities for the detection of autoantibodies.
5. The recombinant antigen display density on T7 phage was shown to be variable – it ranged from 4-70 copies per phage particle; besides, it does not correlate directly with the size of recombinant protein thus implying that signal strength in immunoscreening is not dependent solely upon serum antibody titre and that the usage of this system might result in false negative calls.
6. A T7 phage display-based SEREX technique was elaborated and applied for the identification of comprehensive set of antigens in melanoma, prostate cancer, gastric cancer and gastritis patients that resulted in:
   (i) the assessment of autoantibody responses in 26 melanoma patients – a set of 993 serum-reactive phage clones was isolated and the clone insert sequence analysis revealed that they represent 505 different antigens; from these, 196 antigens were selected by post-vaccination sera;
   (ii) the identification of a prostate cancer-associated antigen set comprising 362 serum-reactive clones that represented 179 different antigens, it was achieved through the analysis of pre- and post-vaccination serum antibody repertoires from patients responding and not responding to immunotherapy (in total, 34 serum samples from 22 prostate cancer patients);
   (iii) the assessment of autoantibody responses in 27 patients with gastric cancer of different subtypes – 423 serum-reactive clones representing 233 different antigens were identified;
   (iv) the assessment of autoantibody responses in 15 patients with atrophic gastritis – 205 serum-reactive clones encoding for 123 individual antigens were identified, which may contribute to the discovery of novel biomarkers for early detection of gastric cancer.

7. The clone insert sequence analysis revealed that only minority of the recombinant proteins (~9%) are expressed in-frame to T7 phage surface protein 10B while the vast majority of them most likely represent mimotopes; serum antibody responses to the selected out-of-frame as well as in-frame antigens were confirmed by Western blot.
8. Approximately 20% of the in-frame antigens represented gene products that have been previously identified by the conventional SEREX studies while ~37% of them belong to protein families, other members of which have been identified by SEREX thus indicating that the antigen repertoire defined by T7 phage display-based SEREX partially overlaps with that of conventional SEREX as well as demonstrates that the developed approach enables the identification of novel TAAs.
9. The data obtained from profiling of autoantibody responses in 121 melanoma, 55 prostate cancer patients' and 136 healthy donors' sera by using a prototype of T7 phage-displayed antigen microarray, which was developed on the basis of all the identified melanoma and prostate cancer antigen clones, showed that 245 and 182 antigens are preferentially recognised by sera form patients with prostate cancer and melanoma, respectively, and not with healthy donors' sera.
10. The preliminary analyses suggest that 91% of prostate cancer and 70% of melanoma patients' sera recognised at least one of the antigens with cancer-specific reactivity thus demonstrating the relevance of autoantibody profiling in the diagnostics of cancer. Moreover, 64% of stage I melanoma patients had autoantibodies against at least one of the antigens indicating that these signatures could be used for the early detection of cancer. The development of the final version of antigen microarray comprising ~1300 antigen clones and survey of autoantibody responses in larger sets of sera is underway to define autoantibody biomarkers for cancer diagnosis and early detection, and prediction of the patients' response to immunotherapy.

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APPENDIX

Appendix 1.

Primers used for the TAA mini-library construction

Antigen	Primer	Primer sequence*
NY-ESO1 N-ter	F	5'-ATT------GCC ATG CAG GCC GAA GGC-3'
	R	5'-TT**GCGGCC GC**T CAG CTC YGC TTC CATGG-3'
NY-ESO1C-ter	F	5'-ATT------GAG AGC CGC CTG CTT GAG TT-3'
	R	5'-TT**GCGGCC GC**T GCG CCT CTG CCC TGAGG-3'
MAGEA N-ter	F	5'-ATT------ATG CCT CTT GAG CAG AGG-3'
	R	5'-TT**GCGGCC GC**T CAG CAT TTC TGC CTT TGT G-3'
MAGEA C-ter	F	5'-ATT------GAG CCG GTC ACA AAG GCA G-3'
	R	5'-TT**GCGGCC GC**T YTC AYG CAG GGR TGG G-3'
SSX	F	5'-ATT------ATG AAC GGA GAC GAC GCC-3'
	R	5'-TT**GCGGCC GC**T CTT GGG CAT GAT CTT CG-3'
BAGE	F	5'-ATT------AGT AAG ATG GCG GCC AGA G-3'
	R	5'-TT**GCGGCC GC**T GAA GAT GAA GCA CAG AGC-3'
GAGE	F	5'-ATT------ATG AGT TGG CGA GGA AGA TC-3'
	R	5'-TT**GCGGCC GC**T TTC TTC AGG CGT TTT CAC C-3'
CT45	F	5'-ATT------ATG ACC GAT AAA ACA GAG AAG-3'
	R	5'-TT**GCGGCC GC**T TTT CTT CTT AAG KTG CT-3'
SPANX	F	5'-ATT------ATG GAC AAA CAA TCC AGT G-3'
	R	5'-TT**GCGGCC GC**T ATA CTT TGC AGG TMT TTC A-3'
MAGEB	F	5'-ATT------ATG CCT CGT GGT CAG AAG AG-3'
	R	5'-TT**GCGGCC GC**T TTT CAG CAT TTC TSC CTT-3'
MAGEC	F	5'-ATT------CTT GCC CTG AKA GAA GTG G-3'
	R	5'-TT**GCGGCC GC**C GTT GCT GGA CAT GAC ACT G-3'
THEG	F	5'-ATT------ATG CCC ATG TCT GAG GTG TC-3'
	R	5'-TT**GCGGCC GC**T GCC AGG CCT GGG TTG ATC-3'
CTAGE1v2	F	5'-ATT------CCT ATG TTT GTC ATC ATA TC-3'
	R	5'-TT**GCGGCC GC**T TCT TAG ATT TAC TCC TTC-3'
CTAGE	F	5'-ATT------TTA GAA GGA GAA AGA AAC C-3'
	R	5'-TT**GCGGCC GC**A ATG CTC TCT GCC AAA TGC TG-3'
CTAGE N-ter (1)	F	5'-ATT------GTT GTG GCA GCA CTG CCTG-3'
	R	5'-TT**GCGGCC GC**G GTT TCT TTC TCC TTC TAA G-3'
CTAGE N-ter (2)	F	5'-ATT------CTT TCT GGA CTA ATT GAA GA-3'
	R	5'-TT**GCGGCC GC**G GTT TCT TTC TCC TTC TAA G-3'
CTAGE C-ter	F	5'-ATT------CCA AAT ACA GCA TTT GGC AG-3'
	R	5'-TT**GCGGCC GC**G GTA AGG AGG AAA ACC CCT-3'
MTA1	F	5'-ATT------TTT GCC ATG AAG ACC AGG C-3'
	R	5'-TT**GCGGCC GC**T GTG CTG CTC GTA GCT GCG-3'
LDHC	F	5'-ATT------ATG GAT CTT CAG CAT GGC AG-3'
	R	5'-TT**GCGGCC GC**T GAT AAT TTC ATA GGC ACT-3'
MLANA[#]	F	5'-ATT**GTCGAC** ATG CCA AGA GAA GAT GCTC-3'
	R	5'-TT**GCGGCC GC**A AGG TGA ATA AGG TGG TGG-3'
TYR[#]	F	5'-ATT**GTCGAC** CTC ACT TTA GCA AAG CAT ACC-3'
	R	5'-TT**GCGGCC GC**A AGG CAG AAA AGC TGG TGC-3'

* The dashes represent the restriction sites introduced in PCR products – i.e. either SalI (GTCGAC) or SpeI (ACTAGT) which enable further cloning into T7Select 10-b or λKM8 vector arms, respectively.

[#] These antigens were cloned into T7Select 10-b vector only and used directly for printing on glass slides

Appendix 2.

Comparison of the antigen repertoire identified by immunoscreening of enriched library by MA002079 serum after the first and the second round of biopanning.

After the first round of biopanning		After the second round of biopanning	
Gene name	**No of isolated clones**	**Gene name**	**No of isolated clones**
C12orf22	3	**ZNF140***	**54**
LDHB	3	BCKDHB/GALNTL2	5
ZNF354B	3	ATF2	1
CTD-2199O4	2	HSF1	1
CTSL	2	TNIK	1
CYCS	2	**TOPORS**	**1**
FLJ22471	2		
LOC400843/EEF2	2		
LANCL1	2		
SPATA3	2		
ALLC	1		
C12orf22	1		
C20orf26	1		
C20orf47	1		
DMRTC2	1		
RP5-1163J12/mtDNA[#]	1		
TCF12/HNRPA2B1	1		
YBX2/MORF4L1	1		
ZNF566/RP11-543N17	1		
IL13RA1	1		
KIF27	1		
LBNL H167	1		
MASK-BP3 (ANKHD1)	1		
mtDNA	1		
POLS	1		
RP11-153P14	1		
RP11-195B21	1		
RP11-232C20	1		
RP11-391P4	1		
RP11-6B7	1		
SAAL1	1		
SE57-1	1		
SHPRH	1		
TOPORS	**1**		
UCP2	1		
ZNF140	**1**		
No of genes: 36	**Total: 49**	**No of genes: 6**	**Total: 63**

***** The overlap of the serum-reactive clone repertoires are marked in bold;
[#] The "/" indicates for hybrid cDNAs generated as a result of cloning artefact.

Appendix 3A. A screenshot of Tumour Antigen database start page.

Sequences | ESTs | BLAST | Patients | Libraries | Serum | Plates | Users | Current user: Zane

[add] [refresh] [log out]

search clear

[add]				Library x	Clone name x	High titre stock x	Screening serum x	Length x	Complete x	Reading frame ^	Gene name x	Gene description x	Protein Structure Motifs x	Serex ID x	CGAP expr.data.
[local]	[Prot]	[exp.data]	[est]	T7-Mel- 525	91	[blank]	Mel-PV Pool 5	33	complete	ND	unknown				
[local]	[Prot]	[exp.data]	[est] (32)	T7-Mel- 525	77	[blank]	Mel-PV Pool 5	310	complete	ND	FLJ25967	hypothetical gene supported by AK098833			
[local]	[Prot]	[exp.data]	[est] (32)	T7-Mel- 525	15	[blank]	Mel 525	266	complete	ND	P18SRP	Novel splice variant of p18 splicing regulatory protein ?			
[local]	[Prot]	[exp.data]	[est] (27)	T7-Mel- 525	67	[blank]	Mel-PV Pool 5	229	complete	correct	OSBPL10	oxysterol binding protein-like 10 [natural STOP]		Not found	
[local]	[Prot]	[exp.data]	[est] (32)	T7-Mel- 525	68	[blank]	Mel-PV Pool 5	298	complete	correct	CCDC128	coiled-coil domain containing 128		Not found	
[local]	[Prot]	[exp.data]	[est] (65)	T7-Mel- 525	59	[blank]	Mel-PV Pool 5	278	complete	correct	ZCCHC9	zinc finger, CCHC domain containing 9 [C-ter]		Not found	
[local]	[Prot]	[exp.data]	[est] (294)	T7-Mel- 525	45	[blank]	Mel 525	272	complete	correct	C6orf111, SFRS18	SR rich protein; splicing factor, arginine/serine-rich 130 [contains STOP]		Not found	
[local]	[Prot]	[exp.data]	[est] (90)	T7-Mel- 525	13	[blank]	Mel 525	380	complete	correct	SMARCC1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 1	Leucine zipper	Related to 988 (SMARCA4)	
[local]	[Prot]	[exp.data]	[est] (3000)	T7-Mel- 525	7	[blank]	Mel 525	196	complete	correct	EEF1A1	eukaryotic translation elongation factor 1 alpha 1 (CTCL tumor antigen; EF1a-like protein; cervical cancer suppressor 3)		2672	
[local]	[Prot]	[exp.data]	[est]	T7-Mel- 525	85	[blank]	Mel-PV Pool 5	215	complete	incorrect	gDNA	gDNA RP11-424N23			
[local]	[Prot]	[exp.data]	[est] (58)	T7-Mel- 525	80	[blank]	Mel-PV Pool 5	546	complete	incorrect	gDNA	BAC clone RP11-901L6			
[local]	[Prot]	[exp.data]	[est] (1)	T7-Mel- 525	35	[blank]	Mel 525	935	complete	incorrect	gDNA	BAC clone RP11-115L11			
[local]	[Prot]	[exp.data]	[est]	T7-Mel- 525	24	[blank]	Mel 525	334	complete	incorrect	gDNA	WI2-3130B22, Alu Sx			

Appendix 3B. A screenshot of individual antigen entry of the Tumour Antigen database.

SEQUENCE	
Additional info:	ESTs: (90) , Local: (0) , Prot: (0) , Expression data: (0)
Library name:	T7-Mel- 525
Clone name:	13
High titre stock:	Yes
Screening serum:	Mel 525
Biopanning serum:	Mel 525
Complete:	complete
Reading frame:	correct
Notes:	without EcoRI adapter
Gene name:	SMARCC1
Alternative names:	BAF155, CRACC1, Rsc8, SRG3, SWI3
mRNA sequence:	NM_003074.2|
Chromosomal localization:	3p23-p21
Serex ID:	Related to 988 (SMARCA4)
Protein Structure Motifs:	Leucine zipper
Gene description:	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 1
Summary:	The protein encoded by this gene is a member of the SWI/SNF family of proteins, whose members display helicase and ATPase activities and which are thought to regulate transcription of certain genes by altering the chromatin structure around those genes. The encoded protein is part of the large ATP-dependent chromatin remodeling complex SNF/SWI and contains a predicted leucine zipper motif typical of many transcription factors. SMARCC1, SMARCC2, and SMARCD1 are assigned to chromosomal regions that are frequently involved in somatic rearrangements in human cancers.
Creation Date:	31.01.2007 18:07
Created by:	Aija
Sequence:	1: TCAGATGCTT CCCTTGGGCC TTTGGCCTAC CAGCCTGTCC CCTTCAGTCA GTCAGGAAAT 61: CCAGTTATGA GTACTGTTGC TTTTTTGGCA TCTGTGGTGG ACCCTCGCGT GGCATCTGCT 121: GCAGCAAAAG CGGCTTTGGA GGAGTTTTCT CGGGTCCGGG AGGAGGTACC ACTGGAATTG 181: GTTGAAGCTC ATGTCAAGAA AGTACAAGAA GCAGCACGAG CCTCTGGGAA AGTGGATCCC 241: ACCTACGGTC TGGAGAGCAG CTGCATTGCA GGCACAGGGC CCGATGAGCC AGAGAAGCTT 301: GAAGGAGCTG AAGAGGAAAA AATGGAAGCC GACCCTGATG GTCAGCAGCC TGAAAAGGCA 361: GAAAATAAAG TGGAAAATGA
Protein Sequence:	1: SDASLGPLAY QPVPFSQSGN PVMSTVAFLA SVVDPRVASA AAKAALEEFS RVREEVPLEL 61: VEAHVKKVQE AARASGKVDP TYGLESSCIA GTGPDEPEKL EGAEEEKMEA DPDGQQPEKA 121: ENKVEN
ContigExpress project:	13, Mel 525.cep

Appendix 4.

Sera used for melanoma antigen identification.

Serum code	Melanoma sera* used for the immunoscreening of		
	T7-T1 library	TAA mini-libraries	Melanoma libraries
LGP-Mel 143	✓		
LGP-Mel 150	✓		
MA002079	✓	✓	
MA00GG	✓	✓	
MA00AM	✓ (Mel-Pool-5)	✓	
MA00SK	✓ (Mel-Pool-5)	✓	
MA00WF	✓ (Mel-Pool-5)	✓	
UKRV-Mel -06	✓ (Mel-Pool-5)	✓	
MA00BL	✓ (Mel-Pool-5)		
UKRV-Mel-31		✓	
MA00550		✓	
MA001111		✓ (PV)	
MA001404		✓ (PV)	
MA001643		✓	
MA000273		✓	
MA000155		✓ (PV)	
MA000148		✓ (PV)	✓ (Mel-PV-Pool-5)
MA000703		✓ (PV)	✓ (Mel-PV-Pool-5)
MA000951		✓ (PV)	✓ (Mel-PV-Pool-5)
MA000161		✓ (PV)	✓ (Mel-PV-Pool-5)
MA000513		✓ (PV)	✓ (Mel-PV-Pool-5)
MA000445		✓	✓
MA000525		✓	✓
MA001816		✓	✓
LGP-Mel-T2			✓
LGP-Mel-T4			✓
Mel-BRS59-PV[#]			✓
Mel-MS49-PV[#]			✓
Mel-SK42-PV[#]			✓
Total (∑=29):	**9**	**21**	**13**

***** If not otherwise indicated, pre-vaccination (Mel-TYR trial) sera was used;

[#] These melanoma sera were not reacting with any of the melanoma-specific antigens on the antigen microarray, and were chosen for additional immunoscreening of T7-diplayed cDNA expression libraries (see section 3.4.2.).

Abbreviations: PV – post-vaccination (Mel-TYR trial).