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Vaccination in gastrointestinal cancer

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To my family

Abstract

Advances in immunology have increased the possibility to develop therapeutic cancer vaccines (TCV), as a complementary approach to standard treatment. The goal of a successful cancer vaccine is to induce a potent long-lasting immune response against the tumour with limited toxicity on normal cells. Most tumour cells express tumour-associated antigens (TAA), which can act as targets for the immune system. However, most TAAs evade recognition by the immune system to avoid auto-immunity, as many TAAs coexist in normal tissues. Commonly expressed TAAs in gastrointestinal malignancies are Carcinoembryonic antigen (CEA) and telomerase which both have been used as targets in cancer immunotherapy.

The aim of this thesis was to explore the immunogenicity and safety of a CEA based protein and DNA TCV in patients with colorectal cancer (CRC) in the adjuvant setting and telomerase vaccination (GV1001) in patients with advanced pancreatic adenocarcinoma (PC).

A long-term follow-up of CRC patients immunized with recombinant (rCEA) ± Granulocyte-macrophage colony-stimulating factor (GM-CSF) was conducted. Induction of anti-CEA IgM, IgA and IgE antibodies was monitored from 36 months after start of immunization. GM-CSF significantly augmented the anti-CEA response for all three classes *($p<0.05$)*. A significant correlation between survival and high IgA anti-CEA titers was noted *($p=0.02$)*. Anti-CEA IgA antibodies could lyse CEA positive cells in antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) assays.

The type, severity and duration of side-effects of CEA66-DNA vaccination in combination with cyclophosphamide and GM-CSF, was evaluated in 10 CRC patients. CEA66-DNA was delivered by a needle-free device system (Biojector). Adverse events (AE) were mild and transient, without any grade 3 or 4 AEs. No clinical signs of autoimmunity were seen.

In an explorative study using CEA66-DNA (producing unglycosylated CEA) and wild type (tetwt)-CEADNA (producing glycosylated CEA) for immunization in combination with cyclophosphamide and GM-CSF immune responses (proliferation assay, ELISPOT, cytokine secretion assay) were analyzed in the adjuvant setting of CRC patients. 10 patients received intradermal (i.d.) or intramuscular (i.m.) CEA66-DNA by Biojector at weeks 0, 2 and 6 (part 1). 10 patients; (part 2), received tetwt-CEADNA 400 µg i.d. by needle followed by electroporation at weeks 0 and 12. Part 3 (n=6) included patients primed with CEA66-DNA and boosted with tetwt-CEADNA. GM-CSF and cyclophosphamide was also included. In total, 16 out of 20 (80%) patients mounted a single assay cellular response; 10/10 (100%) in part 1 and in 6/10 (60%) of the patients in part 2 *($p=0.025$)*. Immune responses were weak but durable.

We also assessed the safety and immunogenicity in advanced PC patients using a 16 aa telomerase peptide (GV1001) for vaccination in combination with GM-CSF and gemcitabine as first line treatment. Three different vaccine treatment schedules (groups A, B, C) were used. In groups A and B, differing only in the dose of GM-CSF, a total of 67% of the patients showed an induced telomerase response. An induced ras (antigenic spreading) specific immune response was noted. All responses were weak and transient. A significant decrease in regulatory T cells over time was noted in patients in groups A and B.

In conclusion, durable weak anti-CEA immune responses were seen following rCEA and CEA-DNA vaccination in CRC patients in the adjuvant setting. Weak and transient anti-telomerase responses following peptide vaccination were induced in patients with advanced PC. To develope a therapeutic concept of clinical significance measures have to be taken to optimize vaccine strategies.

Keywords: CEA, hTERT, immunotherapy, cancer vaccine

List of publications

I. **Staff C**, Magnusson CGM, Hojjat-Farsangi M, Mosolits S, Liljefors M, Frödin JE, Wahrén B, Mellstedt H, Ullenhag G J. Induction of IgM, IgA and IgE antibodies in colorectal cancer patients vaccinated with a recombinant CEA protein. *J Clin Immunol. 2012, 32:855-865*.

II. **Staff C**, Mozaffari F, Haller K, Wahren B, Liljefors M. A phase I safety study of DNA immunization targeting carcinoembryonic antigen in colorectal cancer patients. *Vaccine. 2011, 29:6817-6822*.

III. **Staff C**, Mozaffari F, Haller KB, Frödin JE, Wahren B, Mellstedt H, Liljefors M. DNA immunization targeting carcinoembrynic antigen in colorectal cancer patients. *(Manuscript to be published)*

IV. **Staff C**, Mozaffari F, Frödin JE, Mellstedt H, Liljefors M. Telomerase-peptide vaccination (GV1001) together with gemcitabine in advanced pancreatic cancer patients. *(Manuscript to be submitted)*

List of abbreviations

5-FU	5-Fluorouracil
ADCC	Antibody-dependent cellular cytotoxicity
AE	Adverse events
Ag	Antigen
AJCC	American Joint Committee on Cancer
APC	Antigen presenting cell
Bev	Bevacizumab
CDC	Complement-dependent cytotoxicity
CEA	Carcinoembryonic antigen
Cet	Cetuximab
CFC	Cytokine flow-cytometry
CR	Complete remission
CRC	Colorectal carcinoma
CT	Computed tomography
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic lymphocyte antigen 4
Da	Dalton
DC	Dendritic cell
DTH	Delayed type hypersensitivity
ELISA	Enzyme linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot
EGFR	Epidermal growth factor receptor
EP	Electroporation
EpCAM	Epithelial cell-adhesion molecule
ER	Endoplasmatic reticulum
F(ab)	Fragment antigen binding
FasL	Fas ligand
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HD	Healthy donor
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
hTERT	Human telomerase reverse transcriptase
i.d.	Intradermal
IFN	Interferon
Ig	Immunoglobulin
i.m.	Intramuscular
IL	Interleukin
KRAS	Kirsten rat sarcoma viral oncogene
LPS	Lipopolysaccarid
mAb	Monoclonal antibody
mCRC	metastatic colorectal cancer
MDSC	Myelo-derived suppressor cells
MHC	Major histocompatibility antigen
MRI	Magnetic resonance imaging
NF-κβ	Nuclear factor κβ
NK	Natural killer cell
NKT	Natural killer T cells
NO	Nitric oxide

OIIR	Overall induced immune response
OS	Overall survival
Pan	Panitumumab
PBMC	Peripheral blood mononuclear cell
PFS	Progression free survival
PD1	Programmed death-1
PDL1	Programmed death ligand-1
PHA	Phytohemagglutinin
PPD	Purified protein derivate of tuberculin
PSA	Prostate specific antigen
rCEA	Recombinant carcinoembryonic antigen
RNA	Ribonucleic acid
SAE	Serious adverse events
s.c.	Subcutaneous
SD	Stable disease
SFU	Spotforming units
TAA	Tumor associated antigen
TAM	Tumor infiltrating macrophage
TCR	T cell receptor
TCV	Therapeutic cancer vaccine
tetwt	Tetanus wild type
Th	T helper
TGF-β	Transforming growth factor-β
TIL	Tumor infiltrating lymphocytes
TLR	Toll-like receptor
TME	Total mesorectal excision
TNF	Tumor necrosis factor
TT	Tetanus toxoid
TTP	Time to progression
VEGF	Vascular endothelial growth factor

Contents

1 THE IMMUNE SYSTEM

"Den som är väldigt stark måste också vara väldigt snäll" Ur Känner du Pippi Långstrump

The immune system is crucial to human survival and can be defined as our defence towards pathogens. The word immune stems from the Greek word *immunitas* meaning "liberated or excluded from" for example the effects of a specific pathogen. In brief, two groups of the immune system coexist and interact constantly; the innate (non-adaptive) and the acquired immune system. The innate is quick to respond to pathogens, the acquired is slow but on the other hand it gives rise to a specific memory towards the pathogen – rendering a quicker and more precise immune response at the next encounter. *Thus, the innate immune system carries the memory of the species whereas the acquired immune system carries the memory of the individual.*

The *innate responses* use phagocytic cells (neutrophils, monocytes and macrophages), cells able to release inflammatory mediators (basophils, mast cells, and eosinophils), natural killer (NK) cells and natural killer T cells (NKT cells). Besides NK cells, interdigitating dentritic cells (DCs) comprise essential links between innate and acquired immunity [1] The molecular compartments of innate responses include complement, acute-phase proteins and cytokines. Cytokines are proteins made by cells that affect the behaviour of other cells and bind to specific receptor on their target cells. Cytokines made by lymphocytes are often called interleukins (IL).

Acquired responses involve the proliferation of antigen-specific B and T cells, which occurs when the surface receptors of these cells bind to the cognate antigen. Specialized antigen-presenting cells (APCs) display the antigen to the major histocompability complex (MHC) molecules and cooperate with them in response to the antigen. B cells respond to the immunogens by secreting immunoglobins (Ig), the antigen-specific antibodies mainly responsible for elimination of extracellular agents. There are two main types of T cells: helper T cells (Th) (CD4+) and cytotoxic T cells (CD8+). Although T cells can help the B counterpart to make antibodies, they also contribute to the eradication of intracellular pathogens by activating macrophages and directly killing infected cells.

1.1 Antigen presentation to T cells

The immune system recognizes antigens presented by two types of MHCs, Class I and II. In humans, the MHC Class I molecule corresponds to the human leukocyte antigen (HLA)-A,- B, and C- molecules, and MHC Class II molecules correspond to HLA-D molecules.

MHC Class I molecules are expressed on all nucleated cells and in general present 8-11 amino acid-long peptides derived from *intracellular proteins* digested in the proteasome complex. These MHC class I peptide complexes are recognized by CD8+ T cells. If a CD8+ cell finds a MHC Class I peptide -complex, with its unique T cell receptor (TCR), it undergoes clonal expansion and differentiates into mature CD8+ and memory CD8+ cells. Mature CD8+ cells migrate through the body, searching for cells that possess peptide-

complexes, to which the TCR will bind and destroy. Memory CD8+ cells persist and will multiply if they are re-exposed to the same MHC peptide complex again.

MHC Class II molecules are mainly expressed on APCs, such as dendritic cells (DCs), macrophages and activated B cells. These cells engulf proteins and present small peptides, in conjunction with MHC Class II to CD4+ T cells. These peptides are longer, usually 13 to 17 amino acids (or more), and derive from *exogenous proteins* endocytosed into the cell. If a CD4+ cell recognizes such a MHC class II-peptide complex, it will divide and mature producing many cytokines and express many surface elements, thereby regulating other aspects of the immune system.

1.2 Activation of naïve T cells

All types of acquired immune responses are started by the activation of antigen-specific T cells and the whole process of activation and differentiation occurs in the immediate nursing environment of APCs. Two signals are necessary for the initial activation of naïve T cells. The "*two signal model*" predicts that when an antigen is presented by an MHC molecule (signal 1) together with costimulatory molecules (signal 2) expressed by APCs, an immune response will be generated [2, 3]. Several costimulatory molecules have been described; one important complex is CD28 on T cells binding to B7´s on the APC. According to the danger theory, signal 1 in the absence of signal 2 leads to tolerance irrespective if the antigen is self or non-self. In the absence of danger signals, the APCs are not induced to express costimulatory molecules and the result of such presentation will be tolerance rather than activation. Any cell, however, when stressed and in danger, sends alarm signals to activate APCs.

The not-ever present expression of B7´s on APCs ensures that naïve T cells do not respond to their specific antigens in the absence of danger. This provides a mechanism for peripheral tolerance that prevents naïve T cells with receptor that bind self-antigens from being activated and differentiate into auto reactive effector T cells.

1.3 CD4+ Th1 and Th2 cells

Towards the end of their proliferation, activated T cells, acquire the capacity to synthesize the proteins they need to perform the specialized functions as effector T cells.

For CD4+ cells these proteins comprise cell-surface molecules and soluble cytokines that activate and help other types of cells to participate in the immune response. Because of its facilitating functions, CD4+ cells are called T helper (Th) cells. Defining the range of behaviour are two types of helper cells called CD4+ Th1 or CD4+ Th2. The main cytokines secreted by Th1 cells, IL-2 and INF-γ, promote cytotoxic T cell lymphocytes (CTL) generation and activate NK cells. They may also secrete Granulocyte-macrophage colony-stimulating factor (GM-CSF), which promote a more efficient antigen presentation. The main cytokines secreted by Th2 cells, IL-4, IL-5, IL-6 and IL-10, mainly lead to B cell differentiation. Thus an immune response biased towards Th1 cells is described as being *cell-mediated immunity*, dominated by CD8+ T effector cells whereas an immune response biased towards Th2 cells is dominated by antibodies, described as a component of the *humoral immunity*.

1.4 CD8+ T cells

CD8+ T cells (CTL) are the principle effector cells of the acquired immune response, which mediate antigen-specific, MHC-restricted, cytotoxic effects. Antigen-specific CD8+ T cells become activated by the TCR-MHC Class I peptide interaction on an APC, together with help from activated T cells. The activation of CTL occurs either via direct recognition of antigen on the tumor cell, or by presentation of tumor antigens on APC's (cross presentation) (see Section 1.6), which subsequently prime CTL. This leads to clonal expansion of antigen-specific CTL that will lyse cells that express the same peptide-MHC Class I complex. CTL mediated cytotoxicity is mediated primarily by cytotoxins (perforin, granzymes) or via interactions between via interactions between cell surface molecules on the CTL (Fas ligand) and the target cell (Fas molecules) [4].

1.5 B cells

Each B cell has a receptor molecule with a single specificity called B cell receptor (BCR) or immunoglobulin in its secreted form. Five major Ig classes exist; IgM, IgD, IgG, IgA and IgE and these determine the functional activity of the antibody. After developing B cells leave the bone marrow, they begin to recirculate between the blood, secondary lymphoid tissue and the lymphatic vessels. At this stage the B cells are not fully mature, expressing surface IgM and IgD. Naïve B cells (that have not yet met their specific antigen) passing through the secondary lymphoid tissue pick up, (via surface immunoglobulins), process and present their specific antigens if present there. Upon meeting Th2 cells at the same site, the T cell receptor screen the peptides presented by the MHC Class II molecule on the B cell. Th2 cells then provide signals that activate the B cell to proliferate and differentiate. Some differentiate into plasma cells (the effector cell), which secrete IgM in the secreted form rather than the surface-bound form. Other activated B cells differentiates and undergo isotype switching and hypermutation, producing other classes of antibodies. Cytokines secreted by Th2 cells – IL-4, IL-5 and transforming growth factor-β (TGF-β) influence B cells to switch their isotype. Some cells will develop into resting memory B cells, capable of a quicker and stronger immune response at the next encounter with the antigen. In humans IgG can be further subdivided into four subclasses of human IgG (IgG1, IgG2, IgG3 and IgG4), whereas IgA antibodies exist as two subclasses, (IgA1 and IgA2). IgG1 and IgG3 are the most potent mediators of antibody dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). Circulating antibodies can bind to antigens on microorganisms or transformed cells and destroy them either directly or by ADCC and CDC. Some antigens, notably components of bacterial cell walls and capsules, are capable of inducing a rapid antibody response that does not require T cell help.

1.6 Cross-presentation and cross-priming

As a general rule, exogenous antigens enter the MHC Class II processing pathway and are presented to CD4+ T cells. By a process known as cross-presentation exogenous antigens are delivered into the MHC Class I processing pathway and presented to CD8+ T cells [5].

Cross-presentation is involved in responses to viral infections, transplanted organs and in tumors [5, 6]. TAAs, released by tumor cell or administered as a therapeutic cancer vaccine (TCV), are taken up by APCs, which process and present them on APC cell surface restricted by their own MHC Class I or II molecules.

APCs such as DCs can then efficiently prime naïve T cells if they display MHC-antigen complexes (signal 1) together with costimulatory molecules (signal 2). When an immune response is initiated by cross-presentation this is known as a cross-priming of the immune response [6].

2 THE INTERACTION BETWEEN THE IMMUNE SYSTEM AND CANCER

2.1 Pioneers of modern oncoimmunology

Immunotherapy for cancer was initially used over a century ago. In the 1800s German physician Paul Ehrlich and the American physician William Bradley Coley proposed that vaccination might be used against cancer. Ehrlich delivered the idea of "the magic bullet" that would specifically kill malignant cells but failed to demonstrate that weakened cancer cells, injected in patients, may generate antitumor immunity [7]. On the other hand, inspired by cancer patients who underwent regression following streptococcal fevers, Coley became convinced he could use bacteria to kill cancer cells. Coley created a mixture of heat-killed bacteria (Coley toxin) in 1896 that mediated potent antitumor effects in patients [8, 9]. One of the major hurdles against development of anticancer vaccines, the "self/non-self" dichotomy, was later theorized by Frank Macfarlane Burnet in 1949 and in fact hampered further development for quite some time [10]. According to this theory, tumors – constituting self-tissues – are non-immunogenic and therefore insensitive to immunotherapeutic interventions. In 1994, Polly Matzinger however presented a theory that proposed that the immune system do not react to non-self (while sparing self) constituents, but would rather respond to situations of "danger", either of exogenous (non-self) or endogenous (self) origin i.e. irrespective of the origin [11]. Hence, conditions that have long been thought to be immunologically silent, including cancer and trauma, are now thought to be able to activate the immune system [12-14].

2.2 Tumor immunosurveillance and immunoediting

The long-standing theory of "*immunosurveillance*" suggests that cells are constantly monitored by an ever-alert immune system and that this surveillance is responsible for recognizing and eliminating incipient cancer cells. Accordingly, solid tumors have somehow managed to avoid detection by the immune system or have been able to limit the extent of immunological killing, thereby evading eradication [15].

The developments of carcinogen-induced tumors were assessed in mice deficient for various components of the immune system. It was observed that tumors arose more frequently and grew more rapidly in immunodeficient mice compared to immunocompetent controls. Tumor incidence was, in particular, increased in mice with deficiencies in the development or function of CD8+, CD4+ Th1 helper cells, or natural killer (NK) cells. Results from this experimental mode demonstrated that both the cellular arms of the innate and the acquired immune system contributed significantly to immune surveillance and tumor eradication [16, 17].

Spontaneous antitumor immunity that eliminates tumors or delays their growth involves production of INF-ν , as well as the generation of CTLs [18, 19]. Mice deficient in interferon (IFN-γ) are more susceptible to induced cancers, as are mice lacking T cells and perforin, a key effector protein in T cell cytotoxicity [18, 19].

A number of observations from humans indicate that functional immunosurveillance do exist. Patients with ovarian and colon cancer that are heavily infiltrated by CTLs and NK have a better prognosis than those who lack such tumor infiltrating lymphocytes (TILs) [20-22]. The hypothesis is further strengthened by data that indicate that patients indeed develop immune responses to tumor associated antigens [23, 24] (see Section 3.3.1) and also by the striking evidence of certain cancers in immunocompromised individuals [25].

According to the currently accepted model of immunoediting, neoplasms acquire the ability to develop and grow in spite of the immune system in three sequential steps. Initially, the growth of cancer is efficiently controlled due to robust tumor-specific immune responses (elimination). As the elimination phase is normally unable to completely eradicate malignant cells, some of them may acquire alterations that either reduce their immunogenicity or increase their resistance to the cytotoxic functions to the immune system (equilibrium). Such cells eventually grow uncontrolled (escape), leading to a clinically manifest neoplasm [18].

Editing refers to the changes that occur spontaneously as a tumor develops under the influence of an intact immune system and the understanding of immunoediting mechanisms has important implications for cancer immunotherapy in humans, and *avoiding immune destruction* has recently been proposed as an emerging hallmark of cancer by Hanahan and Weinberg [15].

2.3 The immune response may promote tumorigenesis

The immune system interacts with tumors throughout their development, and the consequences of this interaction have implications for cancer therapy. Some host responses may inhibit tumor growth, as discussed previously, but immune responses can also *promote* cancer by provoking chronic inflammation and drive growth, survival and angiogenesis (see Figure 1).

The proinflammatory cytokine tumor necrosis factor (TNF-α), largely produced by macrophages and mast cells, is implicated in early cancer development as well as in infections. TNF-α promotes cell growth, survival and angiogenesis and the recruitment of immune effector cells. The events downstream of TNF-α are not well known, but nuclear factor-κB (NF-κβ) family transcription factors appear to be linked to TNF-α and cancer [26].

NF-κβ controls the transcription of several proteins involved in cell survival, division, growth and is important for the production of many inflammatory cytokines, including TNF-α itself. In an animal model for colon cancer, ablation of NF-κβ in immune cells led to reduction in tumor growth and ablation in the colonic epithelium decreased tumor incidence [27]. For further details, see Figure 1.

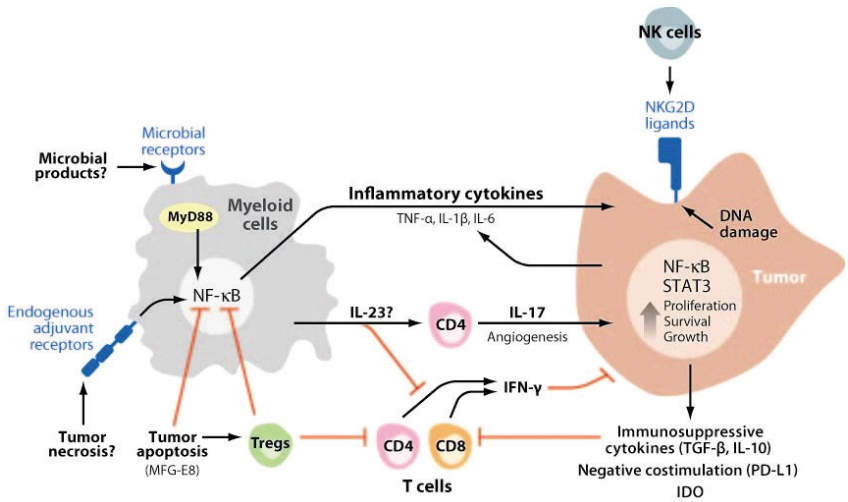

Figure 1. Complex interactions with the immune system shape tumor development. Chronic inflammatory response can be initiated by microbial products or endogenous adjuvants released from necrotic cells. These signals activate nuclear factor-κB (NF-κB) in myeloid cells, leading to the production of inflammatory cytokines (TNF-α, IL-1β, and IL-6), which in turn activate NF-κB in the tumor. Tumor-intrinsic NF-κB activation promotes growth, survival, and proliferation. IL-23 produced by myeloid cells can promote the generation of IL-17-secreting T cells, which can further support tumor growth. Genotoxic stress in tumor cells can activate NK cell ligands, which can synergize with endogenous tumor-specific $CD4^+$ and $CD8^+$ T cells that produce IFN-γ and restrict tumor development. Tumors can suppress nascent immune responses through a variety of mechanisms, including immunosuppressive cytokines (TGF-β and IL-10) and metabolites [indoleamine 2,3-dioxygenase (IDO)] and the expression of negative costimulatory molecules [programmed death ligand 1 (PD-L1)]. Tumors can also promote T_{reg} recruitment and differentiation, in part through the recognition of apoptotic cells by the MFG-E8 (milk fat globule epidermal growth factor 8) pathway.
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2.4 Tumor immune escape mechanisms

Tumors themselves might develop several strategies allowing them to escape immune surveillance and destruction. Tolerance induction is one of the major mechanisms involved and is induced by several steps.

- Mutation or down-regulation of MHC molecules, particularly MHC Class I, has been documented in several tumor types [28]. In many cases, individual HLA alleles are selectively lost, but complete MHC Class I loss has been observed in tumors. The most common mechanism for total MHC Class I loss is mutations in the β2-microglobulin genes. Loss of MHC Class I is an escape mechanisms for CTL recognition.

- Tumor cells often have altered expression of molecules involved in antigen presentation and processing, due to down modulation. Such transporter proteins associated with antigen processing (TAP1), low molecular mass-protein 2 (LMP2) and LMP1 proteasome components, results in suboptimal peptide delivery to MHC Class I [28, 29] and impaired antigen presentation.

- Expression of anti-apoptotic molecules and down-regulation or mutation of pro-apoptotic molecules renders tumor cells resistant to apoptosis [30]. Due to acquired

defects in Fas signal transduction, cancer cells may be resistant to the Fas ligand (FasL)-mediated cytotoxicity of antitumor T and NK cells. Fas resistance enables the tumor cells to express their own FasL, which delivers an apoptotic death signal to activated T and NK cells, thereby inhibiting lymphocyte infiltration into FasL-expressing tumor nests.

- Tumor cells themselves or tumor stroma can secrete immune suppressive cytokines such as IL-10, TGF-β and vascular endothelial growth factor (VEGF). For example, TGF-β inhibits the activation, proliferation and differentiation of T cells, and suppress the activity of CTLs and DC, while inducing differentiation of regulatory T cells (T_{regs}) [31-33]. Tumors also express the enzyme indoleamine 2, 3-dioxygenase (IDO) which prevents proliferation of CD8+ cells and induce apoptosis of CD4+ T cells [34, 35] (see Figure 1).

2.5 Suppressive tumor infiltrating leucocytes

Evidence suggests that, unlike cells found in lymphoid organs that respond to acute infections, immune cells in tumors are dysregulated and functionally impaired and use immune-regulatory patterns to generate an *immune-suppressive environment*. Tumor infiltrating leukocytes subsets can play strikingly antagonistic functions [36].

The suppressive microenvironment of tumors is further established through the activity of *myeloid derived suppressor cells (MDSC), activated macrophages, and* T_{regs} [37-40].

MDSCs are described as a population of $CD11b^+$ $Gr\text{-}1^+$ cells in mice with the ability to suppress CD8+ T cell antitumor activity [41, 42]. GM-CSF is important in driving the expansion of these cells [43]. Studies have shown that MDSCs are a heterogeneous population of cells consisting of cells of monocytic as well as granulocytic origin [43]. In humans, the markers for MDSCs are not clearly defined, but the phenotype resides within a population of LIN-HLA-DR-CD33+ cells [43].

MDCS`s express high levels of two enzymes called inducible nitric oxidase synthase (iNOS) and arginase, leading to depletion of, for T cells, essential nutrients [43, 44]. Arginase depletes L-arginine from the tumor environment and induces cell cycle arrest in T cells [45]. iNOS leads to increased production of nitric oxide (NO). High levels of NO block specific signal transduction in T cells, leading to suppressed production of IL-2 [43, 44]. Other mechanisms include sequestration of cysteine, an essential amino acid for T cells and secretion of IL-10 which has suppressive properties. The ability to skew the differentiation of CD4+ T cells to regulatory cells has also been suggested [46]. Taken together, MDSCs exercise many functions that blunt effector T cell responses.

Tumor associated macrophages (TAM), are divided into two subsets, M1 and M2. INF-γ drives the polarization towards M1, IL-4 polarizes macrophages towards M2 profile[36] promoting tumor evasion [40, 47, 48]. M2-skewed macrophages produce lower levels of proinflammatory cytokines, such as IL-1β, TNF-α and IL-12 and higher levels of immunosuppressive cytokines, such as IL-10, TGF-β and VEGF. Dysfunctional macrophages have an impaired ability to mediate direct lysis of malignant cells in comparison to M1-type cells [49].The most frequent TAM phenotype seems to be M2 [36].

Regulatory T cells (T_{regs}) also infiltrate many tumors [50], their immune suppressive profile was established in a series of experiments showing that CD25+ T cell depletion improved antitumor immunity in mouse models [51-53]. Characteristics of CD4+CD25highFOXp3+ T cells (T_{regs}) are their anergic state and their capacity to inhibit CD8+, T cells, DCs, NK cells, NKT cells and B cells [54]. T_{regs} constitute 5-10% of human CD4+ T cells and have a pivotal role in maintenance of immunologic self-tolerance and avoiding auto-immunity [55]. They are subdivided into "natural" T_{regs} (nT_{reg}) cells. nT_{reg} develop in the thymus and act through contact-depending mechanisms, maintaining self-tolerance and preventing autoimmunity [56], "induced" T_{reg} (iT_{reg}) cells, are induced to differentiate in the periphery and mediate suppression by contact-independent mechanisms, including production of TGF-β [56, 57]. T_{regs} also secrete suppressive IL-10 creating an environment that blunts effector antitumor responses by CD4+, CD8+ and NK cells and by acting as a competitive sink regarding IL-2, due to high affinity for IL-2, in the tumor environment [58, 59]. Activated T cells become exquisitely sensitive to the lack of IL-2, leading to cell death under conditions of IL-2 deprivation [60].

The transcription factor FoxP3 is critical for the development of the functional characteristics of regulatory T cells [61, 62]. FoxP3-knockout mice, and humans with homozygous mutation of FoxP3 (encoded on the X chromosome) develop autoimmune syndromes involving multiple organs [57]. An increase of T_{regs}, both in the periphery and within tumors, is seen in human cancer [63, 64] and the presence of these cells correlates with poor prognosis in ovarian cancer, breast cancer and hepatocellular carcinoma [50].

2.6 Immunosuppressive checkpoints

Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), is an important immune-checkpoint receptor that normally prevents excessive and uncontrolled immune responses. It is expressed exclusively on T cells where it primarily regulates the amplitude of the early stages of T cell activation.

CTLA-4 counteracts the activity of the T cell co-stimulatory receptor CD28 [57]. CD28 does not affect T cell activation unless the TCR is first engaged by cognate antigen. Once antigen recognition occurs, CD28 signalling strongly amplifies TCR signalling to activate T cells. CD 28 and CTLA-4 share identical ligands; CD80 (also known as B7.1) and CD86 (also known as B7.2). As CTLA-4 has a much higher affinity for both these ligands, it has been proposed that it´s expression on the surface of T cells dampens the activation of T cells by outcompeting CD28 binding to CD80 and CD86 [57], thereby acting *immunosuppressive*. Even though CTLA-4 is expressed by activated CD8+ effector T cells, the major physiological role of CTLA-4 seems to be through distinct effect on two major subsets of CD4+ T cells; downmodulation of helper T cell activity and enhancement of the immunosuppressive activity of regulatory T_{regs} [57].

In contrast to *CTLA-4* the major role of *Programmed death 1 (PD1)* is to limit the activity of T cells in peripheral tissues at the time of an inflammatory response to infection and to limit autoimmunity. PD1 thus predominantly regulates effector T cell activity within tissue and tumors, whereas CTLA-4 predominantly regulates T cell activation [57]. PD1 is highly expressed on T_{regs} where it may enhance its proliferation in the presence of its ligand [57]. PD1 is also expressed on B cells and NK cells – limiting their lytic activity [57]. PD1 is

normally transiently induced following immune activation but chronic antigen exposure, such as occurs with chronic viral infection or cancer, can lead to high levels of persistent PD1 expression, which induces a state of exhaustion or anergy among cognate antigen-specific T cells [57].

The PD1 ligands are commonly upregulated on the tumor cell surface in many human cancers [65]. The major PD1 ligand that is expressed is programmed death ligand 1 (PDL1) [57]. PDL1 (present or not present) status in tumors has been correlated with poor prognosis, better prognosis or without correlation to prognosis, probably due to differences in analyses, tumor types and stages [57].

3 IMMUNOTHERAPY IN CANCER

3.1 Background

General Components

Immunotherapy against cancers includes active, passive or immunomodulatory strategies. While passive immunotherapy administers exogenously produced components such as antibodies, active immunotherapies augment the ability of the patients own immune system to mount an immune response able to recognize and eliminate malignant cells. Immunomodulatory agents are not targeted at specific antigens but enhance general immune responsiveness.

3.2 Passive immunotherapy

Impressive clinical responses to monoclonal antibodies (mAb) therapy have already been achieved and several are part of routine treatment, both in the adjuvant and palliative setting. Several murine, chimeric as well as humanized mAbs have been approved. Of these, no less than 14 mAbs, including naked reagents as well as mAbs coupled to antibiotics or radioactive isotopes, are nowadays authorized for use in cancer patients [66, 67]. mAbs that potentially exert antineoplastic effects belong to one of six classes [68]:

1) mAbs that target cancer cell-intrinsic prosurvival signal transduction cascades, e.g. cetuximab (Cet) targeting epidermal growth factor receptor (EGFR) (in CRC)
2) mAbs that interrupt thropic interaction between malignant cells and stroma, e.g. bevacizumab (Bev) targeting VEGF (in several adenocarcinomas)
3) mAbs that recognize antigens expressed on surface of tumor cells and initiate ADCC as well as CDC, e.g. Rituximab targeting CD20 (in lymphoma)
4) Bispecific mAbs, who can bind two antigenic targets, while retaining ability to exert immune effector functions e.g. catumaxomab, an anti CD3 and anti-epithelial cell-adhesion molecule (EpCAM) mAb (in EpCAM+ tumors)
5) Immunoconjugates e.g ^{90}Y-ibritumomab (radionucleotide coupled) anti CD20 mAb (in lymphoma)
6) Immunostimulatory mAbs, that facilitate immune response by balancing immunosuppressive mechanisms, e.g. ipilimumab targeting CTLA-4 (in melanoma).

3.3 Active immunotherapy

General considerations

Active specific immunotherapy principally involves the use of TCV, with the aim to evoke a tumor specific immune response in cancer patients.

The major challenge in developing a successful TCV strategy is shifting the balance from tolerance to self-antigens towards a long-lasting therapeutic anti-tumor immunity, without inducing autoimmune toxicity to normal cells.

Animal models have played a crucial role in understanding the mechanisms of tumor immunology and different modalities of immunization and experimental models have been employed to determine whether TAAs can induce immune responses that are able to hamper the growth of a clinically manifest cancer.

The promising results have driven the attempts to move these applications from bench to bedside. To this day, however, only three vaccines have been approved by the Food and drug administration (FDA) for use in humans; Cervarix® and Gardasil® (in fact constituting preventative measures towards development of cervical carcinoma) and sipuleucel-T (Provenge®), a cellular preparation for therapy of metastatic hormone-refractory prostate cancer. In the veterinary field an immunotherapeutic DNA vaccine encoding human tyrokinase, Oncept™, has been approved as TCV for malignant melanoma a in dogs. Oncept™ generates an effective antibody response against the dog tyrokinase and prolongs the lives of dogs even with advanced stage disease.

3.3.1 Tumor associated antigen for therapeutic cancer vaccines

Virtually any mutant, overexpressed or abnormally expressed protein in cancer cells, can serve as a target for cancer vaccines. Since the pivotal work done by pioneers in oncoimmunology, characterization and identification of hundreds of TAAs has been conducted and additional insights into the mechanisms whereby TAAs can break self-tolerance and elicit an immune response has been provided. Simply put, TAAs can be classified into four classes, see Table 1.

Table 1. Tumor antigens

Classes of tumor antigens [69]
(1) Truly exogenous, non-self TAAs (invariably of viral origin)
(2) Unique, mutated TAAs (stemming from cancer cell-specific genetic alterations)
(3) Idiotypic TAAs (the unique way whereby the B cell receptor expressed by some clonal hematopoietic malignancies is rearranged)
(4) Shared TAAs (which are also expressed by normal cells, mostly at lower levels)

Shared TAAs include cancer-testis and differentiation antigens that are either silent or expressed at only low levels in normal tissue but are transcriptionally activated in certain tumors e.g. CEA in CRC and human telomerase reverse transcriptase (hTERT) in pancreatic cancer.

Optimally designed TCV should combine the best tumor antigens and delivery strategies to achieve a clinical result. A National Cancer Institute (NCI) immunotherapy workshop was held in 2007 to rank agents with high potential to serve as immunotherapeutic drugs. The ranking was based on the likelihood for efficacy. A priority-ranked list of cancer antigens weighed the criteria for the "ideal" cancer antigen to provide focus for prioritized translational research [70]. The resulting criteria were as follows: (1) therapeutic function, (2) immunogenicity, (3) role of the antigen in oncogenicity, (4) specificity, (5) expression level and percentage of antigen-positive cells, (6) stem cell

expression, (7) number of patients with antigen-positive cancers, (8) number of antigenic epitopes and (9) cellular location of antigen expression.

3.3.2 Desired mechanisms of action of TCV

The processes required to mount an effective anti-tumour response can be subdivided into four different steps [71]. In the first, TAAs must be captured, processed and presented by DCs (or directly presented by tumour cells). The second step requires suitable activation/maturation signals that allow DC to differentiate migrate to lymph nodes and present TAAs to naïve T cells. The third step involves expansion of T cells in sufficient numbers capable of recognizing and eliminating tumour cells. In the final step, antigen-educated T cells must leave the lymph node, traffic to infiltrate the tumour and persist long enough to kill the malignant cells.

Thus CD4+, CD8+ T cells, and B cells can all be activated in the immune response against cancer. For intracellular TAAs, CD8+ CTLs may be the primary effector cell, but the activity and longevity of these will likely be enhanced by CD4+ Th1 cell activation. For cell surface antigens, *both CTL and humoral responses may mediate antitumor activity*, again attesting to the importance of activating both CD8+ and CD4+ T cells [72].

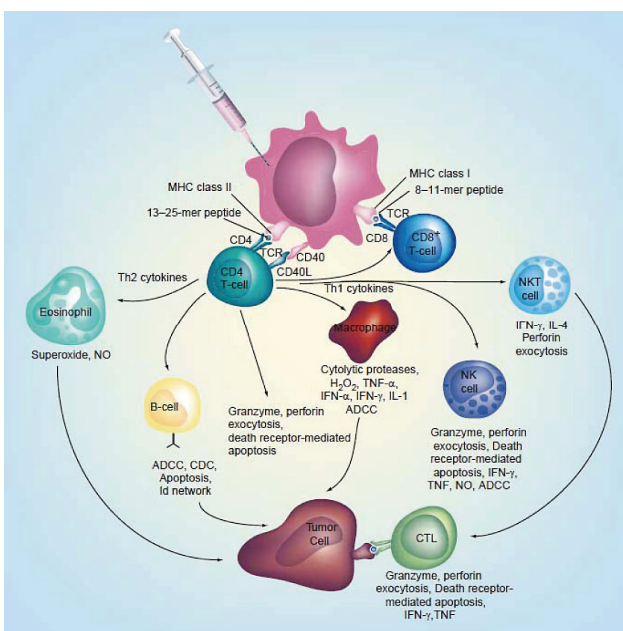

Figure 2. Summary of potential effector mechanisms involved in antitumor immunity induced by vaccine. Immature or intermediate dendritic cells acquire the antigen(s) at the site of vaccination. In response to maturation and activation signals, dendritic cells migrate to draining lymph nodes, upregulate costimulatory molecules, such as B7, and present tumor-associated antigen-derived peptides (13-25-mer) to cognate CD4+ T-cells in the context of MHC class II molecules. The interactions of additional costimulatory signals, such CD40-CD40L, further promote dendritic cell maturation providing help for efficient priming and activation of CD8+ T-cells. [3]. Dendritic cells can present 8-11-mer peptides derived from endogenous antigens (e.g., viral vector-encoded tumor-associated antigens) or exogenous antigens (e.g., recombinant protein vaccine) in the context of MHC class I molecule to CD8+ T-cells. CD4+ T-cells producing Th1 cytokines, such as IL-2, further contribute to the clonal expansion of CD8+ T-cells [73, 74]. Tumor-specific CD8+ T-cells migrate to the sites of tumor metastasis where they encounter peptide-MHC complexes presenting the tumor antigen on tumor cells. CTLs are able to kill tumor cells by perforin-mediated cell lysis or apoptosis mediated through granzymes. CTLs can also kill target cells through death receptor mediated apoptosis via Fas ligand or TNF-related apoptosis inducing ligand (TRAIL). CTLs secreting IFN-γ and TNF-α (or TNF-β) may elicit direct or indirect cytotoxic activity[73]. [5]. Activated CD4+ T-cells may also kill tumor cells by using similar pathways as CTLs. CD4+ T-cells

producing Th1 cytokines may stimulate effector cells of the innate immune system, such as macrophages, NK and natural killer T-cells, which might exert antitumor effects by several mechanisms. CD4+ T-cells secreting Th2 cytokines may attract and activate eosinophils releasing their cytocidal granule content. Th2 cells may also activate B-cells producing antibodies, which may activate B-cells producing antibodies, which may contribute to tumor cell destruction by ADCC and CDC [74, 75]. Antibodies may also induce an idiotypic network cascade or tumor-cell apoptosis.
ADCC: Antibody-dependent cellular cytotoxicity; CDC: Complement-dependent cytotoxicity; CTL: Cytotoxic T-lymphocyte; IFN: Interferon; IL: Interleukin; MHC: Major histocompatibility complex; NK: Natural killer; NO: Nitric oxide; TCR: T-cell receptor; Th: T-helper; TNF: Tumor necrosis factor.

3.4 Adjuvants

Adjuvants are substances or interventions that, combined with an antigen, enhance its immunogenicity and the desired type of immune response. The preferred type of response in cancer is one in which IFN-γ producing Th1 lymphocytes and cytotoxic T lymphocytes predominate. Adjuvants discussed here fall into one of the following categories: biological or chemical adjuvants and cytokines/chemokines.

- → Biological adjuvants are recognized as non-self and known to induce migration of APCs at the site of delivery and hence the induction of an inflammatory response. APCs may then capture and process TAA at the same site. The most commonly used biological adjuvant is the bacillus Calmette-Guerin (BCG), diphtheria toxin and tetanus toxoid.
- → Chemical adjuvants function similarly to biological ones and some, for example, aluminium hydroxide (alum) and incomplete Freud´s adjuvant (IFA) provide a matrix that sequesters antigen at the delivery site, allowing a timed release of antigen to APCs.
- → Cytokines used for decades include GM-CSF and IL-12, known for inducing recruitment, migration, stimulating cross-presentation of DCs and promoting Th-1 differentiation. GM-CSF is known to augment both humoral and cellular immunity, although immune suppression may be seen at high doses [77, 78]. GM-CSF also induces the expansion of NKT cells that may contribute to immunity against tumors [79].
- → Microbes often elicit immune responses by activating pattern-recognition receptors such as members of the Toll-like receptor (TLR) family. TLR-signalling play an important role in both the innate and acquired immune system and TLR agonist has been evaluated as adjuvants. TLR agonists, such as unmethylated CpG-motifs (CpG) and lipopolysaccarid (LPS) derivate monophosphoryl lipid A (MPL) have been in the focus for evaluation lately. However, the use of TLR agonists is often associated with severe toxicity, resulting from non-specific activation of lymphocytes [80].

3.5 Therapeutic cancer vaccines approaches

A plethora of active vaccination strategies exist with attributed advantages and disadvantages.

Discussed in this thesis:

- Whole cell-based vaccines
- Dendritic cell vaccine strategies
- Peptide and protein vaccines
- DNA vaccines

Whole cell-based vaccines

Whole cell-based vaccines have been investigated for decades. They have the advantage to deliver a diverse panel of TAA and to simultaneously provide both CD8+ and CD4+ epitopes, and particularly autologous tumor-cell derivate potentially include unique TAAs. Autologous or allogeneic tumor cells are processed (lysates or irradiated cells) to optimize the release of their antigens, and are injected together with adjuvants or haptens. However, it is difficult to obtain and individually prepare vaccines for each patient. To avoid this problem, tumor cell vaccines have been prepared from allogeneic tumor cell lines. This is feasible as many tumors have overlapping antigen expression [81, 82]. This is attractive as tumor antigen-specific immune responses can be initiated by cross-priming, by-passing the need to match the MHC haplotype of the patient to the vaccine platform [83].

Dendritic cell vaccine strategies

Dendritic cell vaccine strategies are attractive as activated and mature DCs express high levels of both MHC Class I and II molecules for priming of CD8+ and CD4+ T cells, and additionally have co-stimulatory signals for T cell activation. By providing DCs with TAA it is possible to induce tumor-specific immune responses. Vaccination strategies based on DCs can be divided in to three categories.

The **1st** group encompasses strategies where DCs are generated by culturing patient-derived hematopoietic progenitor cells or monocytes with specific cytokine combinations in the presence of adjuvants and loaded with TAAs ex vivo. The DCs are then re-infused to the patient, most often i.d., together with an adjuvant. Ex vivo DC vaccines exhibit a good safety profile and can induce TAA-specific CD8+ and C4+ T cells, as seen with the approval of CD-based vaccine Sipuleucel-T for use in patients with prostate cancer [84].

The **2nd** group of promising DC vaccine strategies comprise TAA delivery to DC in vivo and is achieved by coupling TAAs to monoclonal antibodies or other vectors that recognize specific DC surface receptors [85]. Encouraging results for in vivo – targeted vaccination came from the use of engineered lentiviral vectors encoding the human melanoma antigen NY-ESO-1[86, 87] .

The **3rd** group includes approaches based on DC-derived exosomes [85]. DC derived exosomes (small membrane-surrounded vesicles originating from fusion of plasma-membrane and multivesicular bodies, released by DCs) are 100-fold more enriched with MHC Class II molecules than DCs. They have shown to be capable of inducing immune responses once loaded with TAAs and inoculated in vivo in animal models [85].

Protein and peptide vaccine

Protein and peptide vaccine (Papers I and IV) strategies are based on the administration of high doses of the TAA(s) in order to load empty MCH molecules on APCs.

Tumor–derived *peptide* epitopes constitute the simplest cancer vaccine formulation delivered i.d. together with an adjuvant [88], but this strategy requires prior knowledge to the precise definition of MHC Class I and II epitopes and are thus HLA-restricted. Other drawbacks include the weak immunogenicity of short peptides. Many immunodominant epitopes from TAAs have a low to intermediate binding affinity for the MHC molecule and are subdominant epitopes recognized by low affinity T cells that have escaped central tolerance. The tumor may also have ceased to express the epitope (antigen-variant escape mutants). The immunogenicity may be enhanced by introducing lipid, carbohydrate or phosphate groups, by introducing protease-resistant peptide bounds and thereby prolonging the peptides half-life in vivo [89]. On the other hand, manufacturing on a large scale is affordable and peptide vaccinations are safe with no potential for recombination. The greatest advantage of immunizing with MHC Class II peptides is the concomitant generation of CD4+ cells T cells, particularly the generation the Th1 subtype, essential for the generation of a robust CTL response. It is anticipated that the peptide is intracellulary processed to yield shorter CD8+ epitopes [12]. These are presented by APCs on MHC Class I molecules (cross-presentation) to produce a CTL response, ensuring a maximal immune response.

DNA vaccines

Unlike other vaccines, ***DNA vaccines*** (Papers II and III) do not contain the antigens themselves but are simple vehicles for in vivo transfection and antigen production. The circular DNA plasmid is made of the plasmid backbone, containing the origin of replication, the antibiotic resistance gene, a transcriptional unit that contains the eukaryotic promoter, the transgene(s), and a polyA tail that aids in the stability and translation of the messenger ribonucleic acid (mRNA) [90]. A schematic picture is shown in Figure 3.

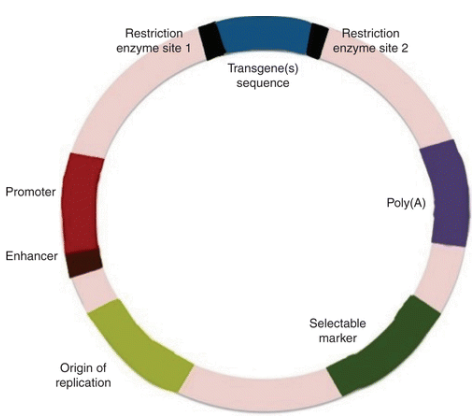

Figure 3. Basic design of plasmid DNA vaccine vector. Therapeutic and prophylactic DNA vaccines for Human immunodeficiency virus (HIV-1). (Reprinted with permission from Copyright Clearance Center (Expert Opinion of Biological Therapy. Ramirez et al [90]. Copyright 2014)

These plasmids are delivered in the form of circular DNA or within appropriate delivery vectors to the intradermally, subcutaneously or intramuscularly by various delivery methods.

In all scenarios, using the host cellular machinery, the plasmid enters the nucleus of transfected local cells, including resident APC [91]. Here, gene expression from the plasmid

is followed by generation of the transgenes(s) [91]. DNA vaccines induce protein synthesis in vivo increasing the possibility to utilize both the MHC Class I and II antigen presenting pathways to induce humoral as well as CD4+ and CD8+ T cell responses [91, 92]. The relevant epitopes do not need to be defined, nor is the vaccine limited to particular HLA genotypes, as the whole protein will be produced and processed in the host cell.

However, whereas professional APCs are very efficient at direct presentation, myocytes and keratinocytes are not, as they express detectable yet rather low levels of MHC Class I and co-stimulatory molecules [92, 93]. The induction of immunity following TAA expression must then proceed via *cross-presentation* (see Section 1.6).

As compared to cell-based and recombinant preparations many advantages with DNA plasmids exist. They are stable at room temperature, can thus be stored without effort and are rapidly produced in bacteria in high amounts at low cost [94]. Additionally, DNA vaccines are inherently immunogenic due to CpG motifs in the DNA backbone, able to bind and activate specific TLR who constitute part of the innate immune system [89, 90] and as mentioned previously DNA plasmids need not be HLA specific to elicit an immune response as the whole protein will be produced in the host cell.

DNA vaccines are also highly flexible and can be designed to encode both TAA and immunological components that may enhance the immune response. They can be engineered to express non-self-proteins (in additions to the TAA gene) that exert adjuvant effects, as fragments of tetanus toxin [95], pseudomonas aeruginosa exotoxin [96] or other immunostimulatory factors, such as cytokines, including IL-2, IL-12 and GM-CSF [69]. Furthermore, the plasmids can be engineered to alter the intracellular routing of the TAAs, resulting in the preferential activating of humoral (when TAAs are targeted to the endoplasmatic reticulum where folding and secretion can occur by adding a gene coding for a signal sequence) or cellular (if TAAs are targeted to the cytosol or – by adding genes encoding for ubiquitin aimed to enhance degradation and peptide production in the proteosome) immunity [97, 98].

A major safety concern has been mutagenesis, which may trigger oncogenes. Studies using DNA vaccines has however not exhibited mutagenesis [99]. No association between plasmid DNA and genomic DNA were seen in mice following DNA vaccination, towards HIV-1 [100]. Vaccine-related adverse events in humans were mild and tolerable using the same DNA vaccine [101].

For plasmid immunization, the aim is to rapidly induce an immune response, while for safety reasons; the plasmid should preferably be cleared rapidly from the body. During the efforts to prove the safety of plasmid DNA, the biodistribution of plasmids have been studied in several animal species, and the results all indicate that the plasmid is rapidly cleared from the body [100] and found exclusively at the site of injection at later time points. Additionally, studies with DNA vaccines have shown that even after multiple immunizations, anti-DNA antibodies are not produced [91].

The vast majority of clinical trials with DNA vaccines to date have utilised naked DNA plasmids.

3.6 Delivery route and delivery systems

Induction of tolerance or activation of immune cells may depend on the nature of the TAA, but also the formulation, dose, route, delivery system and schedule of the vaccine. In particular, delivery of DNA vaccines has been the focus for attention for a long time. Intramuscular injections were commonly used during early tests with large animals and humans resulting in relatively poor efficacy although robust immune responses were seen in mice [69]. This probably stems from the fact that the efficacy of DNA vaccines administered i.m. strictly depends on the injected volume. A high hydrostatic pressure not only augments the uptake of the DNA vaccine by myocytes and resident APCs but also promotes tissue damage, resulting in the release of danger signals [69]. Scaling up the volume needed for DNA vaccines i.m. to humans is not always feasible why alternative routes have been sought for. Some DNA vaccine delivery methods have focused on delivering DNA to skin due to the presence of large quantity of immune cells there, including APCs and lymphocytes, in comparison to muscle [90].

Electroporation (EP) (Paper III) has been used, to enable molecules to be delivered intracellularly ex vivo, for several years and has recently been tested in vivo to aid in transfecting cells. EP consists in the electrical stimulation of skin or muscle immediately after delivery of naked DNA. EP delivers a local controlled electrical field to cells, unstabling the cellmembranes and thereby allowing molecules, such as plasmids, to easily penetrate the cells and enhancing transfection [102, 103] While the electrical field is applied transient pores appear after which the cell can either heal-closing off the pores, or die due to lysis depending on the length and amount of the electrical field exposure [90]. I.m. EP is associated with an increase in transfection efficacy (enabling less injected volume) and local tissue injury resulting in danger signals [69].

EP i.m has been the most commonly used technique in preclinical models for practical reasons [104] but could prove difficult in translational human studies due to painful muscle contractions. In mice skin electroporation was performed after prostate-specific antigen (PSA) DNA administration i.d. A robust PSA-specific CD8+ T cell response was induced after one single DNA injection [104]. Laddy et al conducted a head-to-head comparison of EP-augmented i.m. and i.d delivery of equal amounts of influenza virus-encoding plasmids in monkeys. Results revealed that i.m. EP induced the highest levels of cellular immune responses, whereas i.d. EP was superior for induction of antibodies [105].

The introduction of in vivo electroporation for increasing the uptake and immunogenicity of plasmid vaccines raised concerns that the method might affect the persistence of plasmid DNA in the target tissue and pose a risk of integration into the host genome. In mice, immunized and EP-augmented with a HIV DNA plasmid, no association between plasmid DNA and genomic DNA could be detected [100].

Details about a **needle-free jet-injection device**, Biojector (Paper II, III), will also be discussed within the scope of this thesis. Biojector, is a CO_2-propelled device that injects plasmids as a highly focused liquid stream and is distributed in the i.d. or i.m depending on the amount of pressure used [106]. This has been shown to enhance antigen expression as compared to conventional needle, most probably due to a larger area and thus larger number cells being targeted [107]. In a mice model, a plasmid CEA66-DNA vaccine

construct, was safe and induced both CEA-specific CD4+ and CD8+ T cell responses (IFN-γ) when delivered i.d. by Biojector, however humoral responses were weak [108].

Gen-gun immunization is carried out by propelling DNA-coated gold particles into the cells of the skin and Langerhans cells, which were shown to migrate rapidly to regional lymph nodes [109], generating CD8+ T cell responses.

The potency of the gen-gun approach was shown in a clinical trial in humans; patients had not generated immunity to the licensed recombinant HBsAg vaccine but did produce antibodies following gene gun-delivered hepatitis B surface antigen (HBsAg) DNA[110]. Newly developed i.d. **tattoo DNA vaccine delivery** consist of fine metal needles that oscillate at a high frequency and puncture the skin, leading to transfection of skin associated cells. Robust T cell responses have been seen in a phase I melanoma studies [111].

3.7 Immunomodulating strategies

For TCV to be successful, both sides of the adaptive system must be manipulated. The effector system should be stimulated and the suppressor system inhibited.

Combination therapies may produce synergistic antitumor responses and certain chemotherapeutic agents, rather than being immunosuppressive can, under certain conditions act as strong adjuvants for active immunotherapy [112, 113]. Drugs may induce immunogenic tumor cell death resulting in emission of danger signals and TAA [114]. Secondly, chemotherapy may indirectly stimulate immune components, by inducing transient lymphodepletion, by affecting immunosuppressive mechanisms or by stimulating immune effectors [115].

Cyclophosphamide, a nitrogen mustard alkylating agent, is used for treatment of a variety of tumors. However, the biological activities of cyclophosphamide are dose-dependent [115]. Although regarded as immunosuppressive, cyclophosphamide has been shown to act as a strong immunomodulator for active immunotherapy when used with carefully defined doses [112, 113, 116]. Historically, 300 mg/m2 has been used for enhancing immunotherapies in phase III trials [117]. Treatment with low-dose cyclophosphamide has been shown to transiently reduce T_{reg} levels and enhance tumor-reactive T cell responses when used alone or in combination with active immunotherapy [118, 119].

Novel monoclonal antibodies can directly antagonize immune suppressive check points, (CTLA-4 and PD1), they target lymphocyte receptors or their ligands in order to enhance the antitumor activity [57].

Two fully humanized antibodies; ipilimumab and tremelimumab, exist. Ipilimumab is approved for treatment of advanced melanoma since 2010. In combination with TCV, a randomized three-arm trial of patients with advanced melanoma showed survival benfit for ipilimumab ± vaccine. Patients received either: a peptide vaccine of melanoma-specific pg100 alone; the gp100 vaccine plus ipilimumab; or ipilimumab alone. There was a 3.5 month survival benefit for patients in both groups receiving ipilimumab compared to the group receiving gp100 alone [120].

Clinical experience with PD1 antibodies is less extensive than CTLA-4, but initial results look promising [121].

4 IMMUNOTHERAPY TARGETING CEA AND hTERT

4.1 Carcinoembryonic antigen

CEA, a member of the immunoglobulin family of molecules, is a cell-surface-expressed 180 kDa glycoprotein. Many functions have been linked to CEA. CEAs primary role is in cellular adhesion and it dimerizes through a unique two-point intracellular adhesion mechanism and can interact with several intergrins in the intercellular matrix, suggesting that it may promote malignant cell proliferation and metastasis. It has been shown to inhibit cell death induced by loss of anchorage to the extra cellular matrix. Also, it promotes cells entering G0-like state, thus facilitating accumulation of additional oncogenic events [122-124]. CEA has been shown to induce cellular secretion of cytokines that promote cellular adhesion, thereby increasing the malignant potential of cells overexpressing this protein [125].

In the adult human body, CEA protein expression is low. CEA is normally expressed to a low level in gastrointestinal epithelium and to a higher extent during fetal development. CEA expression in colorectal tissues indicated a CEA tissue content below 300 ng/mg protein in healthy donors, less than 2000 ng/mg protein in normal mucosa of cancer patients, and 200 to >10 000 ng/mg of protein in tumor biopsies [125]. It is overexpressed in nearly all colorectal cancers and to a high extent overexpressed in pancreatic adenocarcinomas, breast cancer and non-small-cell lung cancers [126]. In cancer patients, significantly augmented cell-surface expression of CEA has been associated with more advanced disease and increased relapse rates compared to patients expressing lower levels of CEA. Neoplastic tissues can secrete CEA into the bloodstream, where it can be used as a serological circulating marker, mainly in colorectal cancer [125].

The CEA molecule is synthesized as a precursor protein with N- and C-terminal signal peptides. These signal peptides target the protein through the endoplasmatic reticulum (ER), where it is heavily glycosylated, and subsequently to the cell membrane [125]. More than 50% of the molecule consists of N-linked oligosaccharides on 28 potential sites of N-glycosylation [127]. Both signal peptides are removed during post-translational processing. The mature protein consist of a 107-amino acid N-terminal Ig V-like region; three 178 amino acid C2 Ig-like repeating units called A1 B1, A2 B2 and A3 B3, respectively; and finally, the C-terminal signal peptide has been replaced by a glycophosphatidylinositol (GPI) membrane anchor [126] [72]. Furthermore, CEA may have different molecular weights in normal vs. cancer cells due to different N-glycosylation pattern in cancer cells [128] where hypoglycosylation is associated with tumorigenesis.

CEA protein is processed and presented on major histocompability complex (MHC) proteins for multiple alleles, including HLA A2, A3 and A24. T –lymphocytes from healthy volunteers and cancer patients can recognise processed epitopes of CEA and can become activated to lyse CEA-expressing tumors.

CEA has as a TAA been identified as an attractive target for vaccination approaches against multiple types of cancer due to its pattern of expression and its likely function in

tumorigenesis. In a well-vetted priority-ranked list, composed to prioritize TAA for translational research based on predefined criteria, CEA ranked no.13 of 75 [70].

4.2 Therapeutic cancer vaccines targeting CEA

Several studies have shown that CEA can be used as a target for cancer immunotherapy protocols. It has previously been demonstrated that tolerance to CEA could be interrupted by mouse immunization with recombinant human CEA without leading to autoimmune disease [129-131]. Among the diverse CEA-based cancer vaccines in humans, DCs pulsed with agonist epitopes of CEA and recombinant virus-based vaccines have been most successful [128].

4.2.1 Dendritic based vaccines

Matsuda et al performed a pilot study on eight patients with advanced gastrointestinal malignancy [132]. HLAA24-positive patients received CEA peptide-pulsed DCs subcutaneously (s.c.) every two or three weeks. One patient had a dramatic decrease of CEA-levels. The other patients experienced disease progression, except three who had disease stabilization lasting for 3, 4 and 5 months respectively. In a phase I-II study, the effect of DCs transfected with RNA encoding CEA was evaluated [133]. Twenty-nine patients with advanced cancer expressing CEA were included. Two patients had minor response, 3 patients showed stable disease (SD) lasting more than two years and 18 underwent progression.

4.2.2 Recombinant virus-based vaccines

PANVAC is a cancer vaccine delivered through the viral vectors rV and vF, both of which include transgenes for epithelial mucin 1 (MUC1) and CEA in combination with specific costimulatory molecules (TRICOM). Early clinical trials have demonstrated PANVACs safety and ability to activate antigen-specific T cell responses [134]. PANVAC in combination with GM-CSF was evaluated for efficacy in 25 patients with metastazised CRC, lung, breast gastric or ovarian carcinomas. Multiple previous chemotherapy treatments and short time since last chemotherapy treatment correlated with lack of immune response. Three patients had prolonged SD or improvement and partial response (PR) was seen. Several patients had prolonged survival [135]. A second clinical trial evaluated PANVAC in combination with GM-CSF in 26 patients with metastatic breast or ovarian cancer [136]. SD was seen in four patients, with progression free survival (PFS) of 4-6 months and overall survival (OS) 16.1-40.5 months. Minor response and on case of complete response (CR) was seen. A significant reduction in circulating T_{regs} and increased CD4+ T cell responses were observed in the patient with CR an in another patient with SD.

In a set of Phase I trials, patients with stage III/IV pancreatic cancer who received PANVAC showed a slight increase in survival [134]. This led to a Phase III trial in which PANVAC was compared to best supportive care or second-line chemotherapy in patients who had failed first-line chemotherapy with gemcitabine [134]. The trial did not meet its primary endpoint of improved OS, possibly due to poor trial design that is – administering vaccine as a monotherapy to an inappropriate patient population [134]. An ongoing Phase II

trial is evaluating PANVAC in patients with CRC after complete resection of liver or lung metastasis.

4.2.3 Recombinant protein vaccines

Patients vaccinated with a recombinant CEA protein in combination with GM-CSF mounted a humoral and cellular immune response of a significantly higher magnitude than patients in the non-GM-CSF group. A positive correlation between the anti-CEA IgG titer and overall survival was suggested [137, 138].

4.2.4 Yeast vaccines

Non-pathogenic yeast-strain has been shown to be effective vectors for TCV and can stimulate antigen-specific cellular and humoral immune responses without inducing neutralizing antibodies [139, 140]. They can be administered multiple times to increase immune response with no toxicity and are easy to produce [141]. Twenty-five patients with metastatic CEA-expressing carcinomas were treated with recombinant yeast designed to express CEA. Five patients had SD > 3 months and five patients showed evidence of CEA-specific T cells [142].

4.3 hTERT

Telomeres are specialized structures at the end of human chromosomes composed of 1000-2000 non-coding repeats of TTAGGG nucleotide DNA sequences. They serve as protective "caps" at the end of chromosomes protecting them from DNA degradation and unwanted repair [143-145]. In normal human cells telomeres shorten with each successive cell division, and upon reaching critical lengths they elicit DNA-damage responses, thus activating cell cycle check points, leading to cell apoptosis. In contrast, cancer cells which develop chromosomal aberrations show activation or re-activation of telomerase upon exposure to DNA damage signal, thereby bypassing cell cycle checkpoints and leading to uncontrolled growth and proliferation [145, 146].

Telomerase is a human ribonucleoprotein reverse transcriptase (hTERT) composed of two main subunits: the catalytic protein hTERT and the ribonucleopotein template hTER [143, 144, 147]. Telomerase synthezises telomeric DNA by continually adding single stranded 3′ end of telomere in the 5′ to 3′ direction [143, 145, 148]. Telomerase, and specifically its catalytic subunit hTERT, is overactive in 85-90% of most cancers [147] and has become a widely accepted target for anticancer therapeutics. In normal non-malignant cells telomerase is present in embryonic, male germline and some adult stem cells. In most somatic cells telomerase is present in very low or almost non-detectable levels and is less active compared to cancer cells [149].

One of the advantages of targeting telomerase is that rapidly progressive cancer cells have shorter telomeres compared to normal somatic cells and stem cells, that have not yet reaches critical lengths, due to end replication problem that occurs as a result of aging[150, 151]. By de novo synthesising TTAGGG repeats, telomerase can maintain cancer cell telomeres at stable length at all times, ensuring their immortal capacity. The difference in telomere length and activity in normal cells and cancer cells explains therapeutic

cytotoxicity on cancer cells while having minimal impact on normal cells [152]. Chronic lymphocytic leukemia (CLL) patients have been shown to exhibit spontaneous T cells recognizing GV1001, (a hTERT-peptide, see Section 4.4.1) which could lyse autologous telomerase expressing leukemic cells [153] cells. In a well-vetted priority-ranked list, composed to prioritize TAA for translational research based on predefined criteria, hTERT ranked no.23 of 75 [70].

4.4 Therapeutic cancer vaccines targeting hTERT

There are two general strategies of telomerase targeting in cancer treatment. One is targeting directly by inhibiting the activity of its catalytic subunit (hTERT) or its RNA template (hTER), leading to inhibition of telomerase activity, telomere shortening and inhibition of cell proliferation. Another strategy is to target the telomerase subunit indirectly via G-quadruplex stabilizers, tankyrase or HSP90 inhibitors, thus blocking telomerase access to telomeres or inhibiting binding of telomerase-associated proteins leading to telomere uncapping and cell apoptosis [154, 155]. The latter do not constitute immunotherapeutic modalities. Targeting of hTERT from an immunological standpoint will be discussed in this section.

Since telomerase is present in most cancers, its peptides are considered shared TAAs. They are however capable of producing strong immune response by eliciting both CD4+ and CD8+ T cell responses, potentially leading to tumor cell lysis [155-157].

4.4.1 GV1001- hTERT peptide-based vaccine

GV1001 is a 16 amino acid HLA Class II restricted hTERT-peptide, which consists of amino acids 611-626 of the hTERT active site [158-160] and is capable of binding to molecules encoded by multiple alleles of all three loci of HLA Class II [161]. These characteristics of the hTERT peptide might virtually enable all patients, irrespective of HLA-type, to present one or more immunogenic epitopes to effector cells. GV1001 is administered as an MHC Class II peptide, which is endogenously processed to yield a MHC Class I peptide producing both CD4+ and CD8+ responses. The activity of CD4+ T cells leads to a secretion of IFN-γ and IL-2, further stimulating CD8+ CTL´s and NK cells, which may help to increase the infiltration and the retention of CD8+ T cells into the tumor sites leading to an up regulation and re-expression of MHC Class I molecules. This may have a therapeutic advantage in patients with advanced cancers where loss of MHC Class I is seen [162, 163].

Several strategies are employed in the development of vaccines that may induce hTERTs immunogenicity and eliminate the issue of self-tolerance, such as the use of adjuvans like GM-CSF or TLR-7 agonist (imiquimod), used in GV1001 vaccine strategies [160, 162]. This may prevent the rapid degradation and elimination of anticancer vaccine peptides before recognition by APCs, which may occur due to self-tolerance to self-peptides [160, 164].

GV1001 has completed several phase I and II studies conducted in patients with advanced stage melanoma, hepatic cellular carcinoma and in patients with pancreatic cancer [149]. Forty-eight patients with unresectable pancreatic adenocarcinoma were given

i.d. injections of GV1001 at three dose-levels along with GM-CSF for 10 weeks followed by monthly boosters. Delayed-type hypersensitivity (DTH) and in vitro T cell proliferation was measured. Of the 27 evaluable patients, median survival for the intermediate group (300 nmole GV1001) was 8.6 months, which was significantly longer than the low and high-dose groups. One-year survival in the intermediate group was 25% [165].

Two other phase III studies with GV1001 in patients with nonresectable pancreas cancer were Primo Vax and Telo Vac. The Primo Vax trail examined vaccine monotherapy versus gemcitabine but was terminated due to lack of survival advantage. The Telo Vac had three arms; sequential gemcitabine/capecitabine; and concurrent gemcitabine/capecitabine and vaccine. The results are pending. Another study is evaluating radiation therapy, tadalafil, sargramostim, gemcitabine and GV1001 in patients with unresectable pancreatic cancer and is ongoing, Clinical Trials.gov identifier NCT01342224.

4.5 Immune monitoring

Technologies available for measuring immune responses are numerous. It is critical to select an assay that can accurately measure therapy-induced changes in the frequency and/or function of immune cells. Briefly, phenotypic markers, functional assessments or cellular product may be assayed [166]. Phenotypic assays include measurement of absolute cell numbers and cell frequencies by flow-cytometry and tetramer binding. Functional assays include DTH, proliferation, cytotoxicity assays (ADCC, CDC) and enzyme-linked immunosorbent spot (ELISPOT). Assays for cellular products include enzyme linked immunosorbent assay (ELISA) (immunoglobulin levels, cytokines) and chemokine/cytokine levels (Multiplex).

In patients treated with TCV, three single-cell assays for detection of antigen-specific effector T lymphocytes can be used for monitoring: ELISPOT, cytokine flow-cytometry (CFC) and tetramer binding.

Each of these is based on T cell receptor recognition of cognate peptides presented by MHC Class i/II molecules on the surface of APCs to the responder T cells. They have in many ways replaced “bulk assays” as cytotoxicity assays and proliferation assays. However, no consensus exits as to which of these three should be selected to best monitor vaccination results [166]. ELISPOT measures function of individual responder cells by identifying those that produce and secrete the measured cytokine, CFC measures cytokine expression in a cell and not its actual secretion [166]. Tetramer binding detects peptide-specific T cells expressing the relevant TCR but may also bind non-functional T cells [167]. However, CFC and tetramer binding are flow-cytometry based and can provide information about identification of responding cells.

Simultaneous assessment of multiple biomarkers, for example multiplex assay may provide the investigator with an “immune profile”. Multiplex assays have all but replaced ELISA detection for cytokines allowing simultaneous measurement of, for example, suppressive cytokines, proinflammatory cytokines and Th1 vs Th2 cytokines [166]. For further details see Table 6. Polychromatic flowcytometry enables further examination of cells. mAbs labelled with various chromatophores with various excitation wavelengths is used to identify and determine levels of expression of surface or intracytoplasmic markers.

Table 6. Assays used for immune monitoring in vaccine clinical trials

Assay	Brief description	Advantages	Disadvantages
1 DTH	Ag as soluble protein is injected i.d. and the diameter of erythema or induration is measured after 48-72 h	• only *in vivo* measure available • easy to perform	• no standardized cut-off for a positive response • no standardized dose for DTH testing • Ag-specificity is questionable • Mainly mediated by CD4+ effector memory T cells
2 ELISPOT	Lymphocytes are cultured with the Ag studied in a micro-titer plate coated with a monoclonal Ab to a specific soluble factor (e.g. IFN-γ, IL-4, IL-10, TNF-α). The cells and the Ag are then washed from the wells and replaced with secondary antibody conjugated to a detection reagent. The plate is developed with a chromogen and spots appear where there was a cell secreting the soluble factor being investigated.	• functional assay • allows to measure individual soluble factors secreted by activated T cells and identify the pathway of the immune system activated by the vaccine • Lowest limit of detection (1/100000 Ag-specific T cells) • considerably reliable • relatively rapid	• provides no information on cell phenotype • Responder-stimulator interactions may result in unacceptable high background spots
3 Tetramer staining	Tetramers are composed of four MHC-I molecules, each bound to the epitope of interest. The tag is a fluorescent label, which allows to measure the binding of the tetramer to the TCR at flow-cytometry.	• sensitive (1/100000 Ag-specific T cells) • T cell subset analysis is optimal • allows to identify the peptide sequence or epitopes that bind to the highest number of TCR in a naïve individual • allows to identify the phenotype of the T cell to which the tetramer binds • allows to measure the change in the number of T cells displaying a particular TCR before and after vaccination	• requires knowledge of the epitope • requires availability of the tetramer for the respective epitope/HLA allele • unable to distinguish between functional and dysfunctional T cells
4 Cytokine Flow Cytometry (CFC)	Lymphocytes are cultured with the Ag studied and the presence of intra-cellular cytokines is detected by fluorescein-labelled mAb. The phenotype of the lymphoid cells (CD4+, CD8+, etc) is identified with a second set of fluorescein-labelled mAbs.	• functional assay • sensitive (1/10000 Ag-specific T cells) • provides additional information on cell phenotypes • relatively rapid	• non-specific background staining
5 Lympho-proliferation assay	Lymphocytes are cultured with the Ag studied. ^{3}H thymidine is added to the culture medium. Proliferating (dividing) cells incorporate ^{3}H thymidine, which is quantitated using a beta scintillation counter	• easy to perform • reliable • sensitive • reproducible	• can be influenced by the non-specific immune function of the patient • can be influenced by the *in vitro* stimulation procedure • not qualitative • not quantitative • no information about responding cell population (CD4+, CD8+, etc)
6 Cytotoxicity assay	Lymphocytes previously sensitized to the Ag present on the target cells are cocultured with the target cells. Percentage of lysis of target cells is quantitated by ^{51}Cr release assayor by Flow Cytometry	• functional assay • measures the ability of direct tumor lysis	• low sensitivity • often involves multiple *in vitro* stimulations • not quantitative • often other targets than autologous tumor are used, which may not reflect the capability of effector cells to lyse autologous tumor cells *in vivo*
7 Multiplexing for cytokines	A mixture of an infrared dye with a red dye is incorporated into polystyrene beads. The concentration ratio of both dyes is varied to produce beads which emitt a well-defined fluorescence spectrum upon excitation. Each bead is indentified by its unique colour, and can be coupled with a capture molecule specific for a ligand of interest, e.g. like a protein. The mixture of the beads is quantitatively analyzed in a FACS-like fluorescence-activated bead sorter	• Used as cytokine profiling Th1/Th2 • Small sample volume fluid • High throughput	• Sera measurement may be less useful than in situ measurement

Adapted from [Palma et al. 2007 [168]]

4.6 Clinical endpoints

The gold-standard for confirmatory trials is OS but evaluation of OS in clinical trials is a lengthy process, suggesting the need to identify surrogate endpoints for markers of efficacy. A number of surrogate end-points for OS exist including time to progression (TTP), PFS and disease-free survival (DFS), but such endpoints may not accurately predict the outcome of OS. This was exemplified in the study concerning Sipuleucel-T, previously mentioned above. The primary end-point of TTP was not met, but there was a trend towards improved OS in the treatment group [169].

Furthermore, in order to show clinical efficacy, measurements of tumor progression is often made by using the Response Evaluation Criteria in Solid Tumors (RECIST), which defines the response to treatment according to size [170]. However, this may not always reflect the efficacy of immunotherapy. Anecdotal data suggest that increase in tumor size may be due to increased inflammatory infiltrates. For example, the administration of mAb targeting the immunosuppressive receptor CTLA4 has been shown to double the survival of Stage IV melanoma patients in the absence of early tumor shrinkage [120].

5 COLORECTAL CANCER

5.1 Epidemiology

CRC is the third most common cancer in both men and women, and the fourth leading cause of cancer deaths in the world [171]. The estimated worldwide incidence of CRC is 1.2 million per year [171]. In Sweden approximately 6000 patients are diagnosed with a colorectal cancer every year, see Table 3 [172].

Table 3. Colorectal cancer incidence (total number) in Sweden 2011

Tumour site	Female	Male	Total
Colon	2102	2081	4183
Rectum	821	1158	1979
Total	2923	3239	6162

Mean age at diagnosis was 72 years for colon cancer and 70 years for rectal cancer in patients diagnosed during 2000-2007 [173]. A screening program for early detection commenced in 2008 in the Stockholm-Gotland region, and plans are to further expand this nationwide in late 2013. The vast majority of CRCs, about 95% constitute of adenocarcinomas.

5.2 Clinical staging

To select the optimal therapeutic strategy for a patient with CRC, a correct clinical and pathological staging is of great importance. Furthermore, the clinical and pathological staging at diagnosis is a crucial prognostic factor [174].

Preoperative standard staging involves endoscopy of the rectum and colon, computer tomography (CT) of the chest and abdomen, and in rectal cancer, an additional magnetic resonance imaging (MRI) of the pelvis.

The diagnosis of an adenocarcinoma in the colon or rectum is made by biopsy of the tumor. In order to select the optimal treatment strategy for CRC patients, the results of the staging procedure are discussed at a multidisciplinary team (MPT) conference [175].

5.2.1 Tumor-Node-Metastasis staging

Collected data is condensed according to a staging system, based on the depth of extension of the carcinoma through the bowel wall (T), the presence or absence of lymph node metastases (N) and the presence or absence of distant metastasis (M). Historically, Duke´s classification system has been used, but the TNM staging system by American Joint Committee on Cancer (AJCC) and International Union Against Cancer (UICC) is now recommended. Details on TNM staying system and comparison to Duke classification system are shown in Table 4. In the revision of the TNM system to the 7th edition, further subclassifications were added [176]. In our studies we have used the Dukes classification system in Paper I and AJCC TNM 5th Edition in Papers II and III. In our studies we used

Duke's classification system in Paper I and AJCC TNM 5th Edition in Papers II-III, see Table 4.

Table 4.

TNM Stage 7th Edition				*TNM Stage 5th and6th Edition*				*Duke's*
Stage	T	N	M	***Stage***	T	N	M	
0	Tis	N0	M0	***0***	Tis	N0	M0	–
I	T1-2	N0	M0	***I***	T1-2	N0	M0	A
IIA	T3	N0	M0	***IIA***	T3	N0	M0	B
IIB	T4a	N0	M0	***IIB***	T4	N0	M0	B
IIC	T4b	N0	M0					B
IIIA	T1-2	N1/N1c	M0	***IIIA***	T1-2	N1	M0	C
	T1	N2a	M0					C
IIIB	T3-T4a	N1/N1c	M0	***IIIB***	T3-4	N1	M0	C
	T2-T3	N2a	M0					C
	T1-T2	N2b	M0					C
IIIC	T4a	N2a	M0	***IIIC***	Any T	N2	M0	C
	T3-T4a	N2b	M0					C
	T4b	N1-N2	M0					C
IVA	Any T	Any N	M1a	***IV***	Any T	Any N	M1	D
IVB	Any T	Any N	M1b					D

Tumor-Node-Metastasis Classification

	AJCC 7th Edition	*AJCC 5th and 6th Edition*
	T (primary tumor)	
TX	Primary tumor cannot be assessed	Primary tumor cannot be assessed
T0	No evidence of primary tumor	No evidence of primary tumor
Tis	Carcinoma in situ: intra epithelial or Invasion of lamina propria	Carcinoma in situ: intra epithelial or Invasion of lamina propria
T1	Tumor invades submucosa	Tumor invades submucosa
T2	Tumor invades muscularis propria	Tumor invades muscularis propria
T3	Tumor invades through the muscularis propria into pericolorectal tissues	Tumor invades through the muscularis propria into pericolorectal tissues
T4	Tumor directly invades other organs or structures and/or perforates visceral peritoneum	Tumor directly invades other organs or structures and/or perforates visceral peritoneum
T4a	Tumor penetrates to the surface of the visceral peritoneum	
T4b	Tumor directly invades or is adherent to other organs or structures	
	N (Regional lymphnodes)	
NX	Regional lymph nodes cannot be assessed	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastases	No regional lymph node metastases
N1	Metastases in 1 to 3 regional lymph nodes	Metastases in 1 to 3 regional lymph nodes
N1a	Metastases in one regional lymph node	
N1b	Metastases in 2 to 3 regional lymph node	
N1c	Tumor deposit(s) in the subserosea, mesentery, or non peritonealized pericolic or perirectal tissues without regional nodal metastases	

N2	Metastases in 4 or more regional lymph nodes	Metastases in 4 or more regional lymph nodes
N2a	Metastases in 4-6 regional lymph nodes	
N2b	Metastases in 7 or more regional lymph nodes	
	M (Distant metastases)	
MX	Distant metastases cannot be assessed	Distant metastases cannot be assessed
M0	No distant metastases	No distant metastases
M1	Distant metastases	Distant metastases
M1a	Metastases combined to one organ or site (for example lung, liver, ovary, non-regional node)	
M1b	Metastases in more than one organ/site or the peritoneum	

The TNM system defines the stage, see Table 4, and the 5-year survival for patients with CRC is directly related to the clinical and pathological staging at the time of diagnosis. It varies between 95-100% for stage I, 75-90% for stage II, 45-60% for stage III and about 10% for stage IV, reported in a recently published Swedish follow-up study [177, 178].

5.3 Treatment

5.3.1 Surgery

Surgery is the primary treatment in CRC and the goal is to remove the tumor together with its regional lymphatic drainage and blood supply. The total mesorectal excision (TME) approach for rectal cancer, described in 1982, has reduced the rates of local recurrence considerably [179].

Patients in the palliative patients are offered surgery of the primary tumor if complications such as bleeding, perforation or obstruction may or do occur.

5.3.2 Non-surgical treatment of CRC

Adjuvant treatment/Colon cancer

The aim of adjuvant treatment is to prevent local recurrence, distant metastases and to prolong survival. Postoperative chemotherapy has been advocated for colon cancer in Sweden since the mid nineties. Currently, 6 months of adjuvant chemotherapy is recommended, although ongoing studies are investigating shorter periods of treatment as well as administering part of the chemotherapy preoperatively. Treatment might commence within 8 weeks after surgery [180-182].

Controversy still exists regarding the role of standard adjuvant chemotherapy for ***stage II colon*** cancer disease [183]. Factors associated with a higher risk of recurrence include T4 stage, perforation at presentation and inadequate node sampling (less than 12 nodes). Tumor grade and lymphovascular invasion are more controversial in this regard. All these factors are associated with a higher risk of recurrence but they are not predictive of a benefit from adjuvant chemotherapy, (i.e they are not prognostic markers). The TNM staging system has its limitations. For example, stage II patients who display mismatch repair deficiency (microsatellite instability, which is not included in the TNM system),

have an excellent prognosis following radical surgery and do not appear to benefit from adjuvant 5-fluorouracil (5-FU) [184]. Regarding the use of oxaliplatin in stage II patients, there is no evidence for the addition of this to 5-FU [185]. Stage II patients need to be considered carefully for chemotherapy based on all available clinical individual data.

In ***stage III colon*** cancer, combination of oxaliplatin and 5-FU is recommended for most patients. However, the benefits of oxaliplatin are less clear when the risk of recurrence is low (e.g. low grade, T1/2 or N1 disease) or the treatment related risks are high, such as those with comorbidity, elderly or poor performance status [185-187] and capecetabine monotherapy is a reasonable alternative in these situations [188]. Irinotecan, widely used in metastatic colorectal cancer (mCRC), has failed to improve survival in the adjuvant setting [189, 190].

Likewise, biological agents have been disappointing and are not routinely used in the adjuvant setting. The addition of the antiangiogenic monoclonal antibody targeting VEGF, Bev has not improved 3-year disease free survival (DFS) [191].

Adding the anti-EGFR-targeted monoclonal antibody Cet to adjuvant chemotherapy has also proven disappointing in both Kirsten rat sarcoma viral oncogene (KRAS) wild-type and KRAS mutant tumors [192], thus the identification of a predictive/prognostic biomarker has not yet affected adjuvant therapy selection as it has for mCRC.

5.3.3 Curative treatment of rectal cancer

Neoadjuvant therapy in rectal cancer often involves radiation therapy. The introduction of more precise surgery, TME and the use of pre or postoperative radiation therapy or radiochemotherapy have contributed both to much lower local recurrence rates and improved survival and more accurate preoperative staging with MRI and a series of trials have established radiotherapy as standard care [193, 194]. Radiation therapy, alone or with chemotherapy is given not only to the 10-15% non-resectable but also to much less advanced tumors, and in Sweden the standard is to give RT before surgery.

Two different preoperative approaches are mainly used. The short-course consists of 25 Gy in 5 Gy fractions given during 5 days, followed by surgery the following week. The long-course consist of 45-50.4 Gy given in 1.8-2.0 Gy fractions concomitant with 5-FU, either given as bolus or as capecitabine [193, 195, 196].

Patients are grouped according to their MRI-staging results and considered for radiation therapy. Tumors labelled as “good” receive no radiation therapy before surgery, tumors labelled as “bad” receive short-course radiation therapy and tumors labelled as “ugly” receive long-course radiation therapy, see Table 5 [197].

Table 5.

	Good	Bad	Ugly
Low tumor	T1-2, N0,mrf-	T3,N1-2,mrf-	T4,T3 mrf+
High tumor	T1-T3b,N0,mrf-	T3c-d,N1-2,mrf-	Lateral lymphnodes+

Cut-off between low/high tumors is at app. 8 cm from the anal verge
mrf; Mesorectal fascia
mrf+ Tumors growing adjacent to the mesorectal fascia
mrf- Tumors not growing adjacent to the mesorectal fascia
Extramural vessel invasion (not mentioned above) is labelled as at least "bad"

Adapted from Blomqvist, Glimelius. Acta Oncologica, 2008; 47:5-8.

Opinions on adjuvant chemotherapy vary a great deal in the literature. Some authors advocate that chemotherapy ought to be given applying the same criteria as in colon cancer, whereas some advocate that although surgery and preoperative (chemo)radiotherapy was adequately performed – the value of adjuvant chemotherapy has not been established [198]. Swedish National Guidelines suggest the following recommendations:

- Adjuvant chemotherapy is recommended to patients with highrisk-criteria rectal cancer stage II and stage III patients to whom preoperative radiation therapy was not given.
- Adjuvant chemotherapy may be considered in patients with highrisk-criteria stage II and stage III patients who received short-course radiation therapy and surgery the following week.
- Adjuvant chemotherapy may be considered in patients with highrisk-criteria stage II and stage III patients operated 4-6 weeks after preoperative short-course radiation therapy.

The uncertainty is even greater regarding patients who receive long-course radiation therapy and 5-FU preoperatively. The addition of oxaliplatin in the adjuvant setting has not been evaluated. A small retrospective study compared adjuvant 5-FU-based chemotherapy with no treatment in patients with T3-4 rectal cancers [199]. The results showed no significant impact on adjuvant chemotherapy on PFS or OS, although a difference seemed to emerge at approximately, respectively, 2 and 5 years after start of preoperative treatment. Patients in whom no down staging was seen did not benefit from adjuvant chemotherapy, indicating that the same prognostic factors may drive both tumor sensitivity for the primary treatment and long-term benefit from further adjuvant chemotherapy.

An alternative approach is to administer the CT preoperative, as is currently evaluated in the Rapido trial, Clinicaltrial.gov identifier NCT01558921. Primarily "ugly" tumors are included. To guarantee control of the rectal tumor short course radiation therapy is given followed by systemic chemotherapy (capecitabine and oxaliplatin) before surgery. The investigators hypothesize that with this protocol both the local tumour and possible micro metastases are effectively treated and that this will result in an increased survival. The investigators will compare this with the standard treatment of long-course radiation therapy

followed by TME surgery and adjuvant chemotherapy (capecitabine and oxaliplatin). I.e all patients in the standard arm receive adjuvant chemotherapy.

5.3.4 Metastatic CRC

In selecting the optimal treatment for mCRC, the initial consideration is whether the aim is curative (initially or potentially resectable disease) or noncurative.

At the time of diagnosis approximately 15% of the patients with stage IV CRC have metastatic disease in the liver and less than 10% have pulmonary metastases that are resectable [200, 201]. The majority of patients with mCRC and liver metastases are considered unresectable at presentation, although a minority of these patients may eventually be able to be treated with curative intent if a good response to chemotherapy is achieved. Complete resection of limited liver metastases and/or lung metastases is the only potentially curative treatment for mCRC with a survival rate after hepatectomy of 40-60% if patients are carefully selected [202, 203].

Affecting the choice of treatment are factors relating to the patient (age, performance status, comorbidities, patients preferences) and those relating to the tumor (potential resectability, disease burden, symptoms, rate of progression, prognostic biomarkers and prior treatment history).

Doublet chemotherapy is recommended if an aggressive approach is indicated, i.e to stabilize rapidly progressive disease, in order to reduce metastasis where surgery is possible and in first-line resectable patients. 5-FU is the backbone in doublet chemotherapy. There are little differences in efficacy between doublets of 5-FU-infusion/capecitabine and oxaliplatin (FOLFOX and XELOX) or 5-FU-infusion/capecitabine and irinotecan (FOLFIRI and CAPIRI) regimens [174]. Single chemotherapy (5-FU) is recommended in non-rapidly progressive nonresectable disease.

Patient outcome is further improved by the addition of a biological agent to these chemotherapy regimens, why this may be added to the treatment of potentially resectable mCRC patients and non-resectable patients.

The addition of Bev to both irinotecan and oxaliplatin based doublet chemotherapy significantly improves PFS and with irinotecan, OS in mCRC patients, but with smaller effects on resection rates [204, 205].

The benefit of EGFR-antibody (Cet or panitumumab (Pan)) treatment to KRAS wildtype (WT) tumors are highly variable in trials. Unlike antiangiogenics, a predictive marker exists for EGFR-targeted therapy. KRAS gene mutation (MT) is predictive of nonresponse to EGFR-targeted therapy either as a single agent or in combination with irinitecan and oxaliplatin based chemotherapy and in standard care EGFR-antibodies are only recommended to KRAS WT patients. KRAS mutations in codon 12 are most common, but mutations in codon 13 constitute approximately 15% of all KRAS mutations [174]. Conflicting data exists on whether KRAS MT in codon 13 are eligible for treatment with EGFR-targeted therapy [174]. Furthermore, CRC tumors, defined as KRAS WT may harbour other oncogenic alterations (BRAF, NRAS) in which the benefit of EGFR-targeted therapy is yet unknown [174]. However, large Phase III studies have shown OS benefit provided by Cet and PFS benefit for both Cet and Pan in KRAS WT patients. EGFR-

targeted therapy with Cet or Pan also generally improves RR in KRAS WT patients by over 10% when added to doublet chemotherapy [206, 207].

The use of triplet chemotherapy, 5-FU/irinotecan and oxaliplatin, (FOLFOXIRI) for first line mCRC has been investigated in Phase III studies with an unclear survival benefit compared with double chemotherapy. FOLFOXIRI significantly increased resection rate, complete resection rate after surgery, PFS and OS compared [208] to FOLFIRI in one study, while in another study, FOLFOXIRI was not superior to FOLFIRI [209]. Toxicity is a major concern and FOLFOXIRI is therefore not part of standard treatment, but may be considered in selected patients.

5.3.5 Second-line treatment and beyond

For most patients, progression following an oxaliplatin doublet will result in a switch to irinotecan-based therapy and vice versa. PFS is shorter than with first-line therapy [210]. If single chemotherapy was given, registry data suggest oxaliplatin based doublet chemotherapy [211]. KRAS WT patients, not previously exposed to Cet or Pan, may receive this as third-line therapy alone or in combination with irinotecan. The choice of biological agent in second line therapy is an area of active research with ongoing Phase III studies. Aflibercept in second-line treatment combined with FOLFIRI and regorafenib in late-stage disease has shown survival gains in recently reported trials and may become part of standard treatment [174]. Summary of proposed management for mCRC is seen in Figure 4.

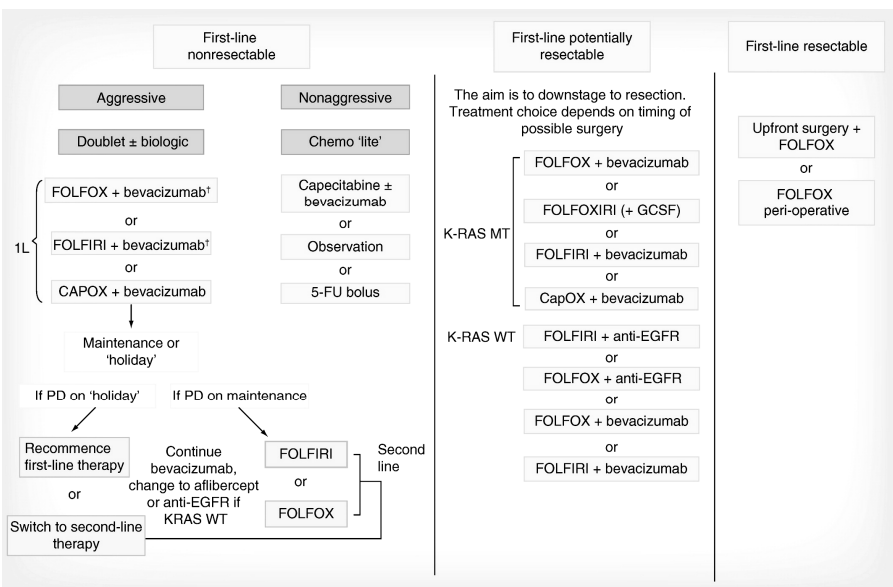

Figure 4. Proposed chemotherapy management algorithm for metastatic colorectal cancer. †If aim is downstaging or bulky and symptomatic disease is present in KRAS WT patients, anti-EGFR should also be considered.EGFR: EGF receptor; MT: Mutation; PD: Progressive disease; WT: Wild-type.
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6 PANCREATIC CANCER

6.1 Epidemiology and prognosis

Pancreatic cancer is currently one of the deadliest of the solid malignancies. The annual incidence in Sweden is approximately 11 cases per 100 000 inhabitants. In Sweden, 1580 people died due to pancreatic cancer in 2011 [172]. Approximately 85% of the patients have advanced spread disease at the time of diagnosis and only 15-20% of the patients are eligible for curative surgery. Following curative surgery, 90 % of the patients will relapse. Despite decades of effort, the 5-year survival rate remains at only 5% and the median survival reported for resected pancreatic cancer patients ranges from 17-27 months [212].

In the sections that follow, "pancreatic cancer" (PC) will refer to invasive ductal adenocarcinoma of the pancreas**.**

6.2 Risk factors

Both environmental and inherited factors contribute to the development of PC. The most common risk-factor is smoking. Studies has shown that current smokers have a 2.2 fold increased risk of PC than never smokers and approximately 25% of PC are attributable to smoking [213, 214]. Long standing type 2 diabetes of >10 years duration have a 1, 5 foldincrease compared with nondiabetics [215]. Increased body mass index (BMI) is an independent risk factor [216]. Heavy alcohol consumption and chronic pancreatitis also elevates the risk of PC, the latter 2.7-fold. Individuals with a family history of PC have a 1.9-13-fold increased risk, based on case-control and cohort studies [212]. Inherited (germline) mutations in the BRCA2 gene, PALB2 and Lynch syndrome are all associated with higher risks [212].

6.3 Staging

A contrast-enhanced thin-slice CT scan of the thorax, adomen and pelvis is performed in the staging procedure. Optimal treatment depends on careful staging and all patients should be discussed in a multidisciplinary team. In the absence of metastatic disease, the relationship of the tumor to the adjacent major vessels defines resectability. Resectable stages include stage I and II, and the subset of stage III that is defined as borderline resectable. Less than 180-degree involvement of the celiac axis or superior mesenteric artery is considered stage III borderline resectable. Greater than 180-degree involvement, is considered locally advanced or unresectable PC [217, 218]. AJCC/UICC and TNM definitions for pancreatic exocrine cancer are shown in Table 6.

Table 6. AJCC/UICC stage groupings and TNM definitions for pancreatic exocrine cancer

AJCC/UICC stage	TNM
0	Tis,N0,M0
1A	T1,N0,M0
IB	T2,N0,M0
IIA	T3,N0,M0
IIB	T1,N1,M0
	T2,N1,M0
	T3,N1,M0
III	T4,any N,M0
IV	Any T, Any N, M1

TNM Classification for Exocrine pancreatic cancer. Adapted from AJCC Cancer Staging manual.7th ed, NY: Springer, 2010, pp 241-9

Primary tumor (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ
T1	Tumor limited to the pancreas, less than 2 cm in greatest dimension
T2	Tumor limited to the pancreas, more than 2 cm in greatest dimension
T3	Tumor extends beyond pancreas but without involvement of the celiac axis or the superior mesenteric artery
T4	Tumor involves the celiac axis or the superior mesenteric artery
Regional lymph nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Regional lymph node metastasis
Distant metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis

6.4 Treatment

6.4.1 Surgery

Patients with Stage I/II disease are recommended immediate surgical resection followed by adjuvant chemotherapy. The low chance of long-time survival after surgery, mentioned above, and considerable morbidity in 40-60% of the patients following major surgery needs to be considered when selecting patients [212]. Complete recovery can take 2-3 months even in the absence of complications.

6.4.2 Neoadjuvant therapy

The advantage with neoadjuvant chemotherapy is 1; down-staging of some locally advanced tumors, (conversion rates vary between 15-40%) [219], 2; it spares the 15-35% of patients who develop metastatic disease during treatment the risks of a major operation [212] and 3; guarantees that almost all patients receive some form of chemotherapy or radiation

therapy as postoperative complications may prevent postoperative treatment. In resectable patients neoadjuvant chemotherapy delays the curative surgery and systemic chemotherapy is therefore given postoperatively.

Stage III borderline patients should be recommended neoadjuvant therapy prior to surgery. Patients with stage III locally advanced disease should be treated with chemotherapy or chemoradiotherapy – a few of these patients may be considered for surgery.

Similar neoadjuvant regimens are often used for locally advanced and unresectable disease, i.e. Stage III with greater vessel involvement. The combination of 5-FU, LV, irinotecan and oxaliplatin (FOLFIRINOX) , the combination of gemcitabine, docetaxel and capecitabine and gemcitabine alone are used, typically followed 5-FU infusion, capecitabine or gemcitabinebased chemoradiation to 45-54 Gy in 1,8 to 2,5 Gy fractions or 36 Gy in 2,4 Gy fractions [212]. Surgery is performed within 6-8 weeks following completion of neoadjuvant therapy. Neoadjuvant chemotherapy to patients with Stage I/II is controversial [212].

6.4.3 Adjuvant therapy

Six months of adjuvant chemotherapy with either 5-FU or gemcitabine is recommended [212]. When gemcitabine was compared with observation in PC patients, DFS and OS were 6,9 months and 20,5 months for the observation arm, and 13,4 months and 24,2 months for the treatment arm [220]. A randomized trial between adjuvant elongated bolus 5-FU or gemcitabine showed similar OS of 23 months, with higher but acceptable rates of diarrhea in the 5-FU group and haematological toxicity in the gemcitabine group [221]. Chemoradiation adjuvant to the tumor bed is being explored, but has not reached standard practice.

Other adjuvant regimens are being evaluated. The combination of interferon-alfa, cisplatin and 5-FU with adjuvant chemoradiation had a median OS of 32 months compared to 28,5 months in the treatment arm with 5-FU alone [212]. Erlotinib has been studied and was safe to combine with gemcitabine as well as with concomitant capecitabine and chemoradiation adjuvant [212].

In a phase II setting immunotherapy was combined with a pancreatic cancer vaccine of irradiated GM-CSF transfected allogenic whole-cell tumor lines. The median OS was 24, 8 months and patients who demonstrated a CD8+ T cell response to mesothelin had a higher likelihood of remaining disease-free [222].

6.4.4 Metastatic disease

Since 1997, gembitabine has been the standard treatment in the metastatic patient and hence tested with various other agents in doublet chemotherapy regimens [223]. Capecitabine coupled with gemcitabine has not shown superiority over gemcitabine alone, but may be considered in frail patients not eligible for single gemcitabine [224]. Nor has gemcitabine plus oxaliplatin or cisplatin shown significantly improved OS in phase III trials [212]. Adding docetaxel and capecitabine to gemcitabine has albeight in small studies, demonstrated good disease control rates and may be considered for patients with good performance status [225].

Gemcitabine has also been tested with targeted therapy, the combination of gemcitabine and erlotinib improved OS minimally [226]. The addition of Cet to gemcitabine, or of Bev both demonstrated no survival benefit and added toxicity [227, 228].

FOLFIRINOX has emerged as a new promising regimen for fit patients in comparison to gemcitabine. Disease control rate and median OS were 70% and 11.1 months in the FOLFIRINOX arm, compared to 51% and 6, 8 months in the gemcitabine arm with intact quality of life [229].

As for second line, patients treated with gemcitabine should be considered for a 5-FU based treatment, either alone or coupled with oxaliplatin or irinotecan [212].

7 AIMS

The overall aim of this thesis was to investigate immunogenicity of rCEA-protein, CEA-DNA and hTERT-peptide vaccination.

- To analyse the induction of IgM, IgA and IgE anti-CEA response in CRC patients without macroscopic disease, immunized with rCEA ± GM-CSF.
- To determine the safety of CEA66-DNA vaccination delivered by needle-free injection in combination with GM-CSF in CRC patients.
- To investigate the cellular and humoral immune response following CEA66-DNA vaccination delivered by Biojector or tetwtCEA-DNA-vaccination followed by electroporation as adjuvant treatment in CRC patients.
- To analyse the cellular and humoral immune response after priming with CEA66-DNA vaccination and boosting with tetwtCEA-DNA vaccination in CRC patients.
- To explore the immunogenicity and safety of GV1001 vaccination combined with GM-CSF and gemcitabine as first-line treatment in patients with advanced pancreatic adenocarcinoma.

8 MATERIAL AND METHODS

8.1 Patients

All patients included in this thesis were recruited and treated at the Department of Gastrointestinal Cancer at the Karolinska University Hospital, Sweden.

8.1.1 Healthy donors

Healthy donors were recruited from the staff at the laboratory at Cancer Centre Karolinska (CCK). In Paper I, four healthy donors were analysed in the flow cytometry, the CDC and the ADCC analysis. No healthy donors were included in Paper II.

In Paper III and IV, nine healthy donors respectively were analysed in the proliferation assay, ELISPOT assay and flow-cytometry.

8.2 Methods

8.2.1 Immune assays

Blood sampling was performed in the clinic and handled in a standardized fashion. All assays were carried out at the adjacent CCK laboratory – except for ELISA in Paper I and III. In Paper I, ELISA was performed at the Department of Clinical Chemistry at Ängelholm Hospital and in Paper III ELISA was performed at the Swedish Institute for Infectious Control. Used immune assays are displayed in Table 7.

Table 7

Assays used	Prolif.	ELISPOT (INF-γ)	Cytokine secretion	DTH	ELISA	Flowcytometry	CDC	ADCC
Paper I					X	X*	X*	X*
Paper III	X	X	X		X	X**		
Paper IV	X	X	X	X		X**		

*In one patient only. **T cell subsets, NK cells, NKT cells and MDSC.

8.2.2 Proliferation assay

Peripheral blood mononuclear cell (PBMC), $1x10^5$/well, were cultured with the rCEA protein (Paper I), or GV1001, the ras-peptide and the HIV-peptide (Paper IV), purified protein derivative of tuberculin (PPD) (Papers III, IV), phytohemagglutinin (PHA) (Papers III, IV) and tetanus toxoid (TT) (Paper III) in 96-well culture plates for 6 days. During the last 18 hours of incubation, 1 μCi/well [^{3}H] thymidine was added. Cells were harvested and incorporated radioactivity was measured by a beta-counter. Results are shown as a stimulation index (SI) calculated by dividing mean radioactivity (cpm) of 6 replicates of experimental wells by that of the background value (cells with medium alone). Cells

stimulated with PPD, PHA, TT served as positive controls. Cells stimulated with HIV served as a negative control.

A change in ratio ≥ 2 on at least two occasions, compared to baseline, was considered significant [230].

8.2.3 ELISPOT (IFN-γ)

PBMCs were cultured in 48-well plates with rCEA (Paper III), a pool of CEA peptides (Paper III), PHA and PPD and TT (Papers III, IV) for 5 days. A millipore 96-well filter plate was coated with anti-IFN-γ antibody. Cultured PBMC were transferred to the coated plate and incubated for 20 h with the antigens as above. Cells were washed and incubated with a secondary biotinylated anti-IFN-γ antibody for 2 hours at room temperature. After washing, Streptavidin-ALP conjugate was added to the cells and incubated for 1 hour at room temperature. Cells secreting IFN-γ were developed by adding substrate BCIP/NBT plus and incubated at room temperature for 5 min. The reaction was stopped at the appearance of dark spots. Spots were counted by an automatic ELISPOT assay reader.

Paper III: A vaccine induced IFN-γ response in ELISPOT was defined as an increase in delta value (experimental value minus background) at least two-fold compared to baseline [231].

Paper IV*:* A vaccine induced IFN-γ response was defined if all of the following criteria were fulfilled: i) Spotforming units (SFU) of stimulated wells significantly higher ($p<0.05$) than of unstimulated wells (background) and at least twice that of the background, ii) SFU of cells stimulated with the control peptide not significantly ($p>0.05$) higher than background; iii) SFU of a post-vaccination test at least twice that of the pre-vaccination test [230]. The non-parametric Mann Whitney two-tailed rank sum test was used for comparison of unstimulated vs stimulated cells in Paper IV.

8.2.4 Cytokine secretion assay

Supernatants were collected (20 μl/well) after 24 and 120 hours of incubation from the proliferation assay and stored at -70°C until analyzed. The volume was replaced with complete medium. IL-4, IL-10, IFN-γ, TNF-α and GM-CSF were analyzed (Paper III, IV). Standard concentration curves were generated. The coefficient of variation (mean ± SD of the individual analytes) of PHA stimulated cells was calculated.

Cytokines concentration (pg/ml) in supernatants of antigen stimulated cells divided by that of cells alone using the highest value at 24 or 120 hours culture periods respectively was used. Post vaccination ratios divided by prevaccination ratios at different time points are shown. A ratio ≥ 1, 5 (Paper III) and ≥ 2 (Paper IV) (relative increase) was considered an antigen induced specific response [232].

8.2.5 DTH

GV1001 (0.112mg) in 0.1ml saline (groups A/B) was injected intradermally in the volar part of the forearm. GV1001 (0.105mg) in 0.22 ml saline (group C) was injected intradermally in the lower abdominal wall. The skin test was read, by the patient, after 48 hours by measuring the diameter of induration (mm). A positive DTH response was defined as ≥ 5 x 5 mm of induration (Paper IV).

8.2.6 ELISA

The levels of IgM, IgA and IgE antibodies against rCEA were assayed by conventional isotype-specific sandwich ELISA (Paper I) [233]. Wells were coated with recombinant human CEA (2 ug/ml) and serum samples were assayed at 1:500 (IgM), 1:200 (IgA) and 1:10 (IgE) dilutions. Following overnight incubation in the wells antibodies were detected with alkaline phosphatase (ALP)-conjugated rabbit polyclonal isotype-specific F(ab')2-fragments (IgM and IgA) and biotinylated anti-IgE mAb/ALP-conjugated streptavidin (19) after 2h/2h incubations, respectively.

The antibody levels were calculated from standard curves established with chimeric IgM, IgA2 and IgE anti-NIP (5-iodo-4-hydroxy-3-nitro-phenacetyl acid) hapten antibodies [233, 234] using BSA-NIP conjugate (10 ug/ml) as coating antigen. This procedure permits comparison of results between runs in a semi-quantitative way where 1 AU (arbitrary ELISA unit) approximates to 1 ng of antibody as previously described [233]. The sensitivities of the IgA, IgM and IgE antibody assays in serum are 0.1 ug/ml, 2 ug/ml and 0.4 ng/ml, respectively.

In Paper III, conventional isotype-specific sandwich ELISA was also performed. Plates were coated with 0.1 µg per well of rCEA or purified hCEA diluted in 0.05 M Na^2CO^3 (pH 9, 6) and incubated at room temperature overnight followed by 24 h in 4°C. After washing in ELISA buffer (0.05% Tween20, 0.15 M NaCl in distilled water), the plates were blocked by 5% milk in PBS for 2 h. Sera from immunized patients were diluted in 2.5% milk in PBS, and added to the plates (1:4 – 1:10000). Following incubation over night at room temperature, excess serum was removed and the plates were washed with ELISA buffer. To detect CEA-specific IgG antibodies, a rabbit anti-human HRP conjugate (1:3000 in 1.25% milk) was added to the plates and incubated at 37°C for 2 h. Plates were washed with ELISA buffer and developed by adding O-phenylene diamine substrate activated with H_2O_2. The reaction was stopped by adding 2.5 M H2SO4. Optical density (OD) was read at 490 and 650 nm. A net absorbance of 0.5 was used as a cutoff. A monkey anti-CEA serum served as a positive control.

A patient was considered to have developed a significant response if IgM, IgA or IgE (Paper I) and IgG (Paper III) antibodies could be detected at at least two different time points and was at least twice that of the pre-immunization value for that patient.

8.2.7 Flowcytometry

Indirect immunofluorescence using flow cytometry

In paper I, SW48 (a human cancer cell line expressing CEA) and A549 (a human lung cancer cell line not expressing CEA) were used for surface staining of a patient serum, pooled sera from healthy donors (HD) and a non-relevant IgA isotype control (22). Briefly, 10^6 cells were washed in PBS containing 0.01% sodium azide and incubated with the IgA isotype control, HD and the patient serum (1:10–1:1000) at 4°C for 1 h followed by 3 washings. A polyclonal rabbit anti human IgA serum (1:100) in washing buffer was then added and incubated at 4°C for 1 h. The cells were finally washed 3 times and fixed by adding 1% paraformaldehyde in PBS. Cells were analyzed by flow cytometry.

Cellular staining and flow cytometry

Briefly conjugated antibodies were added to cells ($5x10^5$ cells per tube) and incubated for 30 min. Intracellular staining was performed using a $T\text{-}_{reg}$ staining kit. After a final wash, cells were resuspended in PBS and events acquired using LSRll and analyzed by the FlowJo software (Paper III, IV).

8.2.8 Complement dependent cytotoxicity (CDC)

Stored sera (-70°C) from a patient who had developed high IgA anti-CEA antibody titers were used (Paper I). Two sampling times were pooled to obtain a sufficient amount of serum. IgA from the patient and pooled sera from healthy donors were isolated using peptide M/Agarose affinity column chromatography yielding highly purified IgA.

Briefly, as previously described [235], SW48 (a human cancer cell line expressing CEA) and A549 cells (a human lung cancer cell line not expressing CEA) (5×10^4) were plated in V-bottomed microtiter plates in 100 µl RPMI-1640 containing 10% FCS (complete medium). Cells were incubated with 20 µg/ml of purified IgA, from the patient and HD, respectively for 30 min at room temperature followed by twice washing with RPMI1640. After washing, 20% normal human serum in complete medium was added to the cells and incubated at 37°C in humidified air with 5% CO2 for 1 h. Finally, cells were collected, washed twice with 1xPBS and resuspended in 100 µl of 1x binding buffer. Five µl of propidium iodine was added to the cells, vortexed and incubated at room temperature in the dark for 15 minutes. 100 µl of 1x binding buffer was added to the cells which were analyzed by flow cytometry.

Results are presented as percent lysis (mean ± SEM), in four different groups; 1; cells alone, 2; cells incubated with human complement, 3; cells plus isolated IgA from the immunized patient +-human complement and 4; cells plus isolated IgA from HD ± human complement. Statistical calculations were done comparing groups by unpaired t-test.

8.2.9 Antibody dependent cellular cytotoxicity (ADCC)

ADCC was performed as previously described [236]. In brief, cell lines (same as used in CDC) were labeled with 2.8 MBq sodium 51Cr for 2 h. After 3 washings with DMEM medium 10000 cells in 100 µl medium were added to each round-bottomed microtiter well (Nunc) and Ficoll-isopaque isolated PBMC of healthy donors to yield target:effector cell ratios of 1:25 and 1:50 to a final volume of 200 µl. The test was run in sixplicate. After 24 h at 37°C the reaction was stopped by centrifugation. ^{51}Cr release was measured.

The percentage of target cell lysis was calculated according to the following formula: % specific lysis = (experiment cpm-spontaneous cpm) / (maximal cpm-spontaneous cpm) x 100. Maximal ^{51}Cr release was determined by adding RIPA lysis buffer (150 mM NaCl, 25 mM Tris PH=7, 5 mM EDTA, 0.5 % SDS and 1 % Triton X-100) to target cells and spontaneous release was measured in the absence of antibodies and effector cells (Paper I). In each cell line, results were compared between three groups; 1; Isolated IgA from immunized patient, 2; Isolated IgA from HD and 3; no antibody. Statistical calculations were done comparing groups by unpaired t-test.

8.3 Follow-up

Paper I

The first patient was included in Dec 1994 and the last in April 1996. Patients were contacted in July 2007 and their medical history was taken. Data was cross-checked with patient's journals. Data on deceased patients were taken from patients journals. All patients were accounted for. Patients were monitored immunologically for 36 months and clinically for 154 months.

Paper and II and III

Systemic medical events occurring after the injection were assessed by the investigators and considered as product-related events only if their relation to the product was judged possible, probable or definite. AE were graded as to their seriousness, severity and relationship to the immunization by the investigators. NCI Common toxicity criteria (version 3.0) grades were applied (http://ctep.cancer.gov/reporting/ctc.html*)*. Serious adverse events (SAEs) were defined as life threatening AE´s, or AE´s that resulted in significant or persistent disability, hospitalization, or death and were collected throughout the study period.

Injection site and systemic symptoms were recorded in a memory aid up to the next scheduled visit. Patients were asked to measure erythema and oedema (only Paper II). Standard urine analysis and extensive laboratory tests were taken.

The study period was 72 weeks during which the patients were monitored for safety, in total seven doctors visits. This was followed with clinical follow-up visits every 6 months, over a 5-year period, from the first immunization for long-term adverse events with special attention to autoimmune diseases, second malignancies, DFS and OS.

Paper IV

During the study, patients were regularly checked for performance status, routine blood haematology and chemistry analyses and serum tumour markers. AEs were assessed once weekly throughout the study period.

AE were assessed according to the National Cancer Institute Common Toxicity Criteria versions 2.0 (Groups A/B) and 3.0 (Groups C/D) resp. and considered related to treatment if a relationship was reported as possible or probable. SAEs were defined as life threatening AE´s, or AE´s that resulted in significant or persistent disability, hospitalization, or death and were collected throughout the study period.

8.4 Criteria for immune response

Paper I

ELISA: A patient was considered to have developed a significant response if IgM, IgA or IgE antibodies could be detected at at least two different time-points and was at least twice that of the pre-immunization value for that patient.

Paper III and IV

- A patient was considered to have a *single assay immune response* if a response in one of the assays (proliferation, ELISPOT, Th-1 like cytokine secretion) was noted at one time point only against rCEA and/or CEA derived peptides (Paper III) or telomerase and/or ras (Paper IV). DTH was performed and included in overall induced immune response (OIIR) in Paper IV. Results of the ELISA assay were used descriptively (Paper IV).
- A patient was considered to be an *overall induced immune responder OIIR* if a positive response (see above) was noted in at least one of the assays at at least at two time points.

8.5 Statistical analysis

Statistical analyses were done using Statview® (SAS Institute Inc. version 5.0 USA) (Papers I, II, III, IV) as well as IBM SPSS statistics (version 19) (Papers I, III).

Paper I

Comparison of pre-immunization anti-CEA titers of deceased and alive patients between groups were analysed by unpaired t-test. The relationship between survival and anti-CEA Ig-titers were analyzed by Cox-Regression analysis where the through mean values of IgM, IgA and IgE levels respectively for all patients at pre-immunization and at month 1, 2, 4, and 6 were calculated and listed from lowest to highest value. Patients with a through mean value below the median of all patients were labelled value 0. Patients with a mean value above the median of all patients were labelled value 1. This factor was analyzed in Wilcoxon Gehan exact test in relation to survival. As development of antibodies is "time dependant", analysis of the prognostic impact of antibodies was performed after a fixed observation time [237]. As one patient died early, after 7 months, the 6 first months were used as observation time. The observation period and follow-up period were then held apart, avoiding "the pitfall of overlapping exposure and follow-up periods" [238]. Survival in relation to both anti-CEA IgA and anti-CEA IgG titers were also analysed. Patients were grouped according to those having both IgA and IgG anti-CEA antibody levels (through value) above the median (n=9) and the remaining patients (n=15). The relationship between anti-CEA IgA titers, GM-CSF and survival were analysed by Cox-regression analyses. The influence on survival by GM-CSF was assessed by univariate Wilcoxon Gehan exact life table test.

Paper II

Changes in size of erythema over time were analyzed using non-parametric tests (Friedman test and repeated measure ANOVA). Continuous parameters (differences in size between Cohort A and B) were compared by the Mann-Whitney U test.

Paper III

The non-parametric Friedman test for multiple comparisons was used to calculate relation of cell subsets at different time points. The Chi-square, Fisher test and one-way ANOVA tests were used to analyze differences in prognostic factors between groups and relation to immune responses. A p-value <0.05 was considered statistically significant. Life table landmark analysis with Wilcoxon Gehan exact test was used analyze a relationship

between OS as well as DFS to immune responses; landmark was set at 72 (part 1) and 52 (part 2/3) weeks, i.e. close to the end of immunological evaluation. The landmark method for evaluating OS and DFS by immune response selects a fixed time, chosen by the investigator prior to the start of the study. Patients still on study at the landmark time are separated into two immune response categories (non-responders and responders) depending on immune response at the landmark time point. Patients were then followed onwards in time from the landmark [239, 240]. One way ANOVA and Friedmans test (non-parametric) were used to assess PPD and PHA differences at baseline between patients and healthy donors.

Paper IV

The non-parametric Mann Whitney two-tailed rank sum test for comparison of independent variables and the two-tailed non-parametric Wilcoxon signed rank test for dependent observations were applied.

8.6 Ethics

All studies were approved by the Regional Ethical Review Board in Stockholm.

9 RESULTS AND DISCUSSION

9.1 Paper I

Induction of IgM, IgA and IgE Antibodies in Colorectal Cancer patients vaccinated with Recombinant CEA protein. *(J Clin Immuno. 32:855-856, 2012. Staff et al)*

Previous clinical studies have indicated that IgG antibodies have the ability to induce apoptosis of tumor cells but IgM, IgE and IgA may also mediate tumor cell killing [241-246]. The presence of spontaneously induced IgM anti-CEA antibodies has been associated with improved survival in CRC patients [247].However, there is scanty information on the Ig subclasses response after vaccination against cancer antigens. The clinical significance of the different Ig subclasses antibodies is not clear.

In a previously reported study, twenty-four resected CRC patients without macroscopic disease were immunized seven times with the tumor associated protein rCEA ± GM-CSF [138, 248]. Four different dose schedules were used over a 12-month period. A significant anti-rCEA-specific IgG1, IgG2 and IgG4 antibody response was shown and the induction of IgG correlated to improved survival.

Hence, a follow-up study of patients previously enrolled in this vaccination study done by our group was carried out. The aim of this study, reported in **Paper I**, was to assess the induction of IgM, IgA and IgE anti-CEA antibodies, during 36 months after vaccination with rCEA with or without GM-CSF as an adjuvant. Isolated IgA from one vaccinated patient containing high IgA anti-CEA titers was tested in ADCC and CDC, using CEA positive and negative cell lines. Patients were evaluated for DFS and OS. The findings were correlated to DFS and OS.

Most patients (83%) had a detectable IgM, a majority (54%) IgA and one patient had IgE anti-CEA antibodies before vaccination. Pre-immunization IgA, IgM and IgE anti-CEA titers did not differ significantly comparing deceased and alive patients.

GM-CSF significantly augmented the anti-CEA response for all three antibody classes ($p< 0.05$). A strong IgM response was observed initially during vaccination with maximum titers at 3 months from start of vaccination. A rapid induction of IgA antibodies was noted already after one immunization, followed by gradual increase with a peak at 12 months. Repeated immunizations induced a gradual increase in the IgE anti-CEA antibody levels. Maximum IgE levels were noted at 15 months. The antibody curves over time (through values) in relation to GM-CSF were highly significant for IgM p=0.003; IgA p=0.006 and IgE p=0.007. The IgM-titers declined as expected much earlier than the IgA and IgE antibody levels. However, IgA and IgE anti-CEA levels clearly above pre-vaccination levels were still detectable 2 years after the last immunization. The dose of CEA had no significant effect on the IgM, IgA or IgE anti-CEA antibody responses.

Six patients in the CEA alone group and six patients in the GM-CSF group relapsed and died during follow-up. Two further patients in the CEA alone group died from other causes than CRC.

A significant correlation between survival and high IgA anti-CEA titers was noted (p=0.02) irrespective of GM-CSF treatment.

Anti-CEA IgA antibodies could significantly lyse CEA positive tumor cells in ADCC (p<0. 0001) as well as in CDC (p<0.0001).

The addition of GM-CSF was practically mandatory to elict a vaccine induced IgM, IgA and IgE anti-CEA response as compared to patients receiving the vaccine alone. This finding corroborates our previous studies where GM-CSF was found to strongly enhance both the T cell and IgG responses [138, 248]. Fc receptors for IgA are found on monocytes/macrophages, polymorphonuclear neutrophils and NK-cells, i.e cells participating in ADCC [249]. In colon cancer patients elevated serum IgA immune complexes have been observed [241]. Moreover, IgA and IgG antitumor antibodies were shown to be equally effective in killing tumor cells in ADCC [241]. In conclusion, the observation that IgA anti-CEA antibodies were cytotoxic and associated with improved survival might indicate that also these antibodies may exert a clinical anti-tumor effect. Also, the duration of the humoral responses seen in Paper I, warrants further studies to evaluate rCEA as a therapeutic complement in patients with CRC.

9.2 Paper II

A phase I safety study of plasmid DNA immunization targeting carcinoembryonic antigen in colorectal cancer patients. *(Vaccine. 29:6817-6822, 2011. Staff et al)*

The feasibility, safety and immunogenicity of therapeutic vaccination in CRC have been established. Different vaccination approaches targeting CEA in over 350 patients, including plasmid DNA as well as prime-boost vaccination strategies (74 patients) have only shown grade 2, and occasionally grade 3, toxicity [72, 138, 250-252].

In the present study, a plasmid DNA vaccine, encoding a truncated form of human CEA fused to a T-helper epitope (CEA66 DNA) was delivered three times i.d (2mg) or i.m (8mg), by a needle-free injector device, Biojector® to patients with colorectal cancer in the adjuvant setting. Five patients were included in each cohort (Cohorts A and B). Prior to the first vaccination, all patients received cyclophosphamide (300 mg/m2) intravenously (i.v). GM-CSF was administered s.c with each vaccination.

Safety and tolerability were evaluated in all ten patients. All patients reported local injection site reactions but the majority was mild. 89% of the total local AE in Cohort A were grade 1; the corresponding figure for cohort B was 85% The frequency of patients with local grade 2 AEs increased during the 1st, 2nd and 3rd vaccinations and was 0%, 20% and 50% respectively in all patients. There was a significant increase in size of erythema at the injection sites from week 0 to week 6 in Cohort A, (p-value=0.015).

Systemic AEs were also mild and transient (grade 1-2). There was a tendency that fatigue, headache and myalgia were more frequent in the i.d cohort. No grade 3 or 4 adverse events possibly, probably or definitely associated with vaccination were reported. One SAE in one patient, in Cohort A, was not considered related to the study drugs. No signs of autoimmunity were seen.

During follow-up time, one patient (Cohort A) died at week 99 due to urine bladder cancer, diagnosed 72 weeks after start of treatment. One patient (Cohort B) had disease

recurrence at week 52 and was alive at data collection. Eight out of ten patients have no evidence of disease. A concern for DNA vaccination is the integration into hosts cellular DNA that could result in mutagenesis. DNA vaccines currently in test do not show of integration into the host genome [253, 254]. In light of the short time period between the initiation of immunization in this study and diagnosis of the bladder cancer in two patients, as well as the coexistence of risk factors as smoking and previous pelvic radiotherapy, integration of CEA66 DNA could not have resulted in this new malignancy [255]. No signs of autoimmunity were seen.

In conclusion, this phase I trial represents the first clinical trial on safety of a therapeutic vaccination strategy with plasmid DNA encoding human truncated CEA (CEA66 DNA), delivered by needle-free injection in combination with GM-CSF and cyclophosphamide in humans.

Skin contains higher numbers of immunoreactive cells than muscle [90], which may explain the more frequent AE in the i.d cohort. The intradermal low dose DNA route indicated that skin may be a preferred site for immunization. Furthermore, the vaccine strategy was well tolerated, confirming the results of a preclinical mode [256].

9.3 Paper III

Plasmid DNA immunization targeting carcinoembryonic antigen in colorectal cancer patients. *(Manuscript, Staff et al)*

The method of DNA delivery as well as the localization of the expressed antigen can impact the outcome of DNA vaccination. The CEA66-DNA vaccine construct, evaluated in Paper II, encodes a modified, full-length, non-glycosylated form of CEA that is retained inside the cell. In preclinical studies, a T cell mediated response to CEA but weak antibody response was seen, using a needle-free pneumatic device (Biojector) [256, 257]. In contrast to the CEA66-DNA construct, DNA encoding wild type CEA (glycosylated) fused to a tetanus T-helper epitope (tetwtCEA-DNA), which is expressed as a membrane protein, induced a strong T cell as well as a antibody response [257].

Delivery of DNA vaccine either i.m. or i.d by EP, may increase DNA uptake and antigen expression compared to needle injections [258, 259]. Short electrical pulses, at the site of vaccination, create transient pores in the cell membrane augmenting non-viral transfection of plasmid DNA. EP also causes a mild inflammation, with recruitment of APC at the site of injection without affecting persistence or integration of the plasmid [260, 261].

In **Paper III**, we report immune data in an explorative study using CEA66-DNA and tetwt-CEADNA for immunization in combination with cyclophosphamide and GM-CSF in the adjuvant setting of CRC patients. Ten patients received i.d or i.m (cohorts I/II) CEA66-DNA by Biojector (for details see Paper II) at weeks 0, 2 and 6 (part 1). Ten patients; cohorts III/IV (part 2), received tetwt-CEADNA 400µg i.d by needle followed by electroporation at weeks 0 and 12. Cohort V (part 3) (n=6) included patients primed with CEA66-DNA and boosted with tetwt-CEADNA. GM-CSF and cyclophosphamide was included in the vaccination schedules.

Adverse events regarding part 1 has previously been published [262] (Paper II), AEs regarding part 2 and 3 are shown in Table 8. The most frequently reported AEs were local injection site reactions, fatigue and nausea, but the majority was mild. No signs of autoimmunity were seen.

Table 8. Frequency (%) of patients with AE treated with cyclophosphamide and vaccinated with tetwtCEA DNA without GM-CSF (part 2, cohort III; part 3, cohort V) and with GM-CSF (part 2, cohort IV) from baseline to week 24 (NCI Common toxicity criteria version 3.0 grades 1-3) [a].

Toxicity	**Cohort III (n=5)**		**Cohort IV (n=5)**		**Cohort V (n=6)**		**Total (n=16)**	
	G* 1-2 No** (%)	G 3 No (%)	G 1-2 No (%)	G 3 No (%)	G 1-2 No (%)	G 3 No (%)	G 1-2 No (%)	G 3 No (%)
Local AE								
Pain/discomfort	3 (60)	0 (0)	2 (40)	0 (0)	2 (33)	0 (0)	7 (44)	0 (0)
Erythema	1 (20)	0 (0)	4 (80)	0 (0)	2 (33)	0 (0)	7 (44)	0 (0)
Oedema	0 (0)	0 (0)	1 (20)	0 (0)	1 (17)	0 (0)	2 (13)	0 (0)
Warmth	0 (0)	0 (0)	0 (0)	0 (0)	0 (00)	0 (0)	0 (0)	0 (0)
Pruritus	2 (40)	0 (0)	1 (20)	0 (0)	5 (83)	0 (0)	8 (50)	0 (0)
Induration	0 (0)	0 (0)	0 (0)	0 (0)	0 (00)	0 (0)	0 (0)	0 (0)
Systemic AE								
Fatigue	2 (40)	1 (20)	2 (40)	0 (0)	4 (67)	0 (0)	8 (50)	1 (6)
Headache	1 (20)	0 (0)	1 (20)	0 (0)	0 (0)	0 (0)	2 (13)	0 (0)
Dizziness	1 (20)	0 (0)	2 (40)	0 (0)	0 (0)	0 (0)	3 (19)	0 (0)
Myalgia	1 (20)	0 (0)	0 (0)	0 (0)	1 (17)	0 (0)	2 (13)	0 (0)
Nausea	4 (80)	0 (0)	3 (60)	0 (0)	2 (33)	0 (0)	9 (56)	0 (0)
Anorexia	0 (0)	0 (0)	1 (20)	0 (0)	0 (0)	0 (0)	1 (6)	0 (0)
Arthralgia	0 (0)	0 (0)	1 (20)	0 (0)	1 (17)	0 (0)	2 (13)	0 (0)
Shivering	1 (20)	0 (0)	3 (60)	0 (0)	0 (0)	0 (0)	4 (25)	0 (0)
Swelling	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (17)	0 (0)	1 (6)
Chest tightness	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Diarrhoea	1 (20)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (6)	0 (0)
Pruritus general	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (17)	0 (0)	1 (6)
Rash/urticaria	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (17)	0 (0)	1 (6)
Microscopic hematuria	0 (0)	0 (0)	0 (0)	0 (0)	1 (17)	0 (0)	1 (6)	0 (0)
Metabolic								
ALAT elevated	0 (0)	0 (0)	1 (20)	0 (0)	0 (0)	0 (0)	1 (6)	0 (0)
ASAT elevated	0 (0)	0 (0)	1 (20)	0 (0)	0 (0)	0 (0)	1 (6)	0 (0)

a. No grade 4 AE was reported. *G=Grade. ** Represents the number of subjects experiencing adverse events.

In total, 16 out of 20 (80%) patients mounted a single assay cellular response; 10/10 (100%) in part 1 and in 6/10 (60%) of the patients in part 2. The difference between the CEA vaccine naïve patients was statistically significant (p = 0.025) in favour of part 1. OIIR was seen in 6/10 (60%) of the patients in part 1 and in 4/10 (40%) of the patients in part 2. The difference was statistically not significant.

In part 3, 5/6 (83%) of the patients mounted a single response in one assay. In part 3 OIIR was noted in 2/5 (40%) of the patients. All patients mounted a single assay cellular immune response in either part. In total, humoral responses were seen in two patients only.

There were no significant differences in either OS or DFS comparing single assay immune responders or OIIR in parts 1 and 2.

In the present study, the frequency of patients mounting a single assay cellular immune response was significantly higher following Biojector delivery (part 1) compared to vaccination with electroporation in CEA vaccine naïve patients (part 2).

An explanation might be a higher number of injections transfecting a larger number of cells and that Biojector delivery may evoke a substantial cell-trauma eliciting danger signals, enhancing an immune response induction [11, 12]. Immune responses are also higher when a higher dose of DNA plasmid is delivered [263]. Another explanation for a better immunogenicity in part 1 is the form of CEA-DNA used. The CEA-DNA construct used in part 1 generates a non-glycosylated protein that to a higher extent might be retained in the cytoplasm in comparison to the DNA construct used in part 2 yielding a glycosylated CEA protein which localizes to the plasma membrane. Thus, the CEA66-DNA might be more foreign compared to wild type CEA than the tetwtCEA-DNA used in part 2 [264, 265]. Likewise, in melanoma, a more heterogenous substance compared to the wild-type were more effective in breaking immune tolerance [266].

Mostly a Th-1 like cellular immune response was seen and only few individuals developed an antibody response, confirming the results in mice where CEA66-DNA primarily induced a cellular immune response[256]. Priming with CEA66-DNA and boosting with tetwtCEA-DNA did not augment the humoral response contradicting the results in mice by Brave et al [255]. Immune responses against CEA-derived peptides may suggest that CEA-DNA vaccination might induce both a CD4 and CD8 response. These results are supported by a preclinical study in mice vaccinated with CEA66-DNA, where a response against the B3 domain was mediated by CD4+ cells and towards the CTL-epitopes by CD8+ cells [256].

The results in Paper III indicate that self-tolerance against the tumor-associated antigen CEA could be broken, although weak but long-lasting. Similar to CEA66-DNA, tetwtCEA-DNA, combined with cyclophosphamide and GM-CSF was safe [262].The present study also indicates that T cell responses could best be induced against CEA-DNA delivery by Biojector, which in this respect seemed to be superior to CEA-DNA injection followed by electroporation for immune induction. Extended studies are warranted to discriminate dose and delivery devices to determine the clinical significance of CEA-DNA vaccination in patients with CRC.

9.4 Paper IV

Telomerase-peptide vaccination (GV1001) together with gemcitabine in advanced pancreatic cancer patients. *(Manuscript, Staff et al)*

In **Paper IV**, we assess safety and immunogenicity in non-resectable pancreatic cancer patients using a 16 aa telomerase peptide (GV1001) for vaccination in combination with GM-CSF and gemcitabine as first line treatment.

Three different vaccine treatment schedules were used (A (n=6), B (n=6), C (n=5)). Groups A/B received GV1001, GM-CSF and gemcitabine concurrently. Group C received

initially GV1001 and GM-CSF while gemcitabine was added at disease progression. Group D (n=4) was treated with gemcitabine alone.

AE related to vaccination were mild (grades 1-2). Grade 3 AEs were few and transient. A higher dose of GM-CSF induced a higher frequency and severity of injection site reactions. Gemcitabine related side-effects were as expected [267] and without overlapping toxicity with the vaccine treatment. One SAE was initially suspected to be related to GV1001 or GM-CSF in one patient in group C, who developed hepatic dysfunction (grade 3) due to liver metastasis. Both median time to progression and overall survival were most poor in group C.

Four out of six (67%) patients developed a single assay immune response against telomerase and 3/6 (50%) against ras in group A. In group B, 4 out of 6 (67%) patients mounted a telomerase response and one (17%) against ras. A telomerase response in group C was noted in 2/5 (40%) patients and a ras response in 1/5 (20%) patients. In group D, a telomerase response was recorded in 2/4 (50%) patients and a ras response in 1/4 (25%) patients.

OIIR was only seen in group A patients, in one patient against telomerase and in two patients against ras. The cytokine pattern was that of a Th_1-profile. A significant decrease in regulatory T cells over time was noted in patients in groups A and B ($p < 0.05$).

The results might indicate that concomitant treatment with gemcitabine may not hamper the induction of an immune response but that delayed administration of gemcitabine might reduce the capacity to mount an immune response and favour tumor progression (group C).

Telomerase vaccination (GV1001) in combination with chemotherapy appeared to be safe but the immune responses were weak and transient. Based on the experience of the present study and of those of others including immune responses and clinical efficacy, measures have to be taken to augment the magnitude and duration of the immune response to GV1001. Furthermore, advanced pancreatic carcinoma patients might not be a preferred clinical setting for vaccine treatment, as is the case for other tumors and tumor cancer vaccines [268]. Maybe the GV1001 vaccine is not an optimal telomerase vaccine candidate, although it has been shown that immune responders to GV1001 vaccination survived longer than non-immune responders [165] and CLL patients exhibited spontaneous T cells recognizing GV1001, which could lyse autologous telomerase expressing leukemic cells [153]. The study might also support the notion that multiple immune read-out systems might increase the sensitivity to detect antigen specific immune responses [269, 270].

10 CONCLUSIONS AND FUTURE PERSPECTIVES

Active immunotherapy is emerging as an important addition to conventional cancer treatments but many important questions remain. Effective strategies for overcoming immunosuppression need to be developed and patient selection are important. Optimal combinations of antigens, adjuvants and delivery vehicles need to be determined. Validation of surrogate endpoints and the identification of predictive biomarkers are equally important.

A concept that is gaining acceptance in the field of active immunotherapy towards cancer is that vaccine monotherapy is unlikely to succeed in generating a robust and long-lasting memory. Synergism between existing chemotherapy and immunotherapy may lead to more clinical responses [271, 272]. Gemcitabine and 5-Fluorouracil have been implicated in the suppression of MDSC activity [273, 274]. Cyclophosphamide induced leucopenia results in an expansion of immature dendritic cells favouring immune responses [275] and may suppress T_{regs} [119, 276-279].

The development of combination therapies is, however, associated with challenges. There is often little preclinical data on such combinations and phase I trials are required to show non-toxic combinations as well as to demonstrate immunological responses to the TCV. In Paper IV, gemcitabine combined with GV 1001 and GM-CSF did not seem to add to the toxicity but a decrease in the frequency of T_{regs} was seen. As shown in Paper II and III, the combination of cyclophosphamide and CEA-DNA vaccination was well tolerated with mild and transient side-effects. No significant decrease in the frequency of T_{regs} were seen, but the PHA responses increased in about 40% of the patients during vaccination, which might be due to reduced suppression induced by the cyclophosphamide treatment.

Combinations of complementary immunotherapies may further induce sustained anti-tumor responses. By combining plasmid DNA vaccination with other modalities of antigen delivery, such as recombinant proteins, in heterologous prime/boost protocols, both B and T cell immune responses can be enhanced [280].

The results in Papers I and III combined, show a durable induction of anti-CEA antibodies following rCEA protein vaccination and long-lasting cellular responses following CEA-DNA vaccination. The induction of anti-CEA specific IgG antibodies as well as proliferative T cell responses, following rCEA protein vaccination, has previously been published [138, 248]. In a preclinical study designed as a DNA prime/protein boost setting, cellular immune responses were of higher magnitude in animals primed with CEA-DNA than in animals receiving repeated doses of rCEA protein [256]. In light of safety data presented in Paper II, a similar study would be feasible in humans.

Clinical studies have demonstrated the ability of different TCV strategies to induce antigen-specific T cells [281-284]. However, despite expanding antigen-specific T cells, in most clinical trials, tumors continue to grow [285-288]. Most clinical trials have been conducted in patients with advanced metastatic disease, and as tumors grow they evade immune destruction, for example by down-regulating HLA-expression [28, 289, 290]. In the present thesis, treatment with GV1001, GM-CSF and gemcitabine in patients with advanced

pancreatic cancer, mounted an immune response against telomerase in approximately two thirds of the patients in the best schedule. However, although the patients did not seem to be immune-hypo responsive as evaluated by PHA and PPD responses, immune response towards telomerase were weak and transient. In a number of clinical studies in which active immunotherapy failed to prolong survival, subgroup analyses suggested clinical benefit in patients with early or less aggressive disease [291-294].

For cell surface antigens, both CTL and humoral responses may mediate antitumor activity, attesting the importance of activating both CD8+ and CD4+ T cells [72]. Previous studies on rCEA protein vaccination in CRC patients resulted in both cellular and humoral CEA-specific responses, and the IgG antibody responses to CEA correlated with increased survival [137, 138].

Studies have suggested that IgM, IgA as well as IgE antibodies may have a role in eradicating cancer cells [241-246]. IgA antibodies have been proposed to be advantageous in tumor cell killing as compared to IgG [295]. In Paper I, rCEA vaccination combined with GM-CSF as an adjuvans, significantly induced durable antigen –specific humoral responses (IgA, IgM, IgE) against CEA and the induction of IgA was significantly correlated to survival. Furthermore, anti-CEA IgA could significantly lyse CEA positive cells. Most TCV studies focus on the induction of antigen-specific T cells. Future cancer vaccine trials might also assess the induction of the humoral response, including different isotypes.

Modifications of the antigen may alter the immune response. In Paper III, the CEA-DNA construct used in part 1 generates a non-glycosylated protein that to a higher extent might be retained in the cytoplasm in comparison to the DNA construct used in part 2 yielding a glycosylated CEA protein which localizes to the plasma membrane. Tumor associated MUC1 is frequently hypoglycosylated on myeloma cells and this is associated by a more efficient antigen processing by dendritic cells, leading to stronger T cell responses [296-298].

DNA potency may also be improved by a) alteration to the plasmid itself to increase expression of immunogenicity (i.e. insertion of immune modulators) or b) placing DNA in/on microparticles acting as adjuvants for APCs [110].

There is a need for development and validation of tools to identify patients who can benefit from a particular form of immunotherapy. Despite effort, we do not know which parameters of immune response, and which assays used to assess these parameters, are optimal for efficacy analysis. According to a workshop held 2011, involving FDA, National Cancer Institute and the International Society for Biological Therapy of Cancer-Society for Immunotherapy of cancer (iSBTc-SITC), several key issues need to be addressed to identify patients and patient-groups who will benefit from treatment [299].

Recommendations included standardized handling of blood samples, use of robust and standardized assays. Furthermore, they recommend that several different assays used – to capture both the magnitude of the response, but also duration, quality and frequency.

The characterization of responders and non-responders is difficult, assays yield plenty data. Distinguishing them from assay variation and normal human variation is difficult. Tight response criteria, requiring positive responses in 2 post-therapy time points is recommended [168]. Obtaining multiple pre-therapy samples, at different time-points, can be

used to assess pre-therapy variability and published raw data enables the reader to interpret data [168]. In addition, new analytes and/or biomarkers needs to be standardized and validated by other investigators before they are correlated to clinical outcome [168].

Looking ahead, an effective immune response that leads to an antitumor response requires not only an increase in immune activation but also reduction of suppressive immune checkpoints [278, 300]. The approval of mAb against CTLA-4 provided proof-of –principle for targeting immune suppressive checkpoints in the treatment of cancer [120] and mAb targeting PD1/PDL1 are in phase III clinical development. Synergy with TCV in mouse models suggests the potential for combining TCV with either of these mAbs [300-302].

Moreover, the mechanisms whereby radiotherapy elicits tumor-specific immune responses have begun to emerge [303, 304]. Well designed trials are required to investigate this combotorial approach [305].

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12 REFERENCES

1. Delves PJ, Roitt IM. The immune system. First of two parts. The New England journal of medicine. 2000; 343(1): 37-49
2. Bretscher P, Cohn M. A theory of self-nonself discrimination. Science. 1970; 169(3950): 1042-9
3. Matzinger P. The danger model: a renewed sense of self. Science. 2002; 296(5566): 301-5
4. Barry M, Bleackley RC. Cytotoxic T lymphocytes: all roads lead to death. Nature reviews Immunology. 2002; 2(6): 401-9
5. Heath WR, Carbone FR. Cross-presentation, dendritic cells, tolerance and immunity. Annual review of immunology. 2001; 19: 47-64
6. Arina A, Tirapu I, Alfaro C, Rodriguez-Calvillo M, Mazzolini G, Inoges S, et al. Clinical implications of antigen transfer mechanisms from malignant to dendritic cells. exploiting cross-priming. Experimental hematology. 2002; 30(12): 1355-64
7. Waldmann TA. Immunotherapy: past, present and future. Nature medicine. 2003; 9(3): 269-77
8. Galluzzi L, Vacchelli E, Eggermont A, Fridman WH, Galon J, Sautes-Fridman C, et al. Trial Watch: Experimental Toll-like receptor agonists for cancer therapy. Oncoimmunology. 2012; 1(5): 699-716
9. Vacchelli E, Galluzzi L, Eggermont A, Fridman WH, Galon J, Sautes-Fridman C, et al. Trial watch: FDA-approved Toll-like receptor agonists for cancer therapy. Oncoimmunology. 2012; 1(6): 894-907
10. Burgio GR. Commentary on the biological self: Toward a "Biological Ego". From Garrod's "chemical individuality" to Burnet's "self". Thymus. 1990; 16(2): 99-117
11. Matzinger P. Tolerance, danger, and the extended family. Annual review of immunology. 1994; 12: 991-1045
12. Vacchelli E, Martins I, Eggermont A, Fridman WH, Galon J, Sautes-Fridman C, et al. Trial watch: Peptide vaccines in cancer therapy. Oncoimmunology. 2012; 1(9): 1557-76
13. Fridman WH, Pages F, Sautes-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. Nature reviews Cancer. 2012; 12(4): 298-306
14. Galluzzi L, Kepp O, Kroemer G. Mitochondria: master regulators of danger signalling. Nature reviews Molecular cell biology. 2012; 13(12): 780-8
15. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011; 144(5): 646-74
16. Teng MW, Swann JB, Koebel CM, Schreiber RD, Smyth MJ. Immune-mediated dormancy: an equilibrium with cancer. Journal of leukocyte biology. 2008; 84(4): 988-93
17. Kim R, Emi M, Tanabe K. Cancer immunoediting from immune surveillance to immune escape. Immunology. 2007; 121(1): 1-14
18. Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. Annual review of immunology. 2004; 22: 329-60
19. Swann JB, Smyth MJ. Immune surveillance of tumors. The Journal of clinical investigation. 2007; 117(5): 1137-46
20. Pages F, Galon J, Dieu-Nosjean MC, Tartour E, Sautes-Fridman C, Fridman WH. Immune infiltration in human tumors: a prognostic factor that should not be ignored. Oncogene. 2010; 29(8): 1093-102
21. Nelson BH. The impact of T-cell immunity on ovarian cancer outcomes. Immunological reviews. 2008; 222: 101-16
22. Leffers N, Gooden MJ, de Jong RA, Hoogeboom BN, ten Hoor KA, Hollema H, et al. Prognostic significance of tumor-infiltrating T-lymphocytes in primary and metastatic lesions of advanced stage ovarian cancer. Cancer immunology, immunotherapy : CII. 2009; 58(3): 449-59
23. Sahin U, Tureci O, Schmitt H, Cochlovius B, Johannes T, Schmits R, et al. Human neoplasms elicit multiple specific immune responses in the autologous host. Proceedings of the National Academy of Sciences of the United States of America. 1995; 92(25): 11810-3
24. Wang RF, Rosenberg SA. Human tumor antigens for cancer vaccine development. Immunological reviews. 1999; 170: 85-100
25. Vajdic CM, van Leeuwen MT. Cancer incidence and risk factors after solid organ transplantation. International journal of cancer Journal international du cancer. 2009; 125(8): 1747-54
26. Dougan M, Dranoff G. Immune therapy for cancer. Annual review of immunology. 2009; 27: 83-117

27. Greten FR, Eckmann L, Greten TF, Park JM, Li ZW, Egan LJ, et al. IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. Cell. 2004; 118(3): 285-96
28. Campoli M, Chang CC, Ferrone S. HLA class I antigen loss, tumor immune escape and immune selection. Vaccine. 2002; 20 Suppl 4: A40-5
29. Marincola FM, Jaffee EM, Hicklin DJ, Ferrone S. Escape of human solid tumors from T-cell recognition: molecular mechanisms and functional significance. Advances in immunology. 2000; 74: 181-273
30. Igney FH, Krammer PH. Immune escape of tumors: apoptosis resistance and tumor counterattack. Journal of leukocyte biology. 2002; 71(6): 907-20
31. Ranges GE, Figari IS, Espevik T, Palladino MA, Jr. Inhibition of cytotoxic T cell development by transforming growth factor beta and reversal by recombinant tumor necrosis factor alpha. The Journal of experimental medicine. 1987; 166(4): 991-8
32. Thomas DA, Massague J. TGF-beta directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. Cancer cell. 2005; 8(5): 369-80
33. Walker MR, Kasprowicz DJ, Gersuk VH, Benard A, Van Landeghen M, Buckner JH, et al. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25- T cells. The Journal of clinical investigation. 2003; 112(9): 1437-43
34. Uyttenhove C, Pilotte L, Theate I, Stroobant V, Colau D, Parmentier N, et al. Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. Nature medicine. 2003; 9(10): 1269-74
35. Terness P, Bauer TM, Rose L, Dufter C, Watzlik A, Simon H, et al. Inhibition of allogeneic T cell proliferation by indoleamine 2,3-dioxygenase-expressing dendritic cells: mediation of suppression by tryptophan metabolites. The Journal of experimental medicine. 2002; 196(4): 447-57
36. Lanca T, Silva-Santos B. The split nature of tumor-infiltrating leukocytes: Implications for cancer surveillance and immunotherapy. Oncoimmunology. 2012; 1(5): 717-25
37. Murdoch C, Muthana M, Coffelt SB, Lewis CE. The role of myeloid cells in the promotion of tumour angiogenesis. Nature reviews Cancer. 2008; 8(8): 618-31
38. Shojaei F, Zhong C, Wu X, Yu L, Ferrara N. Role of myeloid cells in tumor angiogenesis and growth. Trends in cell biology. 2008; 18(8): 372-8
39. Ostrand-Rosenberg S, Sinha P. Myeloid-derived suppressor cells: linking inflammation and cancer. Journal of immunology. 2009; 182(8): 4499-506
40. Sica A, Bronte V. Altered macrophage differentiation and immune dysfunction in tumor development. The Journal of clinical investigation. 2007; 117(5): 1155-66
41. Bronte V, Wang M, Overwijk WW, Surman DR, Pericle F, Rosenberg SA, et al. Apoptotic death of CD8+ T lymphocytes after immunization: induction of a suppressive population of Mac-1+/Gr-1+ cells. Journal of immunology. 1998; 161(10): 5313-20
42. Seung LP, Rowley DA, Dubey P, Schreiber H. Synergy between T-cell immunity and inhibition of paracrine stimulation causes tumor rejection. Proceedings of the National Academy of Sciences of the United States of America. 1995; 92(14): 6254-8
43. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. Nature reviews Immunology. 2009; 9(3): 162-74
44. Bronte V. Myeloid-derived suppressor cells in inflammation: uncovering cell subsets with enhanced immunosuppressive functions. European journal of immunology. 2009; 39(10): 2670-2
45. Highfill SL, Rodriguez PC, Zhou Q, Goetz CA, Koehn BH, Veenstra R, et al. Bone marrow myeloid-derived suppressor cells (MDSCs) inhibit graft-versus-host disease (GVHD) via an arginase-1-dependent mechanism that is up-regulated by interleukin-13. Blood. 2010; 116(25): 5738-47
46. Huang B, Pan PY, Li Q, Sato AI, Levy DE, Bromberg J, et al. Gr-1+CD115+ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host. Cancer research. 2006; 66(2): 1123-31
47. Mantovani A, Sica A. Macrophages, innate immunity and cancer: balance, tolerance, and diversity. Current opinion in immunology. 2010; 22(2): 231-7
48. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. Cell. 2010; 141(1): 39-51
49. Guiducci C, Vicari AP, Sangaletti S, Trinchieri G, Colombo MP. Redirecting in vivo elicited tumor infiltrating macrophages and dendritic cells towards tumor rejection. Cancer research. 2005; 65(8): 3437-46

50. Curiel TJ. Tregs and rethinking cancer immunotherapy. The Journal of clinical investigation. 2007; 117(5): 1167-74
51. Onizuka S, Tawara I, Shimizu J, Sakaguchi S, Fujita T, Nakayama E. Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor alpha) monoclonal antibody. Cancer research. 1999; 59(13): 3128-33
52. Antony PA, Restifo NP. CD4+CD25+ T regulatory cells, immunotherapy of cancer, and interleukin-2. Journal of immunotherapy. 2005; 28(2): 120-8
53. Shimizu J, Yamazaki S, Sakaguchi S. Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity. Journal of immunology. 1999; 163(10): 5211-8
54. Beyer M, Schultze JL. Regulatory T cells in cancer. Blood. 2006; 108(3): 804-11
55. Sakaguchi S. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. Annual review of immunology. 2004; 22: 531-62
56. Bergmann C, Strauss L, Wang Y, Szczepanski MJ, Lang S, Johnson JT, et al. T regulatory type 1 cells in squamous cell carcinoma of the head and neck: mechanisms of suppression and expansion in advanced disease. Clinical cancer research : an official journal of the American Association for Cancer Research. 2008; 14(12): 3706-15
57. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nature reviews Cancer. 2012; 12(4): 252-64
58. Bluestone JA, Abbas AK. Natural versus adaptive regulatory T cells. Nature reviews Immunology. 2003; 3(3): 253-7
59. Fehervari Z, Sakaguchi S. CD4+ Tregs and immune control. The Journal of clinical investigation. 2004; 114(9): 1209-17
60. Lenardo M, Chan KM, Hornung F, McFarland H, Siegel R, Wang J, et al. Mature T lymphocyte apoptosis--immune regulation in a dynamic and unpredictable antigenic environment. Annual review of immunology. 1999; 17: 221-53
61. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nature immunology. 2003; 4(4): 330-6
62. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science. 2003; 299(5609): 1057-61
63. Shevach EM. Fatal attraction: tumors beckon regulatory T cells. Nature medicine. 2004; 10(9): 900-1
64. Zou W. Regulatory T cells, tumour immunity and immunotherapy. Nature reviews Immunology. 2006; 6(4): 295-307
65. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. Annual review of immunology. 2008; 26: 677-704
66. Weiner LM, Surana R, Wang S. Monoclonal antibodies: versatile platforms for cancer immunotherapy. Nature reviews Immunology. 2010; 10(5): 317-27
67. Galluzzi L, Vacchelli E, Fridman WH, Galon J, Sautes-Fridman C, Tartour E, et al. Trial Watch: Monoclonal antibodies in cancer therapy. Oncoimmunology. 2012; 1(1): 28-37
68. Vacchelli E, Eggermont A, Galon J, Sautes-Fridman C, Zitvogel L, Kroemer G, et al. Trial watch: Monoclonal antibodies in cancer therapy. Oncoimmunology. 2013; 2(1): e22789
69. Senovilla L, Vacchelli E, Garcia P, Eggermont A, Fridman WH, Galon J, et al. Trial watch: DNA vaccines for cancer therapy. Oncoimmunology. 2013; 2(4): e23803
70. Cheever MA, Allison JP, Ferris AS, Finn OJ, Hastings BM, Hecht TT, et al. The prioritization of cancer antigens: a national cancer institute pilot project for the acceleration of translational research. Clinical cancer research : an official journal of the American Association for Cancer Research. 2009; 15(17): 5323-37
71. Mellman I, Coukos G, Dranoff G. Cancer immunotherapy comes of age. Nature. 2011; 480(7378): 480-9
72. Berinstein NL. Carcinoembryonic antigen as a target for therapeutic anticancer vaccines: a review. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2002; 20(8): 2197-207
73. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature. 1998; 392(6673): 245-52
74. Belardelli F, Ferrantini M. Cytokines as a link between innate and adaptive antitumor immunity. Trends in immunology. 2002; 23(4): 201-8

75. Dredge K, Marriott JB, Todryk SM, Dalgleish AG. Adjuvants and the promotion of Th1-type cytokines in tumour immunotherapy. Cancer immunology, immunotherapy : CII. 2002; 51(10): 521-31
76. Mosolits S, Nilsson B, Mellstedt H. Towards therapeutic vaccines for colorectal carcinoma: a review of clinical trials. Expert Rev Vaccines. 2005; 4(3): 329-50
77. Warren TL, Weiner GJ. Uses of granulocyte-macrophage colony-stimulating factor in vaccine development. Current opinion in hematology. 2000; 7(3): 168-73
78. Mellstedt H, Fagerberg J, Frodin JE, Henriksson L, Hjelm-Skoog AL, Liljefors M, et al. Augmentation of the immune response with granulocyte-macrophage colony-stimulating factor and other hematopoietic growth factors. Current opinion in hematology. 1999; 6(3): 169-75
79. Gillessen S, Naumov YN, Nieuwenhuis EE, Exley MA, Lee FS, Mach N, et al. CD1d-restricted T cells regulate dendritic cell function and antitumor immunity in a granulocyte-macrophage colony-stimulating factor-dependent fashion. Proceedings of the National Academy of Sciences of the United States of America. 2003; 100(15): 8874-9
80. Aly HA. Cancer therapy and vaccination. Journal of immunological methods. 2012; 382(1-2): 1-23
81. Kawakami Y, Eliyahu S, Delgado CH, Robbins PF, Sakaguchi K, Appella E, et al. Identification of a human melanoma antigen recognized by tumor-infiltrating lymphocytes associated with in vivo tumor rejection. Proceedings of the National Academy of Sciences of the United States of America. 1994; 91(14): 6458-62
82. Van Der Bruggen P, Zhang Y, Chaux P, Stroobant V, Panichelli C, Schultz ES, et al. Tumor-specific shared antigenic peptides recognized by human T cells. Immunological reviews. 2002; 188: 51-64
83. Thomas AM, Santarsiero LM, Lutz ER, Armstrong TD, Chen YC, Huang LQ, et al. Mesothelin-specific CD8(+) T cell responses provide evidence of in vivo cross-priming by antigen-presenting cells in vaccinated pancreatic cancer patients. The Journal of experimental medicine. 2004; 200(3): 297-306
84. Cheever MA, Higano CS. PROVENGE (Sipuleucel-T) in prostate cancer: the first FDA-approved therapeutic cancer vaccine. Clinical cancer research : an official journal of the American Association for Cancer Research. 2011; 17(11): 3520-6
85. Galluzzi L, Senovilla L, Vacchelli E, Eggermont A, Fridman WH, Galon J, et al. Trial watch: Dendritic cell-based interventions for cancer therapy. Oncoimmunology. 2012; 1(7): 1111-34
86. Yang L, Yang H, Rideout K, Cho T, Joo KI, Ziegler L, et al. Engineered lentivector targeting of dendritic cells for in vivo immunization. Nature biotechnology. 2008; 26(3): 326-34
87. Lopes L, Dewannieux M, Gileadi U, Bailey R, Ikeda Y, Whittaker C, et al. Immunization with a lentivector that targets tumor antigen expression to dendritic cells induces potent CD8+ and CD4+ T-cell responses. Journal of virology. 2008; 82(1): 86-95
88. Purcell AW, McCluskey J, Rossjohn J. More than one reason to rethink the use of peptides in vaccine design. Nature reviews Drug discovery. 2007; 6(5): 404-14
89. Cecco S, Muraro E, Giacomin E, Martorelli D, Lazzarini R, Baldo P, et al. Cancer vaccines in phase II/III clinical trials: state of the art and future perspectives. Current cancer drug targets. 2011; 11(1): 85-102
90. Ramirez LA, Arango T, Boyer J. Therapeutic and prophylactic DNA vaccines for HIV-1. Expert opinion on biological therapy. 2013; 13(4): 563-73
91. Fioretti D, Iurescia S, Fazio VM, Rinaldi M. DNA vaccines: developing new strategies against cancer. J Biomed Biotechnol. 2010; 2010: 174378
92. Rice J, Ottensmeier CH, Stevenson FK. DNA vaccines: precision tools for activating effective immunity against cancer. Nature reviews Cancer. 2008; 8(2): 108-20
93. Shirota H, Petrenko L, Hong C, Klinman DM. Potential of transfected muscle cells to contribute to DNA vaccine immunogenicity. Journal of immunology. 2007; 179(1): 329-36
94. Webster RG, Robinson HL. DNA vaccines: a review of developments. BioDrugs : clinical immunotherapeutics, biopharmaceuticals and gene therapy. 1997; 8(4): 273-92
95. King CA, Spellerberg MB, Zhu D, Rice J, Sahota SS, Thompsett AR, et al. DNA vaccines with single-chain Fv fused to fragment C of tetanus toxin induce protective immunity against lymphoma and myeloma. Nature medicine. 1998; 4(11): 1281-6

96. Hung CF, Cheng WF, Hsu KF, Chai CY, He L, Ling M, et al. Cancer immunotherapy using a DNA vaccine encoding the translocation domain of a bacterial toxin linked to a tumor antigen. Cancer research. 2001; 61(9): 3698-703
97. Boyle JS, Koniaras C, Lew AM. Influence of cellular location of expressed antigen on the efficacy of DNA vaccination: cytotoxic T lymphocyte and antibody responses are suboptimal when antigen is cytoplasmic after intramuscular DNA immunization. International immunology. 1997; 9(12): 1897-906
98. Rice J, King CA, Spellerberg MB, Fairweather N, Stevenson FK. Manipulation of pathogen-derived genes to influence antigen presentation via DNA vaccines. Vaccine. 1999; 17(23-24): 3030-8
99. Kutzler MA, Weiner DB. DNA vaccines: ready for prime time? Nature reviews Genetics. 2008; 9(10): 776-88
100. Brave A, Gudmundsdotter L, Sandstrom E, Haller BK, Hallengard D, Maltais AK, et al. Biodistribution, persistence and lack of integration of a multigene HIV vaccine delivered by needle-free intradermal injection and electroporation. Vaccine. 2010; 28(51): 8203-9
101. Sandstrom E, Nilsson C, Hejdeman B, Brave A, Bratt G, Robb M, et al. Broad immunogenicity of a multigene, multiclade HIV-1 DNA vaccine boosted with heterologous HIV-1 recombinant modified vaccinia virus Ankara. J Infect Dis. 2008; 198(10): 1482-90
102. Rols MP. Mechanism by which electroporation mediates DNA migration and entry into cells and targeted tissues. Methods in molecular biology. 2008; 423: 19-33
103. Chu G, Hayakawa H, Berg P. Electroporation for the efficient transfection of mammalian cells with DNA. Nucleic acids research. 1987; 15(3): 1311-26
104. Roos AK, Eriksson F, Walters DC, Pisa P, King AD. Optimization of skin electroporation in mice to increase tolerability of DNA vaccine delivery to patients. Molecular therapy : the journal of the American Society of Gene Therapy. 2009; 17(9): 1637-42
105. Laddy DJ, Yan J, Khan AS, Andersen H, Cohn A, Greenhouse J, et al. Electroporation of synthetic DNA antigens offers protection in nonhuman primates challenged with highly pathogenic avian influenza virus. Journal of virology. 2009; 83(9): 4624-30
106. Johansson S, Ek M, Wahren B, Stout R, Liu M, Hallermalm K. Intracellular targeting of CEA results in Th1-type antibody responses following intradermal genetic vaccination by a needle-free jet injection device. TheScientificWorldJournal. 2007; 7: 987-99
107. Babiuk S, Baca-Estrada ME, Foldvari M, Baizer L, Stout R, Storms M, et al. Needle-free topical electroporation improves gene expression from plasmids administered in porcine skin. Molecular therapy : the journal of the American Society of Gene Therapy. 2003; 8(6): 992-8
108. Hallermalm K, Johansson S, Brave A, Ek M, Engstrom G, Boberg A, et al. Pre-clinical evaluation of a CEA DNA prime/protein boost vaccination strategy against colorectal cancer. Scandinavian journal of immunology. 2007; 66(1): 43-51
109. Porgador A, Irvine KR, Iwasaki A, Barber BH, Restifo NP, Germain RN. Predominant role for directly transfected dendritic cells in antigen presentation to CD8+ T cells after gene gun immunization. The Journal of experimental medicine. 1998; 188(6): 1075-82
110. Liu MA. DNA vaccines: an historical perspective and view to the future. Immunological reviews. 2011; 239(1): 62-84
111. Quaak SG, van den Berg JH, Toebes M, Schumacher TN, Haanen JB, Beijnen JH, et al. GMP production of pDERMATT for vaccination against melanoma in a phase I clinical trial. European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik eV. 2008; 70(2): 429-38
112. Moschella F, Proietti E, Capone I, Belardelli F. Combination strategies for enhancing the efficacy of immunotherapy in cancer patients. Annals of the New York Academy of Sciences. 2010; 1194: 169-78
113. Nowak AK, Lake RA, Robinson BW. Combined chemoimmunotherapy of solid tumours: improving vaccines? Advanced drug delivery reviews. 2006; 58(8): 975-90
114. Zitvogel L, Kepp O, Kroemer G. Decoding cell death signals in inflammation and immunity. Cell. 2010; 140(6): 798-804
115. Sistigu A, Viaud S, Chaput N, Bracci L, Proietti E, Zitvogel L. Immunomodulatory effects of cyclophosphamide and implementations for vaccine design. Seminars in immunopathology. 2011; 33(4): 369-83

116. Proietti E, Moschella F, Capone I, Belardelli F. Exploitation of the propulsive force of chemotherapy for improving the response to cancer immunotherapy. Molecular oncology. 2012; 6(1): 1-14
117. Emens LA. Breast cancer immunobiology driving immunotherapy: vaccines and immune checkpoint blockade. Expert Rev Anticancer Ther. 2012; 12(12): 1597-611
118. Cerullo V, Diaconu I, Kangasniemi L, Rajecki M, Escutenaire S, Koski A, et al. Immunological effects of low-dose cyclophosphamide in cancer patients treated with oncolytic adenovirus. Molecular therapy : the journal of the American Society of Gene Therapy. 2011; 19(9): 1737-46
119. Ghiringhelli F, Menard C, Puig PE, Ladoire S, Roux S, Martin F, et al. Metronomic cyclophosphamide regimen selectively depletes CD4+CD25+ regulatory T cells and restores T and NK effector functions in end stage cancer patients. Cancer immunology, immunotherapy : CII. 2007; 56(5): 641-8
120. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. The New England journal of medicine. 2010; 363(8): 711-23
121. Brahmer JR, Drake CG, Wollner I, Powderly JD, Picus J, Sharfman WH, et al. Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2010; 28(19): 3167-75
122. Benchimol S, Fuks A, Jothy S, Beauchemin N, Shirota K, Stanners CP. Carcinoembryonic antigen, a human tumor marker, functions as an intercellular adhesion molecule. Cell. 1989; 57(2): 327-34
123. Taheri M, Saragovi U, Fuks A, Makkerh J, Mort J, Stanners CP. Self recognition in the Ig superfamily. Identification of precise subdomains in carcinoembryonic antigen required for intercellular adhesion. The Journal of biological chemistry. 2000; 275(35): 26935-43
124. Ordonez C, Screaton RA, Ilantzis C, Stanners CP. Human carcinoembryonic antigen functions as a general inhibitor of anoikis. Cancer research. 2000; 60(13): 3419-24
125. Gameiro SR, Jammeh ML, Hodge JW. Cancer vaccines targeting carcinoembryonic antigen: state-of-the-art and future promise. Expert review of vaccines. 2013; 12(6): 617-29
126. Hammarstrom S. The carcinoembryonic antigen (CEA) family: structures, suggested functions and expression in normal and malignant tissues. Semin Cancer Biol. 1999; 9(2): 67-81
127. Garcia M, Seigner C, Bastid C, Choux R, Payan MJ, Reggio H. Carcinoembryonic antigen has a different molecular weight in normal colon and in cancer cells due to N-glycosylation differences. Cancer research. 1991; 51(20): 5679-86
128. Turriziani M, Fantini M, Benvenuto M, Izzi V, Masuelli L, Sacchetti P, et al. Carcinoembryonic antigen (CEA)-based cancer vaccines: recent patents and antitumor effects from experimental models to clinical trials. Recent patents on anti-cancer drug discovery. 2012; 7(3): 265-96
129. Kaufman H, Schlom J, Kantor J. A recombinant vaccinia virus expressing human carcinoembryonic antigen (CEA). International journal of cancer Journal international du cancer. 1991; 48(6): 900-7
130. Kantor J, Irvine K, Abrams S, Snoy P, Olsen R, Greiner J, et al. Immunogenicity and safety of a recombinant vaccinia virus vaccine expressing the carcinoembryonic antigen gene in a nonhuman primate. Cancer research. 1992; 52(24): 6917-25
131. Greiner JW, Zeytin H, Anver MR, Schlom J. Vaccine-based therapy directed against carcinoembryonic antigen demonstrates antitumor activity on spontaneous intestinal tumors in the absence of autoimmunity. Cancer research. 2002; 62(23): 6944-51
132. Matsuda K, Tsunoda T, Tanaka H, Umano Y, Tanimura H, Nukaya I, et al. Enhancement of cytotoxic T-lymphocyte responses in patients with gastrointestinal malignancies following vaccination with CEA peptide-pulsed dendritic cells. Cancer immunology, immunotherapy : CII. 2004; 53(7): 609-16
133. Morse MA, Nair SK, Mosca PJ, Hobeika AC, Clay TM, Deng Y, et al. Immunotherapy with autologous, human dendritic cells transfected with carcinoembryonic antigen mRNA. Cancer Invest. 2003; 21(3): 341-9
134. Madan RA, Arlen PM, Gulley JL. PANVAC-VF: poxviral-based vaccine therapy targeting CEA and MUC1 in carcinoma. Expert opinion on biological therapy. 2007; 7(4): 543-54
135. Gulley JL, Arlen PM, Tsang KY, Yokokawa J, Palena C, Poole DJ, et al. Pilot study of vaccination with recombinant CEA-MUC-1-TRICOM poxviral-based vaccines in patients with

metastatic carcinoma. Clinical cancer research : an official journal of the American Association for Cancer Research. 2008; 14(10): 3060-9
136. Mohebtash M, Tsang KY, Madan RA, Huen NY, Poole DJ, Jochems C, et al. A pilot study of MUC-1/CEA/TRICOM poxviral-based vaccine in patients with metastatic breast and ovarian cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2011; 17(22): 7164-73
137. Samanci A, Yi Q, Fagerberg J, Strigard K, Smith G, Ruden U, et al. Pharmacological administration of granulocyte/macrophage-colony-stimulating factor is of significant importance for the induction of a strong humoral and cellular response in patients immunized with recombinant carcinoembryonic antigen. Cancer immunology, immunotherapy : CII. 1998; 47(3): 131-42
138. Ullenhag GJ, Frodin JE, Jeddi-Tehrani M, Strigard K, Eriksson E, Samanci A, et al. Durable carcinoembryonic antigen (CEA)-specific humoral and cellular immune responses in colorectal carcinoma patients vaccinated with recombinant CEA and granulocyte/macrophage colony-stimulating factor. Clinical cancer research : an official journal of the American Association for Cancer Research. 2004; 10(10): 3273-81
139. Ardiani A, Higgins JP, Hodge JW. Vaccines based on whole recombinant Saccharomyces cerevisiae cells. FEMS Yeast Res. 2010; 10(8): 1060-9
140. Bernstein MB, Chakraborty M, Wansley EK, Guo Z, Franzusoff A, Mostbock S, et al. Recombinant Saccharomyces cerevisiae (yeast-CEA) as a potent activator of murine dendritic cells. Vaccine. 2008; 26(4): 509-21
141. Wansley EK, Chakraborty M, Hance KW, Bernstein MB, Boehm AL, Guo Z, et al. Vaccination with a recombinant Saccharomyces cerevisiae expressing a tumor antigen breaks immune tolerance and elicits therapeutic antitumor responses. Clinical cancer research : an official journal of the American Association for Cancer Research. 2008; 14(13): 4316-25
142. Bilusic M, Heery CR, Arlen PM, Rauckhorst M, Apelian D, Tsang KY, et al. Phase I trial of a recombinant yeast-CEA vaccine (GI-6207) in adults with metastatic CEA-expressing carcinoma. Cancer immunology, immunotherapy : CII. 2013; Abstract 458 ASCO 2012:
143. Blackburn EH. Telomerase and Cancer: Kirk A. Landon--AACR prize for basic cancer research lecture. Molecular cancer research : MCR. 2005; 3(9): 477-82
144. Blackburn EH, Greider CW, Szostak JW. Telomeres and telomerase: the path from maize, Tetrahymena and yeast to human cancer and aging. Nature medicine. 2006; 12(10): 1133-8
145. Tian X, Chen B, Liu X. Telomere and telomerase as targets for cancer therapy. Applied biochemistry and biotechnology. 2010; 160(5): 1460-72
146. Rankin AM, Faller DV, Spanjaard RA. Telomerase inhibitors and 'T-oligo' as cancer therapeutics: contrasting molecular mechanisms of cytotoxicity. Anti-cancer drugs. 2008; 19(4): 329-38
147. Bisoffi M, Heaphy CM, Griffith JK. Telomeres: prognostic markers for solid tumors. International journal of cancer Journal international du cancer. 2006; 119(10): 2255-60
148. Artandi SE, DePinho RA. Telomeres and telomerase in cancer. Carcinogenesis. 2010; 31(1): 9-18
149. Ruden M, Puri N. Novel anticancer therapeutics targeting telomerase. Cancer treatment reviews. 2013; 39(5): 444-56
150. Kelland LR. Overcoming the immortality of tumour cells by telomere and telomerase based cancer therapeutics--current status and future prospects. European journal of cancer. 2005; 41(7): 971-9
151. Phatak P, Burger AM. Telomerase and its potential for therapeutic intervention. British journal of pharmacology. 2007; 152(7): 1003-11
152. Corey DR. Telomerase: an unusual target for cytotoxic agents. Chemical research in toxicology. 2000; 13(10): 957-60
153. Kokhaei P, Palma M, Hansson L, Osterborg A, Mellstedt H, Choudhury A. Telomerase (hTERT 611-626) serves as a tumor antigen in B-cell chronic lymphocytic leukemia and generates spontaneously antileukemic, cytotoxic T cells. Experimental hematology. 2007; 35(2): 297-304
154. Harley CB. Telomerase and cancer therapeutics. Nature reviews Cancer. 2008; 8(3): 167-79
155. Shay JW, Keith WN. Targeting telomerase for cancer therapeutics. British journal of cancer. 2008; 98(4): 677-83
156. Beatty GL, Vonderheide RH. Telomerase as a universal tumor antigen for cancer vaccines. Expert review of vaccines. 2008; 7(7): 881-7

157. Vonderheide RH. Prospects and challenges of building a cancer vaccine targeting telomerase. Biochimie. 2008; 90(1): 173-80
158. Kyte JA. Cancer vaccination with telomerase peptide GV1001. Expert opinion on investigational drugs. 2009; 18(5): 687-94
159. Middleton G, Ghaneh P, Costello E, Greenhalf W, Neoptolemos JP. New treatment options for advanced pancreatic cancer. Expert review of gastroenterology & hepatology. 2008; 2(5): 673-96
160. Nava-Parada P, Emens LA. GV-1001, an injectable telomerase peptide vaccine for the treatment of solid cancers. Current opinion in molecular therapeutics. 2007; 9(5): 490-7
161. Brunsvig PF, Aamdal S, Gjertsen MK, Kvalheim G, Markowski-Grimsrud CJ, Sve I, et al. Telomerase peptide vaccination: a phase I/II study in patients with non-small cell lung cancer. Cancer immunology, immunotherapy : CII. 2006; 55(12): 1553-64
162. Shaw VE, Naisbitt DJ, Costello E, Greenhalf W, Park BK, Neoptolemos JP, et al. Current status of GV1001 and other telomerase vaccination strategies in the treatment of cancer. Expert review of vaccines. 2010; 9(9): 1007-16
163. Seliger B. Molecular mechanisms of MHC class I abnormalities and APM components in human tumors. Cancer immunology, immunotherapy : CII. 2008; 57(11): 1719-26
164. Wong SB, Bos R, Sherman LA. Tumor-specific CD4+ T cells render the tumor environment permissive for infiltration by low-avidity CD8+ T cells. Journal of immunology. 2008; 180(5): 3122-31
165. Bernhardt SL, Gjertsen MK, Trachsel S, Moller M, Eriksen JA, Meo M, et al. Telomerase peptide vaccination of patients with non-resectable pancreatic cancer: A dose escalating phase I/II study. British journal of cancer. 2006; 95(11): 1474-82
166. Whiteside TL. Immune monitoring of clinical trials with biotherapies. Advances in clinical chemistry. 2008; 45: 75-97
167. Markovic SN, Nevala WK, Uhl CB, Celis E, McKean DJ. A reproducible method for the enumeration of functional (cytokine producing) versus non-functional peptide-specific cytotoxic T lymphocytes in human peripheral blood. Clinical and experimental immunology. 2006; 145(3): 438-47
168. Palma M, Mellstedt H, Choudhury A. Cancer vaccines. In: Mellstedt H, Schrijvers D, Bafaloukos D, Greil R, (Eds.), Handbook of principles of translational research. Oxon, UK: Informa Healthcare, 2007: 157-65
169. Small EJ, Schellhammer PF, Higano CS, Redfern CH, Nemunaitis JJ, Valone FH, et al. Placebo-controlled phase III trial of immunologic therapy with sipuleucel-T (APC8015) in patients with metastatic, asymptomatic hormone refractory prostate cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2006; 24(19): 3089-94
170. Michaelis LC, Ratain MJ. Measuring response in a post-RECIST world: from black and white to shades of grey. Nature reviews Cancer. 2006; 6(5): 409-14
171. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. International journal of cancer Journal international du cancer. 2010; 127(12): 2893-917
172. Socialstyrelsen, Cancerfonden. Cancer i siffror 2013. Cancerfonden och Socialstyrelsen i samarbete, 2013
173. Maringe C, Walters S, Rachet B, Butler J, Fields T, Finan P, et al. Stage at diagnosis and colorectal cancer survival in six high-income countries: a population-based study of patients diagnosed during 2000-2007. Acta Oncol. 2013; 52(5): 919-32
174. Price TJ, Segelov E, Burge M, Haller DG, Ackland SP, Tebbutt NC, et al. Current opinion on optimal treatment for colorectal cancer. Expert Rev Anticancer Ther. 2013; 13(5): 597-611
175. Wille-Jorgensen P, Bulow S. The multidisciplinary team conference in rectal cancer--a step forward. Colorectal disease : the official journal of the Association of Coloproctology of Great Britain and Ireland. 2009; 11(3): 231-2
176. Edge S, Compton C, Fritz A, Greene T, Trotti A. AJCC Cancer Staging Manual 7th ed.: Springer, 2010
177. 19] Nf-urccA. http://www.cancercentrum.se/Global/Diagnoser/kolorektal/rapporter/rekti2011_120629.pdf.
178. 19] Nf-urccA. http://www.cancercentrum.se/Global/Diagnoser/kolorektal/rapporter/colon2011.pdf.

179. Heald RJ, Husband EM, Ryall RD. The mesorectum in rectal cancer surgery--the clue to pelvic recurrence? Br J Surg. 1982; 69(10): 613-6
180. Biagi JJ, Raphael MJ, Mackillop WJ, Kong W, King WD, Booth CM. Association between time to initiation of adjuvant chemotherapy and survival in colorectal cancer: a systematic review and meta-analysis. JAMA : the journal of the American Medical Association. 2011; 305(22): 2335-42
181. Czaykowski PM, Gill S, Kennecke HF, Gordon VL, Turner D. Adjuvant chemotherapy for stage III colon cancer: does timing matter? Diseases of the colon and rectum. 2011; 54(9): 1082-9
182. Des Guetz G, Nicolas P, Perret GY, Morere JF, Uzzan B. Does delaying adjuvant chemotherapy after curative surgery for colorectal cancer impair survival? A meta-analysis. European journal of cancer. 2010; 46(6): 1049-55
183. Quasar Collaborative G, Gray R, Barnwell J, McConkey C, Hills RK, Williams NS, et al. Adjuvant chemotherapy versus observation in patients with colorectal cancer: a randomised study. Lancet. 2007; 370(9604): 2020-9
184. Sargent DJ, Marsoni S, Monges G, Thibodeau SN, Labianca R, Hamilton SR, et al. Defective mismatch repair as a predictive marker for lack of efficacy of fluorouracil-based adjuvant therapy in colon cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2010; 28(20): 3219-26
185. Yothers G, O'Connell MJ, Allegra CJ, Kuebler JP, Colangelo LH, Petrelli NJ, et al. Oxaliplatin as adjuvant therapy for colon cancer: updated results of NSABP C-07 trial, including survival and subset analyses. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2011; 29(28): 3768-74
186. Andre T, Boni C, Mounedji-Boudiaf L, Navarro M, Tabernero J, Hickish T, et al. Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer. The New England journal of medicine. 2004; 350(23): 2343-51
187. Haller DG, Tabernero J, Maroun J, de Braud F, Price T, Van Cutsem E, et al. Capecitabine plus oxaliplatin compared with fluorouracil and folinic acid as adjuvant therapy for stage III colon cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2011; 29(11): 1465-71
188. Twelves C, Scheithauer W, McKendrick J, Seitz JF, Van Hazel G, Wong A, et al. Capecitabine versus 5-fluorouracil/folinic acid as adjuvant therapy for stage III colon cancer: final results from the X-ACT trial with analysis by age and preliminary evidence of a pharmacodynamic marker of efficacy. Ann Oncol. 2012; 23(5): 1190-7
189. Saltz LB, Niedzwiecki D, Hollis D, Goldberg RM, Hantel A, Thomas JP, et al. Irinotecan fluorouracil plus leucovorin is not superior to fluorouracil plus leucovorin alone as adjuvant treatment for stage III colon cancer: results of CALGB 89803. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2007; 25(23): 3456-61
190. Van Cutsem E, Labianca R, Bodoky G, Barone C, Aranda E, Nordlinger B, et al. Randomized phase III trial comparing biweekly infusional fluorouracil/leucovorin alone or with irinotecan in the adjuvant treatment of stage III colon cancer: PETACC-3. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2009; 27(19): 3117-25
191. Allegra CJ, Yothers G, O'Connell MJ, Sharif S, Petrelli NJ, Lopa SH, et al. Bevacizumab in stage II-III colon cancer: 5-year update of the National Surgical Adjuvant Breast and Bowel Project C-08 trial. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2013; 31(3): 359-64
192. Alberts SR, Sargent DJ, Nair S, Mahoney MR, Mooney M, Thibodeau SN, et al. Effect of oxaliplatin, fluorouracil, and leucovorin with or without cetuximab on survival among patients with resected stage III colon cancer: a randomized trial. JAMA : the journal of the American Medical Association. 2012; 307(13): 1383-93
193. Improved survival with preoperative radiotherapy in resectable rectal cancer. Swedish Rectal Cancer Trial. The New England journal of medicine. 1997; 336(14): 980-7
194. Kapiteijn E, Marijnen CA, Nagtegaal ID, Putter H, Steup WH, Wiggers T, et al. Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer. The New England journal of medicine. 2001; 345(9): 638-46
195. Randomised trial of surgery alone versus surgery followed by radiotherapy for mobile cancer of the rectum. Medical Research Council Rectal Cancer Working Party. Lancet. 1996; 348(9042): 1610-4

196. Bentzen SM, Balslev I, Pedersen M, Teglbjaerg PS, Hanberg-Soerensen F, Bone J, et al. A regression analysis of prognostic factors after resection of Dukes' B and C carcinoma of the rectum and rectosigmoid. Does post-operative radiotherapy change the prognosis? British journal of cancer. 1988; 58(2): 195-201
197. Blomqvist L, Glimelius B. The 'good', the 'bad', and the 'ugly' rectal cancers. Acta Oncol. 2008; 47(1): 5-8
198. Bujko K, Glynne-Jones R, Bujko M. Does adjuvant fluoropyrimidine-based chemotherapy provide a benefit for patients with resected rectal cancer who have already received neoadjuvant radiochemotherapy? A systematic review of randomised trials. Ann Oncol. 2010; 21(9): 1743-50
199. Collette L, Bosset JF, den Dulk M, Nguyen F, Mineur L, Maingon P, et al. Patients with curative resection of cT3-4 rectal cancer after preoperative radiotherapy or radiochemotherapy: does anybody benefit from adjuvant fluorouracil-based chemotherapy? A trial of the European Organisation for Research and Treatment of Cancer Radiation Oncology Group. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2007; 25(28): 4379-86
200. Sjovall A, Jarv V, Blomqvist L, Singnomklao T, Cedermark B, Glimelius B, et al. The potential for improved outcome in patients with hepatic metastases from colon cancer: a population-based study. Eur J Surg Oncol. 2004; 30(8): 834-41
201. Negri F, Musolino A, Cunningham D, Pastorino U, Ladas G, Norman AR. Retrospective study of resection of pulmonary metastases in patients with advanced colorectal cancer: the development of a preoperative chemotherapy strategy. Clin Colorectal Cancer. 2004; 4(2): 101-6
202. Lise M, Da Pian PP, Nitti D, Pilati PL. Colorectal metastases to the liver: present results and future strategies. J Surg Oncol Suppl. 1991; 2: 69-73
203. Bismuth H, Adam R, Navarro F, Castaing D, Engerran L, Abascal A. Re-resection for colorectal liver metastasis. Surg Oncol Clin N Am. 1996; 5(2): 353-64
204. Saltz LB, Clarke S, Diaz-Rubio E, Scheithauer W, Figer A, Wong R, et al. Bevacizumab in combination with oxaliplatin-based chemotherapy as first-line therapy in metastatic colorectal cancer: a randomized phase III study. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2008; 26(12): 2013-9
205. Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. The New England journal of medicine. 2004; 350(23): 2335-42
206. Peeters M, Price TJ, Cervantes A, Sobrero AF, Ducreux M, Hotko Y, et al. Randomized phase III study of panitumumab with fluorouracil, leucovorin, and irinotecan (FOLFIRI) compared with FOLFIRI alone as second-line treatment in patients with metastatic colorectal cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2010; 28(31): 4706-13
207. Bokemeyer C, Van Cutsem E, Rougier P, Ciardiello F, Heeger S, Schlichting M, et al. Addition of cetuximab to chemotherapy as first-line treatment for KRAS wild-type metastatic colorectal cancer: pooled analysis of the CRYSTAL and OPUS randomised clinical trials. European journal of cancer. 2012; 48(10): 1466-75
208. Falcone A, Ricci S, Brunetti I, Pfanner E, Allegrini G, Barbara C, et al. Phase III trial of infusional fluorouracil, leucovorin, oxaliplatin, and irinotecan (FOLFOXIRI) compared with infusional fluorouracil, leucovorin, and irinotecan (FOLFIRI) as first-line treatment for metastatic colorectal cancer: the Gruppo Oncologico Nord Ovest. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2007; 25(13): 1670-6
209. Souglakos J, Androulakis N, Syrigos K, Polyzos A, Ziras N, Athanasiadis A, et al. FOLFOXIRI (folinic acid, 5-fluorouracil, oxaliplatin and irinotecan) vs FOLFIRI (folinic acid, 5-fluorouracil and irinotecan) as first-line treatment in metastatic colorectal cancer (MCC): a multicentre randomised phase III trial from the Hellenic Oncology Research Group (HORG). British journal of cancer. 2006; 94(6): 798-805
210. Tournigand C, Andre T, Achille E, Lledo G, Flesh M, Mery-Mignard D, et al. FOLFIRI followed by FOLFOX6 or the reverse sequence in advanced colorectal cancer: a randomized GERCOR study. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2004; 22(2): 229-37
211. Neo EL, Beeke C, Price T, Maddern G, Karapetis C, Luke C, et al. South Australian clinical registry for metastatic colorectal cancer. ANZ J Surg. 2011; 81(5): 352-7

212. Wolfgang CL, Herman JM, Laheru DA, Klein AP, Erdek MA, Fishman EK, et al. Recent progress in pancreatic cancer. CA Cancer J Clin. 2013; 63(5): 318-48
213. Bosetti C, Lucenteforte E, Silverman DT, Petersen G, Bracci PM, Ji BT, et al. Cigarette smoking and pancreatic cancer: an analysis from the International Pancreatic Cancer Case-Control Consortium (Panc4). Ann Oncol. 2012; 23(7): 1880-8
214. Maisonneuve P, Lowenfels AB. Epidemiology of pancreatic cancer: an update. Dig Dis. 2010; 28(4-5): 645-56
215. Huxley R, Ansary-Moghaddam A, Berrington de Gonzalez A, Barzi F, Woodward M. Type-II diabetes and pancreatic cancer: a meta-analysis of 36 studies. British journal of cancer. 2005; 92(11): 2076-83
216. Arslan AA, Helzlsouer KJ, Kooperberg C, Shu XO, Steplowski E, Bueno-de-Mesquita HB, et al. Anthropometric measures, body mass index, and pancreatic cancer: a pooled analysis from the Pancreatic Cancer Cohort Consortium (PanScan). Arch Intern Med. 2010; 170(9): 791-802
217. Tamm EP, Balachandran A, Bhosale PR, Katz MH, Fleming JB, Lee JH, et al. Imaging of pancreatic adenocarcinoma: update on staging/resectability. Radiol Clin North Am. 2012; 50(3): 407-28
218. Vauthey JN, Dixon E. AHPBA/SSO/SSAT Consensus Conference on Resectable and Borderline Resectable Pancreatic Cancer: rationale and overview of the conference. Annals of surgical oncology. 2009; 16(7): 1725-6
219. Gillen S, Schuster T, Meyer Zum Buschenfelde C, Friess H, Kleeff J. Preoperative/neoadjuvant therapy in pancreatic cancer: a systematic review and meta-analysis of response and resection percentages. PLoS Med. 2010; 7(4): e1000267
220. Oettle H, Post S, Neuhaus P, Gellert K, Langrehr J, Ridwelski K, et al. Adjuvant chemotherapy with gemcitabine vs observation in patients undergoing curative-intent resection of pancreatic cancer: a randomized controlled trial. JAMA : the journal of the American Medical Association. 2007; 297(3): 267-77
221. Neoptolemos JP, Stocken DD, Bassi C, Ghaneh P, Cunningham D, Goldstein D, et al. Adjuvant chemotherapy with fluorouracil plus folinic acid vs gemcitabine following pancreatic cancer resection: a randomized controlled trial. JAMA : the journal of the American Medical Association. 2010; 304(10): 1073-81
222. Lutz E, Yeo CJ, Lillemoe KD, Biedrzycki B, Kobrin B, Herman J, et al. A lethally irradiated allogeneic granulocyte-macrophage colony stimulating factor-secreting tumor vaccine for pancreatic adenocarcinoma. A Phase II trial of safety, efficacy, and immune activation. Ann Surg. 2011; 253(2): 328-35
223. Burris HA, 3rd, Moore MJ, Andersen J, Green MR, Rothenberg ML, Modiano MR, et al. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 1997; 15(6): 2403-13
224. Cunningham D, Chau I, Stocken DD, Valle JW, Smith D, Steward W, et al. Phase III randomized comparison of gemcitabine versus gemcitabine plus capecitabine in patients with advanced pancreatic cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2009; 27(33): 5513-8
225. De Jesus-Acosta A, Oliver GR, Blackford A, Kinsman K, Flores EI, Wilfong LS, et al. A multicenter analysis of GTX chemotherapy in patients with locally advanced and metastatic pancreatic adenocarcinoma. Cancer Chemother Pharmacol. 2012; 69(2): 415-24
226. Moore MJ, Goldstein D, Hamm J, Figer A, Hecht JR, Gallinger S, et al. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2007; 25(15): 1960-6
227. Philip PA, Benedetti J, Corless CL, Wong R, O'Reilly EM, Flynn PJ, et al. Phase III study comparing gemcitabine plus cetuximab versus gemcitabine in patients with advanced pancreatic adenocarcinoma: Southwest Oncology Group-directed intergroup trial S0205. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2010; 28(22): 3605-10
228. Kindler HL, Niedzwiecki D, Hollis D, Sutherland S, Schrag D, Hurwitz H, et al. Gemcitabine plus bevacizumab compared with gemcitabine plus placebo in patients with advanced pancreatic cancer: phase III trial of the Cancer and Leukemia Group B (CALGB 80303). Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2010; 28(22): 3617-22

229. Conroy T, Desseigne F, Ychou M, Bouche O, Guimbaud R, Becouarn Y, et al. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. The New England journal of medicine. 2011; 364(19): 1817-25
230. Mosolits S, Markovic K, Frodin JE, Virving L, Magnusson CG, Steinitz M, et al. Vaccination with Ep-CAM protein or anti-idiotypic antibody induces Th1-biased response against MHC class I- and II-restricted Ep-CAM epitopes in colorectal carcinoma patients. Clinical cancer research : an official journal of the American Association for Cancer Research. 2004; 10(16): 5391-402
231. Rossmann E, Österborg A, Löfvenberg E, Choudhury A, Forssmann U, von Heydebreck A, et al. Mucin 1-specific active cancer immunotherapy with L-BLP25 in patients with multiple myeloma: an exploratory study. Karolinska Institutet and Karolinska University Hospital, Stockholm, Sweden; 2University of Queensland, Brisbane, Australia; 3MerckSerono S.A., Geneva, Switzerland, 4Merck KGaA, Darmstadt, Germany, 2013
232. Rossman E. Randomized phase II study of BLP25 liposome vaccine (L-BLP25) in patients with multiple myeloma. Poster no 2927 Presented at ASH 2011:
233. Magnusson CG, Nelson DF, Magnusson MA. Disproportional distribution of isotype and non-isotype-specific IgG subclass anti-IgE autoantibodies in human cord serum. Int Arch Allergy Immunol. 1996; 110(1): 31-40
234. Bruggemann M, Williams GT, Bindon CI, Clark MR, Walker MR, Jefferis R, et al. Comparison of the effector functions of human immunoglobulins using a matched set of chimeric antibodies. The Journal of experimental medicine. 1987; 166(5): 1351-61
235. Teeling JL, French RR, Cragg MS, van den Brakel J, Pluyter M, Huang H, et al. Characterization of new human CD20 monoclonal antibodies with potent cytolytic activity against non-Hodgkin lymphomas. Blood. 2004; 104(6): 1793-800
236. Liljefors M, Nilsson B, Mellstedt H, Frodin JE. Influence of varying doses of granulocyte-macrophage colony-stimulating factor on pharmacokinetics and antibody-dependent cellular cytotoxicity. Cancer immunology, immunotherapy : CII. 2008; 57(3): 379-88
237. Frodin JE, Faxas ME, Hagstrom B, Lefvert AK, Masucci G, Nilsson B, et al. Induction of anti-idiotypic (ab2) and anti-anti-idiotypic (ab3) antibodies in patients treated with the mouse monoclonal antibody 17-1A (ab1). Relation to the clinical outcome--an important antitumoral effector function? Hybridoma. 1991; 10(4): 421-31
238. Enterline PE. Pitfalls in epidemiological research. An examination of the asbestos literature. J Occup Med. 1976; 18(3): 150-6
239. Anderson JR, Cain KC, Gelber RD. Analysis of survival by tumor response. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 1983; 1(11): 710-9
240. Martin-Yuste V, Alvarez-Contreras L, Brugaletta S, Ferreira-Gonzalez I, Cola C, Garcia-Picart J, et al. Emergent versus elective percutaneous stent implantation in the unprotected left main: long-term outcomes from a single-center registry. J Invasive Cardiol. 2011; 23(10): 392-7
241. Baseler MW, Maxim PE, Veltri RW. Circulating IgA immune complexes in head and neck cancer, nasopharyngeal carcinoma, lung cancer, and colon cancer. Cancer. 1987; 59(10): 1727-31
242. Brandlein S, Lorenz J, Ruoff N, Hensel F, Beyer I, Muller J, et al. Human monoclonal IgM antibodies with apoptotic activity isolated from cancer patients. Human antibodies. 2002; 11(4): 107-19
243. Brandlein S, Pohle T, Ruoff N, Wozniak E, Muller-Hermelink HK, Vollmers HP. Natural IgM antibodies and immunosurveillance mechanisms against epithelial cancer cells in humans. Cancer research. 2003; 63(22): 7995-8005
244. Gould HJ, Mackay GA, Karagiannis SN, O'Toole CM, Marsh PJ, Daniel BE, et al. Comparison of IgE and IgG antibody-dependent cytotoxicity in vitro and in a SCID mouse xenograft model of ovarian carcinoma. European journal of immunology. 1999; 29(11): 3527-37
245. Julien S, Picco G, Sewell R, Vercoutter-Edouart AS, Tarp M, Miles D, et al. Sialyl-Tn vaccine induces antibody-mediated tumour protection in a relevant murine model. British journal of cancer. 2009; 100(11): 1746-54
246. Karagiannis SN, Bracher MG, Hunt J, McCloskey N, Beavil RL, Beavil AJ, et al. IgE-antibody-dependent immunotherapy of solid tumors: cytotoxic and phagocytic mechanisms of eradication of ovarian cancer cells. Journal of immunology. 2007; 179(5): 2832-43
247. Albanopoulos K, Armakolas A, Konstadoulakis MM, Leandros E, Tsiompanou E, Katsaragakis S, et al. Prognostic significance of circulating antibodies against carcinoembryonic antigen (anti-CEA) in patients with colon cancer. Am J Gastroenterol. 2000; 95(4): 1056-61

248. Ullenhag GJ, Frodin JE, Strigard K, Mellstedt H, Magnusson CG. Induction of IgG subclass responses in colorectal carcinoma patients vaccinated with recombinant carcinoembryonic antigen. Cancer research. 2002; 62(5): 1364-9
249. Mota G, Manciulea M, Cosma E, Popescu I, Hirt M, Jensen-Jarolim E, et al. Human NK cells express Fc receptors for IgA which mediate signal transduction and target cell killing. European journal of immunology. 2003; 33(8): 2197-205
250. Conry RM, Curiel DT, Strong TV, Moore SE, Allen KO, Barlow DL, et al. Safety and immunogenicity of a DNA vaccine encoding carcinoembryonic antigen and hepatitis B surface antigen in colorectal carcinoma patients. Clinical cancer research : an official journal of the American Association for Cancer Research. 2002; 8(9): 2782-7
251. Marshall J. Carcinoembryonic antigen-based vaccines. Semin Oncol. 2003; 30(3 Suppl 8): 30-6
252. Marshall JL, Gulley JL, Arlen PM, Beetham PK, Tsang KY, Slack R, et al. Phase I study of sequential vaccinations with fowlpox-CEA(6D)-TRICOM alone and sequentially with vaccinia-CEA(6D)-TRICOM, with and without granulocyte-macrophage colony-stimulating factor, in patients with carcinoembryonic antigen-expressing carcinomas. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2005; 23(4): 720-31
253. Ledwith BJ, Manam S, Troilo PJ, Barnum AB, Pauley CJ, Griffiths TG, 2nd, et al. Plasmid DNA vaccines: investigation of integration into host cellular DNA following intramuscular injection in mice. Intervirology. 2000; 43(4-6): 258-72
254. Sheets RL, Stein J, Manetz TS, Duffy C, Nason M, Andrews C, et al. Biodistribution of DNA plasmid vaccines against HIV-1, Ebola, Severe Acute Respiratory Syndrome, or West Nile virus is similar, without integration, despite differing plasmid backbones or gene inserts. Toxicol Sci. 2006; 91(2): 610-9
255. Brave A, Hallengard D, Gudmundsdotter L, Stout R, Walters R, Wahren B, et al. Late administration of plasmid DNA by intradermal electroporation efficiently boosts DNA-primed T and B cell responses to carcinoembryonic antigen. Vaccine. 2009; 27(28): 3692-6
256. Hallermalm K. Pre-Clinical evaluation of a CEA DNA prime/protein boost vaccination strategy against colorectal cancer. Scandinavian journal of Immunology. 2007; 66: 43-51
257. Johansson. Intracellular targeting of CEA results in Th1-type antibody responses following intradermal genetic vaccination by a needle-free jet injection device. The scientific word journal. 2007; 7: 987-99
258. Roos AK, Eriksson F, Timmons JA, Gerhardt J, Nyman U, Gudmundsdotter L, et al. Skin electroporation: effects on transgene expression, DNA persistence and local tissue environment. PloS one. 2009; 4(9): e7226
259. Luxembourg A, Evans CF, Hannaman D. Electroporation-based DNA immunisation: translation to the clinic. Expert opinion on biological therapy. 2007; 7(11): 1647-64
260. Roos AK. Skin electroporation:effects on transgene expression, DNA peristance and local tissue environment. 2009:
261. Gothelf A, Eriksen J, Hojman P, Gehl J. Duration and level of transgene expression after gene electrotransfer to skin in mice. Gene Ther. 2010; 17(7): 839-45
262. Staff C, Mozaffari F, Haller BK, Wahren B, Liljefors M. A Phase I safety study of plasmid DNA immunization targeting carcinoembryonic antigen in colorectal cancer patients. Vaccine. 2011; 29(39): 6817-22
263. Sin JI, Hong SH, Park YJ, Park JB, Choi YS, Kim MS. Antitumor therapeutic effects of e7 subunit and DNA vaccines in an animal cervical cancer model: antitumor efficacy of e7 therapeutic vaccines is dependent on tumor sizes, vaccine doses, and vaccine delivery routes. DNA Cell Biol. 2006; 25(5): 277-86
264. Johansson S, Engstrom G, Winberg G, Hinkula J, Wahren B. Responses of mice immunized with a DNA vaccine encoding carcinoembryonic antigen (CEA). Vaccine. 2006; 24(21): 4572-5
265. Lund LH, Andersson K, Zuber B, Karlsson A, Engstrom G, Hinkula J, et al. Signal sequence deletion and fusion to tetanus toxoid epitope augment antitumor immune responses to a human carcinoembryonic antigen (CEA) plasmid DNA vaccine in a murine test system. Cancer Gene Ther. 2003; 10(5): 365-76
266. Grosenbaugh DA, Leard AT, Bergman PJ, Klein MK, Meleo K, Susaneck S, et al. Safety and efficacy of a xenogeneic DNA vaccine encoding for human tyrosinase as adjunctive treatment for oral malignant melanoma in dogs following surgical excision of the primary tumor. Am J Vet Res. 2011; 72(12): 1631-8

267. Herrmann R, Bodoky G, Ruhstaller T, Glimelius B, Bajetta E, Schuller J, et al. Gemcitabine plus capecitabine compared with gemcitabine alone in advanced pancreatic cancer: a randomized, multicenter, phase III trial of the Swiss Group for Clinical Cancer Research and the Central European Cooperative Oncology Group. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2007; 25(16): 2212-7
268. Hale DF, Clifton GT, Sears AK, Vreeland TJ, Shumway N, Peoples GE, et al. Cancer vaccines: should we be targeting patients with less aggressive disease? Expert Rev Vaccines. 2012; 11(6): 721-31
269. Clay TM, Hobeika AC, Mosca PJ, Lyerly HK, Morse MA. Assays for monitoring cellular immune responses to active immunotherapy of cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2001; 7(5): 1127-35
270. Abdalla AO, Hansson L, Eriksson I, Nasman-Glaser B, Rossmann ED, Rabbani H, et al. Idiotype protein vaccination in combination with adjuvant cytokines in patients with multiple myeloma--evaluation of T-cell responses by different read-out systems. Haematologica. 2007; 92(1): 110-4
271. Zitvogel L, Apetoh L, Ghiringhelli F, Kroemer G. Immunological aspects of cancer chemotherapy. Nature reviews Immunology. 2008; 8(1): 59-73
272. Gulley JL, Madan RA, Arlen PM. Enhancing efficacy of therapeutic vaccinations by combination with other modalities. Vaccine. 2007; 25 Suppl 2: B89-96
273. Ko HJ, Kim YJ, Kim YS, Chang WS, Ko SY, Chang SY, et al. A combination of chemoimmunotherapies can efficiently break self-tolerance and induce antitumor immunity in a tolerogenic murine tumor model. Cancer research. 2007; 67(15): 7477-86
274. Vincent J, Mignot G, Chalmin F, Ladoire S, Bruchard M, Chevriaux A, et al. 5-Fluorouracil selectively kills tumor-associated myeloid-derived suppressor cells resulting in enhanced T cell-dependent antitumor immunity. Cancer research. 2010; 70(8): 3052-61
275. Salem ML, Diaz-Montero CM, Al-Khami AA, El-Naggar SA, Naga O, Montero AJ, et al. Recovery from cyclophosphamide-induced lymphopenia results in expansion of immature dendritic cells which can mediate enhanced prime-boost vaccination antitumor responses in vivo when stimulated with the TLR3 agonist poly(I:C). Journal of immunology. 2009; 182(4): 2030-40
276. Bracci L, Moschella F, Sestili P, La Sorsa V, Valentini M, Canini I, et al. Cyclophosphamide enhances the antitumor efficacy of adoptively transferred immune cells through the induction of cytokine expression, B-cell and T-cell homeostatic proliferation, and specific tumor infiltration. Clinical cancer research : an official journal of the American Association for Cancer Research. 2007; 13(2 Pt 1): 644-53
277. Piccirillo CA, Shevach EM. Cutting edge: control of CD8+ T cell activation by CD4+CD25+ immunoregulatory cells. Journal of immunology. 2001; 167(3): 1137-40
278. Sakaguchi S, Sakaguchi N, Shimizu J, Yamazaki S, Sakihama T, Itoh M, et al. Immunologic tolerance maintained by CD25+ CD4+ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. Immunological reviews. 2001; 182: 18-32
279. Woo EY, Yeh H, Chu CS, Schlienger K, Carroll RG, Riley JL, et al. Cutting edge: Regulatory T cells from lung cancer patients directly inhibit autologous T cell proliferation. Journal of immunology. 2002; 168(9): 4272-6
280. Irvine KR, Chamberlain RS, Shulman EP, Surman DR, Rosenberg SA, Restifo NP. Enhancing efficacy of recombinant anticancer vaccines with prime/boost regimens that use two different vectors. Journal of the National Cancer Institute. 1997; 89(21): 1595-601
281. Chianese-Bullock KA, Irvin WP, Jr., Petroni GR, Murphy C, Smolkin M, Olson WC, et al. A multipeptide vaccine is safe and elicits T-cell responses in participants with advanced stage ovarian cancer. Journal of immunotherapy. 2008; 31(4): 420-30
282. Salgaller ML, Marincola FM, Cormier JN, Rosenberg SA. Immunization against epitopes in the human melanoma antigen gp100 following patient immunization with synthetic peptides. Cancer research. 1996; 56(20): 4749-57
283. Rosenberg SA, Sherry RM, Morton KE, Scharfman WJ, Yang JC, Topalian SL, et al. Tumor progression can occur despite the induction of very high levels of self/tumor antigen-specific CD8+ T cells in patients with melanoma. Journal of immunology. 2005; 175(9): 6169-76
284. Parkhurst MR, Salgaller ML, Southwood S, Robbins PF, Sette A, Rosenberg SA, et al. Improved induction of melanoma-reactive CTL with peptides from the melanoma antigen gp100 modified at HLA-A*0201-binding residues. Journal of immunology. 1996; 157(6): 2539-48

285. Butterfield LH, Comin-Anduix B, Vujanovic L, Lee Y, Dissette VB, Yang JQ, et al. Adenovirus MART-1-engineered autologous dendritic cell vaccine for metastatic melanoma. J Immunother. 2008; 31(3): 294-309
286. Butterfield LH, Ribas A, Dissette VB, Amarnani SN, Vu HT, Oseguera D, et al. Determinant spreading associated with clinical response in dendritic cell-based immunotherapy for malignant melanoma. Clinical cancer research : an official journal of the American Association for Cancer Research. 2003; 9(3): 998-1008
287. Lee KH, Wang E, Nielsen MB, Wunderlich J, Migueles S, Connors M, et al. Increased vaccine-specific T cell frequency after peptide-based vaccination correlates with increased susceptibility to in vitro stimulation but does not lead to tumor regression. Journal of immunology. 1999; 163(11): 6292-300
288. Ribas A, Glaspy JA, Lee Y, Dissette VB, Seja E, Vu HT, et al. Role of dendritic cell phenotype, determinant spreading, and negative costimulatory blockade in dendritic cell-based melanoma immunotherapy. J Immunother. 2004; 27(5): 354-67
289. Tsioulias G, Godwin TA, Goldstein MF, McDougall CJ, Ngoi SS, DeCosse JJ, et al. Loss of colonic HLA antigens in familial adenomatous polyposis. Cancer research. 1992; 52(12): 3449-52
290. Sers C, Kuner R, Falk CS, Lund P, Sueltmann H, Braun M, et al. Down-regulation of HLA Class I and NKG2D ligands through a concerted action of MAPK and DNA methyltransferases in colorectal cancer cells. International journal of cancer Journal international du cancer. 2009; 125(7): 1626-39
291. Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ, Penson DF, et al. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. The New England journal of medicine. 2010; 363(5): 411-22
292. Choudhury A, Mosolits S, Kokhaei P, Hansson L, Palma M, Mellstedt H. Clinical results of vaccine therapy for cancer: learning from history for improving the future. Advances in cancer research. 2006; 95: 147-202
293. Testori A, Richards J, Whitman E, Mann GB, Lutzky J, Camacho L, et al. Phase III comparison of vitespen, an autologous tumor-derived heat shock protein gp96 peptide complex vaccine, with physician's choice of treatment for stage IV melanoma: the C-100-21 Study Group. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2008; 26(6): 955-62
294. Hanna MG, Jr., Hoover HC, Jr., Vermorken JB, Harris JE, Pinedo HM. Adjuvant active specific immunotherapy of stage II and stage III colon cancer with an autologous tumor cell vaccine: first randomized phase III trials show promise. Vaccine. 2001; 19(17-19): 2576-82
295. Dechant M, Valerius T. IgA antibodies for cancer therapy. Critical reviews in oncology/hematology. 2001; 39(1-2): 69-77
296. Hiltbold EM, Alter MD, Ciborowski P, Finn OJ. Presentation of MUC1 tumor antigen by class I MHC and CTL function correlate with the glycosylation state of the protein taken Up by dendritic cells. Cell Immunol. 1999; 194(2): 143-9
297. Hanisch FG, Schwientek T, Von Bergwelt-Baildon MS, Schultze JL, Finn O. O-Linked glycans control glycoprotein processing by antigen-presenting cells: a biochemical approach to the molecular aspects of MUC1 processing by dendritic cells. European journal of immunology. 2003; 33(12): 3242-54
298. Purcell AW, van Driel IR, Gleeson PA. Impact of glycans on T-cell tolerance to glycosylated self-antigens. Immunol Cell Biol. 2008; 86(7): 574-9
299. Butterfield LH, Palucka AK, Britten CM, Dhodapkar MV, Hakansson L, Janetzki S, et al. Recommendations from the iSBTc-SITC/FDA/NCI Workshop on Immunotherapy Biomarkers. Clinical cancer research : an official journal of the American Association for Cancer Research. 2011; 17(10): 3064-76
300. Curran MA, Montalvo W, Yagita H, Allison JP. PD-1 and CTLA-4 combination blockade expands infiltrating T cells and reduces regulatory T and myeloid cells within B16 melanoma tumors. Proceedings of the National Academy of Sciences of the United States of America. 2010; 107(9): 4275-80
301. Duraiswamy J, Kaluza KM, Freeman GJ, Coukos G. Dual blockade of PD-1 and CTLA-4 combined with tumor vaccine effectively restores T-cell rejection function in tumors. Cancer research. 2013; 73(12): 3591-603

302. Hurwitz AA, Yu TF, Leach DR, Allison JP. CTLA-4 blockade synergizes with tumor-derived granulocyte-macrophage colony-stimulating factor for treatment of an experimental mammary carcinoma. Proceedings of the National Academy of Sciences of the United States of America. 1998; 95(17): 10067-71
303. Kroemer G, Galluzzi L, Kepp O, Zitvogel L. Immunogenic cell death in cancer therapy. Annual review of immunology. 2013; 31: 51-72
304. Krysko DV, Garg AD, Kaczmarek A, Krysko O, Agostinis P, Vandenabeele P. Immunogenic cell death and DAMPs in cancer therapy. Nature reviews Cancer. 2012; 12(12): 860-75
305. Vacchelli E, Vitale I, Tartour E, Eggermont A, Sautes-Fridman C, Galon J, et al. Trial Watch: Anticancer radioimmunotherapy. Oncoimmunology. 2013; 2(9): e25595