Thesis for doctoral degree (Ph.D.) 2008

Thesis for doctoral degree (Ph.D.) 2008

NOVEL APPROACHES FOR CANCER IMMUNOTHERAPY

Fredrik Eriksson

Karolinska Institutet

NOVEL APPROACHES FOR CANCER IMMUNOTHERAPY

Fredrik Eriksson

Karolinska Institutet

From the Department of Oncology and Pathology
Karolinska Institutet, Stockholm, Sweden

NOVEL APPROACHES FOR CANCER IMMUNOTHERAPY

Fredrik Eriksson

Karolinska Institutet

Stockholm 2008

Published by Karolinska Institutet.

ISBN 978-91-7409-043-7

Printed by


www.reproprint.se
Gårdsvägen 4, 169 70 Solna

Abstract

Cancer immunotherapy, *i.e.* activation of the patient's own immune system to combat cancer, represents a treatment strategy which is being clinically tested to complement surgery, radiotherapy and chemotherapy – the current cornerstones of our fight against cancer. It has become clear now, that tumors not only escape immune recognition but also actively suppress antitumor immune responses. Cancer immunotherapy therefore has to overcome several obstacles. Since the immune system has evolved to fight infections there are intrinsic mechanisms that limit the ability to react against self antigens. If however, antitumor immune responses are generated, the genetic instability of tumor cells and the immunosuppressive state of the tumor microenvironment create a second barrier that hampers immune mediated tumor eradication. In order to improve cancer immunotherapy, effective manipulation of the immune system to break self-tolerance need to be designed and approaches that counteract immunosuppressive mechanisms need to be developed.

We show that treatment with tumor-specific phage display particles leads to eradication of established mouse melanoma tumors and long-term survival. The effect is initiated by tumor associated macrophages (TAMs), which after bacteriophage encounter reverse their typical immunosuppressive state and create an environment that promotes recruitment of neutrophils and potentiates neutrophil-mediated tumor destruction. The pro-tumorigenic phenotype of prostate cancer-TAMs can be also modified using zoledronic acid (ZA). We show that ZA suppresses the expression of MMP-9 by TAMs and, in combination with IL-12, enhances their tumor-eliminating functions. Furthermore, ZA drives the proliferation and activation of γδ T cells which lyse ZA-pulsed prostate cancer cells. Finally, we developed a more clinically suitable protocol for delivery of DNA vaccines using electroporation. Shortening of electroporation pulse intervals, resulting in a 10-fold reduction of total pulse length, and application of local anesthesia do not negatively affect the vigor of antigen-specific cytotoxic T lymphocytes responses normally observed after DNA electrovaccination.

This study has contributed to the identification and development of novel immunotherapeutic approaches which have a significant potential for use in cancer immunotherapy.

List of publications

I. **Eriksson F**, Culp WD, Massey R, Egevad L, Garland D, Persson MAA, Pisa P. Tumor specific phage particles promote tumor regression in a mouse melanoma model. *Cancer Immunology Immunotherapy*, 2007, May; 56(5): 677-687

II. **Eriksson F**, Tsagozis P, Persson MAA, Harris RA and Pisa P. Tumor-specific bacteriophages induce tumor destruction through activation of tumor associated macrophages. (*Manuscript submitted*)

III. Tsagozis P, **Eriksson F**, Pisa P. Zoledronic acid modulates antitumoral responses of prostate cancer-tumor associated macrophages. *Cancer Immunology Immunotherapy*, 2008*, In press*

IV. Roos A-K *****, **Eriksson F ***, Pisa P, King AD. Optimization of skin electroporation to increase tolerability of DNA vaccine delivery to patients. (*Manuscript submitted)*

* Shared first authorship

Table of contents

Abbreviations

1ME	1-methyltryptophan
ACAD	activated cell autonomous death
AICD	activation-induced cell death
APC	antigen presenting cell
ARG	arginase
BAGE	B antigen
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2-associated X protein
BCG	Bacillus Calmette-Guerin
Bcl-2	B cell lymphoma-2
Bcl-xL	Basal cell lymphoma-extra large
Bim	Bcl-2 interacting mediator of cell death
CCL	chemokine (C-C motif) ligand
CCR	chemokine (C-C motif) receptor
CD	cluster of differentiation
CEA	carcinoembryonic antigen
CM	complete medium
CpG	Cytosine-phosphate-Guanine
CRP	C-reactive protein
CSF-1	colony stimulating factor-1
CTL	cytotoxic T lymphocyte
CTLA-4	CTL associated antigen 4
DC	dendritic cell
ECM	extracellular matrix
EBNA2	Epstein-Barr virus nuclear antigen 2
ER	endoplasmatic reticulum
ERBB2	erythroblastic leukemia viral oncogene homolog 2
FasL	Fas ligand
FGF-2	fibroblast growth factor-2
FLIP	FLICE-like inhibitory protein
Foxp3	forkhead box protein 3
GAGE	G antigen
GM-CSF	granulocyte macrophage-colony stimulating factor
HBsAg	hepatitis B surface antigen
Her-2	human epidermal growth factor receptor 2
HP-NAP	*Helicobacter pylori* neutrophil activating protein
HPV	human papilloma virus
HSP	heat shock protein
hTERT	human telomerase reverse transcriptase
ICAM	intracellular adhesion molecule
IDO	indoleamine 2, 3-dioxygenase
IFN	interferon
Ig	immunoglobulin
Ii	invariant chain
IL	interleukin
LFA-1	lymphocyte function-associated antigen-1
MAGE	melanoma antigen
MART-1	melanoma antigen recognized by T cells
MBL	mannan-binding lectin
MCP-	monocyte chemoattractant protein-
M-CSF	macrophage colony stimulating factor
MDSC	myeloid-derived suppressor cell
MHC	major histocompatibility complex
MIF	macrophage migration inhibitory factor
MMP	matrix metalloproteinase
MUC-1	mucin-1
NF-κB	nuclear factor-kappa B

N-BP	nitrogen-containing bisphosphonate
NK	natural killer
NO	nitric oxide
NOHA	N-hydroxyl-L-Arginine
PAMP	pathogen-associated molecular pattern
PAP	prostate acid phosphatase
PC	prostate cancer
PDGF	platelet-derived growth factor
PD-L	programmed cell death ligand
PRR	pathogen recognition receptor
PSA	prostate specific antigen
PSMA	prostate specific membrane antigen
PUMA	p53-upregulated modulator of apoptosis
-R	receptor
RAG	recombination activating gene
RNS	reactive nitrogen species
ROS	reactive oxygen species
SLPI	secretory leukocyte protease inhibitor
TAA	tumor associated antigen
TAM	tumor associated macrophage
TAP	transporter associated with antigen processing
tapasin	TAP-associated protein
TCR	T cell receptor
TGF	transforming growth factor
Th	helper T cell
TIL	tumor infiltrating lymphocyte
TLR	toll-like receptor
TNF	tumor necrosis factor
Treg	regulatory T cell
TSAG	tumor stroma associated antigen
VEGF	vascular endothelial growth factor
ZA	zoledronic acid

I Introduction

1.0 The Immune System

In all vertebrates the ability to resist and survive pathogenic infections relies on a functioning immune system. The immune system consists of several distinct components, whose close interaction, under optimal conditions, results in elimination of the pathogen and establishment of an immunological memory. In the following section I will give a simplified view of the immunological mechanisms relevant to the work described in this thesis.

1.1 The Innate Immune System

After a pathogen has breached the surface barriers of the body, *i.e.* skin and epithelia, the innate immune response is the first line of defense. The innate immune system can be divided into a humoral and a cellular arm.

The major humoral component is the **complement system** which consists of more than 30 plasma proteins that can bind to the surfaces of pathogens. Complement proteins are involved in killing and opsonization (coating) of pathogens (to facilitate phagocytosis) and promotion of **inflammation** (1).

The second arm of the innate immune system is the cellular arm which consists of mainly myeloid lineage white blood cells called **leukocytes**. These include the phagocytic cells (**macrophages, dendritic cells (DCs)** and **neutrophils**) which phagocytose and eliminate pathogens. They also secrete **cytokines** and **chemokines** which trigger the inflammatory response as we will see below. Additionally, macrophages and DCs are involved in antigen presentation and thereby they serve as a link between the innate and the adaptive immune response (2). Other cells of the cellular arm of the innate immune system are **basophils**, **mast cells** and **eosinophils** and also the lymphoid lineage-derived **natural killer (NK)** and **γδ T cells**.

1.2 Inflammation

Inflammation is a cascade of both molecular and cellular events which serve to 1) limit the spread of the invading pathogen; 2) eliminate the invading pathogen and; 3) to repair the injured tissue. It is characterized by redness (*rubor*), heat (*calor*), swelling (*tumor*) and pain (*dolor*) which are the cardinal signs of inflammation. A fifth consequence of inflammation may be loss of function (*functio laesa*) (3).

The inflammatory cascade is triggered when tissue resident leukocytes, primarily macrophages and mast cells, are stimulated by either structural motifs of invading pathogens or, in the case of tissue damage, molecules released from injured cells (4). The microbial motifs are often referred to as **pathogen-associated molecular patterns (PAMPs)** and include bacterial lipopolysaccharide, non-methylated CpG DNA and double-stranded viral RNA (5) and are shared by many infectious agents. These are recognized by either soluble receptors such as complement, mannan binding lectin (MBL), and C-reactive protein (CRP) or cell-surface receptors such as **Toll-like receptors (TLRs)** and scavenger receptors (5). These receptors are germline-encoded and are commonly called **pattern-recognition receptors (PRRs)**. Their main functions include opsonization, phagocytosis and activation of proinflammatory signaling pathways (6). Recognition of pathogens by membrane bound PRRs leads to the production of cytokines such as tumor necrosis factor-α (TNF-α), interleukin 1 (IL-1), IL-6 and IL-12 which have several effects (7). TNF-α and IL-1 interact with endothelial cells to induce expression of adhesion molecules, e.g. ICAM-1 and E-selectin (8). Other effects of these cytokines are increased vascular permeability, induction of acute phase protein synthesis in the liver and activation of NK cells (8).

Guided by chemokines such as IL-8 (CXCL8) released by activated tissue resident macrophages, the first cell to enter the site of infection is the neutrophil (9). After adhering to the endothelium through its leukocyte adherence molecules it disrupts the endothelial cell-cell junctions by secretion of proteases and extravasates to the tissue (10). It enters the site of infection and initiates several effector mechanisms to kill the invading organisms. Following activation, neutrophils release intracellularly stored

antimicrobial peptides, e.g. defensins, which are toxic to several pathogens such as bacteria and fungi (11, 12).

Attracted by factors released in the first phase of the inflammatory response, the next cells to enter the scene are the monocytes, which differentiate into mature macrophages or immature dendritic cells (13). As long as microbial and proinflammatory stimuli prevail, the macrophages will continue to attract more neutrophils.

An important mechanism used by neutrophils and activated macrophages to eliminate pathogens is **phagocytosis**. This process is aided by soluble PRRs (e.g. complement, CRP, MBL), which act as opsonins and label the pathogen for engulfment. After recognition by membrane bound PRRs (14) the pathogens are ingested and delivered into phagosomes, which after fusion with lysosomes mature into phagolysosomes (15). Inside the phagolysosome the pathogen is exposed to an array of toxic agents such as proteases, reactive oxygen (O_2^-, H_2O_2) and nitrogen (NO) species (ROS/RNS) (16) which eventually results in pathogen death. Activated neutrophils and macrophages also release these agents to kill extracellular pathogens (17) which together with secreted proteinases e.g. elastase, **matrix matalloproteinases (MMP)** and cathepsins degrade extracellular matrix (ECM). The MMPs released from neutrophils cleave TNF-α from macrophages and recruited monocytes, the TNF-α then activates more neutrophils (4). Furthermore, TNF-α, chemokines, neutrophil-derived defensins and leukotrienes collaborate to initiate an adaptive immune response by recruitment of memory T cells and dendritic cells (18, 19). Unfortunately the inflammatory response can cause more damage to the surrounding tissues than the microbe itself (20, 21) and since excessive tissue damage can lead to chronic inflammation it is important that the inflammatory process is properly terminated.

The switch from inflammation to tissue repair starts when the levels of proinflammatory stimuli decrease and macrophages start to produce more secretory leukocyte protease inhibitor (SLPI). SLPI is a serine protease inhibitor with both anti-inflammatory and wound-healing effects (22). These include inhibition of the action of elastase (and consequently MMPs), inhibition of ROS release, suppression of neutrophil activation and migration, and prevention of transforming growth factor β

(TGF-β) breakdown (23, 24). TGF-β is a cytokine with dual actions: after cleavage from the surface on macrophages by MMPs it is a potent neutrophil chemoattractant. However, after the acute phase of inflammation its actions turn into promotion of tissue repair (ECM synthesis) and immunosuppression (8) as will be discussed below. The residual neutrophils at the inflammatory site undergo apoptosis and are ingested by macrophages which will induce their expression of TGF-β (4). Once phagocytosis is complete the macrophages exit the inflammatory site and enter the lymphatic system (25).

1.3 Adaptive immunity

In some cases the innate immune system is unable to handle the infectious agent and thus the induction of an adaptive immune response is necessary. As for the innate immune system the adaptive immune system also consists of a humoral and a cellular arm. In the adaptive immune system there are two types of antigen recognition receptors: B-cell receptors, which are membrane bound **immunoglobulins (Ig)**, and **T cell receptors (TCR)**. In contrast to the receptors of the innate immune system which have broad specificities for conserved motifs of microbes, the antigen receptors of the adaptive immune system are highly specific. These receptors are assembled from variable and constant regions by recombination-activating gene (RAG) -mediated somatic recombination. Through mechanisms such as nucleotide addition and somatic hypermutation (Ig only) (26, 27) a highly diverse Ig/TCR receptor repertoire with a potential to recognize a vast number of antigens is generated.

1.3.1 Antigen presentation to T cells

The specificities of the lymphocyte receptors are not predetermined and neither is their site of antigen encounter or effector response after activation. Lymphocytes patrol the body through the lymph nodes, which drain the body's tissues, and the spleen which filters the blood, until encounter of their cognate antigen occurs. While the Ig-receptors recognize their specific antigens by its three-dimensional structure, recognition by TCR requires antigen presentation in the context of **major histocompatibility complex (MHC)** (28).

There are two types of T cells: **cytotoxic T cells**, expressing the **CD8** co-receptor, and **helper T cells (Th)** which express the **CD4** co-receptor. Via their TCR these

recognize antigenic peptides bound to **MHC class I** and **II** respectively. The TCR is a complex of the α and β chains which recognize the antigen, the **CD3** complex (ε, δ and γ chains) and the ζ chain which are required for TCR signaling (29).

Peptides presented on MHC I are derived from self proteins or intracellular pathogens such as viruses. Intracellular proteins are continuously degraded to peptide fragments in a protease complex known as the proteasome (30). After degradation the peptide is transported to the endoplasmatic reticulum (ER) by transporters associated with antigen processing (TAP) proteins (31) where it, via TAP associated protein (tapasin) binds to the MHC I molecule complex (32). After binding, the peptide-MHC I complex leaves the ER and is transported to the surface where it can be recognized by CD8 T cells.

Antigenic peptides derived from extracellular or intravesicular pathogens are primarily presented on MHC II molecules (33). While MHC I is expressed by all nucleated cells, MHC II is mainly expressed by **antigen presenting cells (APCs)** such as macrophages and DCs (34). After phagocytosis the pathogens are contained in endosomes (phagosomes) which eventually fuse with lysosomes (see above). Proteases in the lysosome degrade the pathogen-derived proteins into peptide fragments. The MHC II molecule is assembled in the ER where it is associated with the invariant chain (Ii). Ii prevents binding of peptides to MHC II in the ER and also directs the delivery of MHC II molecules to endosomal/lysosomal compartments where the Ii is dissociated and peptide binding can occur. After binding, the peptide-MHC II complex travels to the cell surface where it can be recognized by CD4 T cells (35).

To maintain self-tolerance *i.e.* to remove or inactivate those T cells which bear a TCR with the ability to recognize self-peptides and at the same time assuring the ability to recognize and respond to foreign antigens, immature T cells undergo the processes of **positive and negative selection** in the thymus. Positive selection results from TCR recognition of self-peptide–MHC molecule complexes generally presented by thymic cortical epithelial cells; if the T cells fail to recognize such complexes they die by neglect. On the other hand, if the recognition of the complexes is too strong, the self-reactive T cells are negatively selected and clonally deleted by apoptosis (36, 37).

1.3.2 Dendritic cells and T cell priming

Since TCRs are of random specificities and cannot determine the origin of its specific antigen, the induction of a T cell response requires translation of innate signals of infection or damage. For T cell activation the central "translator" is the dendritic cell (38). Immature DCs are highly efficient phagocytes that reside at sites of interaction with the environment, such as mucosa and skin, where they screen the tissue for the presence of pathogens using PRRs (39). After pathogen ingestion its proteins are processed and, upon DC maturation, presented via the MHC. DC maturation is triggered by pathogen recognition by PPRs and cytokines (e.g. TNF-α and IL-1) and is characterized by 1) enhanced antigen presentation through upregulation of MHC I and II (40, 41), 2) cross-presentation *i.e.* the ability to present phagocytosed antigens on MHC class I, 3) proinflammatory cytokine production, and 4) expression of co-stimulatory molecules B7.1 and B7.2 (CD80 and CD86) (42). Following maturation DCs migrate from the peripheral tissues to lymph nodes. The migration is dependent on expression of chemokine receptor 7 (CCR7) which directs the DCs to the T cell areas in lymph nodes in response to secreted CCL19 and CCL21 chemokines. When the DCs reach the lymph node they present the pathogen-derived antigens to naïve T cells *i.e.* T cells which have not yet encountered their cognate antigen in the periphery.

The signal from DCs to naïve T cells is mediated through **"the immunological synapse"** which is the area of contact between APCs/DCs and T cells (43) (Fig. 1). It involves interaction of 1) TCR with peptide-MHC I/II complexes; 2) CD8 or CD4 interaction with MHC I or II respectively and; 3) LFA-1 interaction with ICAM-1. Effective activation of T cells also requires a **co-stimulatory signal**. This signal is mediated through ligation of CD28, which is expressed on T cells, with CD80 and CD86 expressed by activated DCs (44). TCR stimulation in the absence of co-stimulation results in T cell tolerance (45). In conjunction with the signal provided by the TCR, CD28 ligation leads to expression of IL-2 and its receptor IL-2R. Binding of IL-2 to its receptor results in clonal proliferation and differentiation into antigen-specific effector T cells (46).

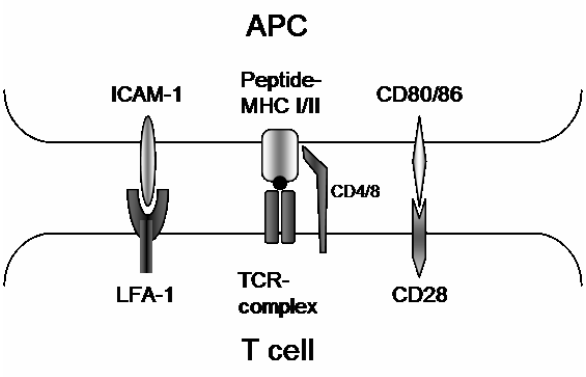

Figure 1. The immunological synapse. A simplified view.

1.3.3 T cell effector functions

Upon stimulation CD4 T cells differentiate into either **T helper 1 (Th1)** or **T helper 2 (Th2)** cells and these subsets are identified by their cytokine repertoire (47). A third subset of Th cells are the **regulatory T cells (Tregs)** which will be described later. Simplistically, Th1 cells produce interferon-γ (IFN-γ), activate monocytes and macrophages and thereby support cell-mediated immunity. They also promote production of opsonizing antibodies (IgG class) by B-cells. The main inducer of Th1 responses is IL-12 which is produced after TLR-ligation on DCs (48). Th2 cells produce IL-4, IL-5, and IL-13, promote immunity against multicellular pathogens *i.e* parasites, and provide help for B cells to produce antibodies of the IgM, IgA and IgE classes (humoral immunity) (49). The key determinant of Th2 cell differentiation is IL-4 (50). However, the initial source of IL-4 is not entirely known.

Naïve CD8 T cells differentiate into **cytotoxic T lymphocytes (CTLs)** whose main function is to eliminate virus infected, and in some cases, tumor cells in the body. As for CD4 T cells they need co-stimulation in order to be activated upon antigen recognition. The classical view of how this co-stimulation occurs was described as a three-cell interaction where the CD4 and CD8 T cells simultaneously recognize antigens presented on the same APC. After activation the Th1 cell secretes cytokines such as IL-2 which stimulate CD8 activation. However, since CTL responses can be induced in the absence of Th cells (51) an alternative model has gained support (52). According to this the CD4/CD8-APC interaction is divided into two steps, 1) the Th1 cell interacts with antigen-MHC II on an APC whereby the Th1 cell becomes

activated and consequently able to condition the APC through interactions of CD40 (on APCs) (53) and CD40 ligand (CD40L) (on Th) (54); 2) conditioned APCs acquire the competence to activate CD8 T cells directly through upregulation of CD80 and CD86. However, some viruses can directly induce upregulation of co-stimulatory molecules on DCs and thereby the need for CD4 help for CD8 activation is redundant (52, 55).

While Th cells mainly act by influencing the action of other cells of the immune system, CTLs have the capacity to directly kill cells expressing viral or cancer antigens. CTL-mediated killing can occur by at least three different pathways where two involve cell-cell-contact and the third is mediated by cytokines. Upon TCR stimulation CTLs secrete cytokines such as IFN-γ and TNF-α. Besides having direct antiviral effects (56), IFN-γ induces transcription of MHC class I thus making the target cell more susceptible for CTL recognition (57), it also induces expression of Fas (CD95) which increases Fas-mediated apoptosis (58). TNF-α binds to its receptor and triggers apoptosis via the caspase cascade (59). Cell-cell-mediated target killing occurs either by Fas ligand (FasL) expression on activated CTLs which induces apoptosis of Fas expressing target cells (60) or through release of cytotoxic mediators. By exocytosis, CTLs secrete a membrane-disrupting protein known as perforin and a family of structurally related proteases (granzymes) with various substrate specificities and together these induce apoptosis of the target cell (61). Granulysin is another cytotoxic molecule released by CTLs that causes target cell apoptosis by disrupting mitochondrial potential (62).

Although being favorable for the host at most times, T cell responses can cause immunopathology if left uncontrolled. Thus, the magnitude and duration of the T cell response need to be tightly regulated. It is well characterized that the peak of expansion is followed by a death phase where most of the antigen-specific T cells are eliminated (63). However, a certain number of death resistant, antigen-specific T cells may survive, forming a pool of **memory T cells** which respond rapidly upon a second exposure to the same antigen (64). Several mechanisms cooperate to regulate the magnitude of the response e.g. activated cell-autonomous death (ACAD) and activation-induced cell death (AICD). ACAD is also known as death by neglect or by cytokine deprivation and occurs in the absence of appropriate survival signals (63).

After cytokine deprivation there is an increased expression of the pro-apoptotic proteins PUMA and Bim. These bind to the antiapoptotic Bcl-2 or Bcl-xL causing release of Bax and Bak which mediate apoptosis via the intrinsic pathway (65). Stimulation of the TCR on already activated T cells may lead to AICD which includes stimulation through Fas and TNF receptor 1 (66). IL-2 appears to play a role in AICD as increased levels decrease the expression of FLIP, an inhibitor of the Fas signaling pathway (67). AICD seems to be the major cause of death upon chronic antigen stimulation and is therefore considered to be a major mechanism in maintaining peripheral tolerance (68, 69). The presence of immunosuppressive factors, regulatory cells and access of nutrients also regulate T cell responses and will be described in chapter 3.

2.0 Tumor Immunology

In 1909 the German immunologist Paul Ehrlich predicted that the immune system continuously represses spontaneously arising cancers. The hypothesis could be experimentally proven in the mid 1900's when it was demonstrated that immunization with chemically or virally induced tumors could stimulate tumor-specific responses that were able to reject the original tumors upon re-challenge (70, 71). The results from these experiments suggested the existance of tumor associated antigens and gave rise to the field of cancer immunotherapy.

2.1 Cancer Immunotherapy

Classical cancer treatments such as surgery, chemotherapy, radiation and hormonal treatment not only lead to severe side effects but are also mainly effective in early stage cancer. Despite major advances in the understanding of cancer biology and in the treatment of several types of cancer the disease remains a major cause of death. Therefore there is a great medical need for alternative treatment strategies. Cancer immunotherapy, *i.e.* activation of the patient's own immune system to combat cancer, represents one such treatment strategy.

In the 1890s William Coley observed that patients with *Streptococcus* infections had spontaneous regression of certain cancers. This led him to perform some pioneering experiments where he intentionally infected cancer patients with bacteria or bacterial extracts (Coley's toxin) and some therapeutic success was achieved. He is therefore considered to be the "father of immunotherapy" (72). Due to the intense research in the field of immunology including the mapping of **tumor antigens** (73) and the development of new technologies, the field of cancer immunotherapy has, since then, developed into complex molecular targeted therapies. Cancer immunotherapy is commonly classified into two major categories, **passive** and **active** which can further be divided into **specific** and **non-specific** depending on the strategy used.

2.1.1 Passive cancer immunotherapy

Passive cancer immunotherapy administers ready-made effector molecules or cells e.g. tumor antigen-specific antibodies or effector T cells. In contrast to active cancer

immunotherapy (see section 2.1.2), the passive approach does not always require an active participation by the patient's immune system. It is also short-lived and relies on repeated administration of the effectors. While the majority of the active immunotherapeutic approaches are still in pre-clinical evaluation or in early phase clinical trials, several of the antibody-based therapies have resulted in very successful clinically available agents such as trastuzumab (74), which targets the Her-2 antigen expressed on breast cancer cells, and the anti-CD20 antibody rituximab (75), which is used for B cell lymphoma treatment.

Another technique of passive specific immunotherapy is adoptive transfer of effector T cells. This involves isolation of the patient's T cells, either from the tumor or from blood, which after *ex vivo* processing are re-administered to the patient. This approach demonstrated clinical responses in melanoma patients (76). An example of a passive non-specific approach used clinically is Bacillus Calmette-Guerin (BCG) which is used for treatment of superficial bladder cancer (77).

2.1.2 Active cancer immunotherapy

Active specific cancer immunotherapies, or **cancer vaccines**, aim at the induction of tumor antigen-specific immune responses, both cellular and humoral and also at the generation of immunological memory. By using non-specific immune stimulators such as cytokines e.g. IFN-α and IL-2 (78, 79), or CpG (80), tumor-specific immune responses can be further enhanced. Despite the strong antitumor immune responses observed in animal models, only limited responses have been observed in clinical trials. In a review article summarizing 35 clinical trials with a total of 765 patients receiving various cancer vaccines, only 29 objective responses were reported. (81). A better understanding of the mechanisms of tumor escape, immunological tolerance, negative immune regulation and the influence of the tumor microenvironment is critical in order to improve the efficacy of active specific cancer immunotherapy. A schematic overview of active and passive cancer immunotherapy is illustrated in figure 2.

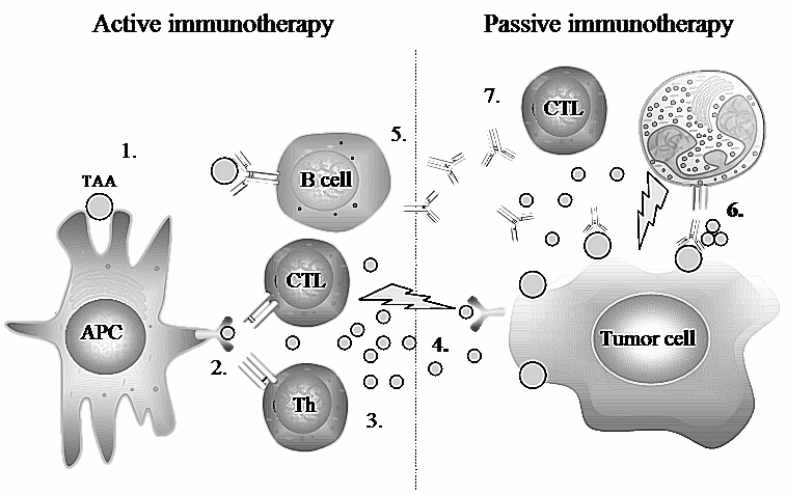

Figure 2. Active and passive cancer immunotherapy. Following vaccination the tumor associated antigen (TAA) is taken up by antigen presenting cells (APC) (1). After processing the antigenic peptide is presented to T cells (2) which leads to cytokine release (3) and subsequently CTL-mediated tumor killing (4). Alternatively, B cells are activated and produce antibodies (5) leading to lysis of tumor cells by either antibody dependent cytotoxicity or complement dependent cytotoxicity (6). The passive approach administers ready made effectors such as tumor-specific CTLs or antibodies (7). Figure adapted from Schuster et al. Biotechnology Journal (2006), 1, 138-146

2.2 Cancer Vaccines

The major focus in the field of cancer immunotherapy has been on cancer vaccines as this is an attractive option for long-term cure of cancer. The majority of cancer vaccines aim at the generation of tumor antigen-specific CTLs. While antibodies only target membrane bound antigens, CTLs can mediate direct killing of tumor cells by recognition of peptides derived from intracellular tumor antigens presented in the context of MHC I (see section 1.3.1). Moreover, the presence of tumor infiltrating lymphocytes (TILs) is correlated to increased survival in some cancers e.g. renal cell carcinoma (82). Increasing significance has also been given to CD4 Th, which can augment the development of tumor-specific CTLs (83), antibody production by B cells (84) or directly eliminate tumors (85). Cancer vaccines are intended to be utilized in a minimal disease setting, *i.e.* after therapy of bulky tumors, to prevent tumor recurrence and/or to eliminate metastases. Alternatively, cancer vaccines can be used in a prophylactic setting to prevent the development of cancer, which could be appropriate against virus-induced tumors. A vaccine against human papilloma virus-(HPV) induced cervical cancer was recently approved for clinical use (86).

2.2.1 Tumor antigens

The tumor antigen is the specific target against which the immune response is generated after vaccination and several tumor antigens that can be recognized by T cells have been identified and used in clinical trials (87). Due to their genetic instability, tumor cells express atypical proteins, *i.e.* tumor associated antigens which have low or limited expression on non-malignant cells. Another group of tumor antigens are the tumor specific antigens, which are uniquely expressed by tumor cells.

Tumor specific antigens are relatively rare and among these are 1) antigens that arise from point mutations in oncogenes or tumor suppressor genes such as *ras* in pancreatic and colorectal cancer (88) or *p53* in lung cancer (89), these antigens expose new, potentially immunogenic, epitopes which have never been encountered by the host's immune system and thus have the potential of being recognized by T cells and subsequently being targets of an immune attack; 2) antigens belonging to the cancer testis family which are the result of reactivation of genes that are normally silent in adult tissues, such as the antigens of the MAGE, BAGE and GAGE families (73) and; 3) antigens derived from virally induced tumors, which constitute almost 20 % of all human cancers (90). Examples of the latter are the E7 antigen from HPV which causes cervical carcinoma (91) and the EBNA2 antigen derived from Epstein-Barr virus which is associated with B cell lymphomas (92).

More common than the uniquely expressed antigens are the **tumor associated antigens (TAA)**. These are shared by the tumor and the tissue from which the tumor originates. This group comprises differentiation antigens and has mainly been found in melanomas and melanocytes, e.g. Melan-A (93) and tyrosinase (94), but also in other epithelial tissues such as prostate specific antigen (PSA) found in prostate (95). Other TAAs, such as HER-2/neu (96), hTERT (97) and survivin (98), are overexpressed in tumors but also expressed at lower levels in normal tissues.

2.2.2 Approaches of active immunization

Generally tumor cells do not induce immune responses by themselves and for an antitumor immune response to occur, tumor antigens need to be properly presented to the immune system. Several approaches to deliver tumor antigens and stimulate antitumor immune responses have been investigated in both animal models and

patients, e.g. cell-based vaccination using either inactivated tumor cells (99) or DCs loaded with antigen (100), protein/peptide vaccines (101), genetic vaccines (DNA or RNA encoding the antigen) (102, 103), viral vectors (104) or combinations of these with or without adjuvants.

Since vaccination with tumor antigens alone has shown to be inefficient, adjuvants are frequently used to enhance immune responses. As reviewed by Dredge *et al*. (105), adjuvants act through a wide range of mechanisms such as direct activation of T cells by IL-2 administration (106) or recruitment and stimulation of DCs by TLR ligands (107).

DCs are considered to play a central role in antitumor immune responses. As ligation of the TCR without the secondary co-stimulatory signals may render T cells anergic (108) it is of utmost importance that the DCs are stimulated to express co-stimulatory molecules when the vaccine is delivered. Since DCs are activated to express co-stimulatory ligands by stimulation with PAMPs or endogenous "danger signals", the use of molecules recognized by PRR such as TLRs (109), have been extensively used as adjuvants to activate and maturate DCs and consequently to induce more powerful immune responses. Among those adjuvants are BCG and CpG oligodeoxynucleotides. BCG has been used in the treatment of melanoma (110, 111) and CpG has shown promising results in a pre-clinical mouse model of colon cancer (112) and is currently in clinical testing (113). Chemokines have also been evaluated as adjuvants, and monocyte chemotactic protein 3 (MCP-3) fused to a B cell lymphoma tumor antigen induced T cell dependent protective immunity in mice (114). Among the endogenous signals which are capable of DC stimulation, the heat shock proteins (HSPs) are the best characterized (115) and the use of HSPs as adjuvants has demonstrated clinical benefit in patients with various cancers (116).

2.2.3 DNA vaccines

A DNA vaccine is a plasmid containing a cDNA which encodes the desired antigen to be targeted *in vivo*. The cDNA is inserted between a eukaryotic promoter and a polyadenylation signal. To allow amplification in bacteria the plasmid also contains a prokaryotic origin of replication and an antibiotic resistance gene. DNA vaccines offer several advantages over other vaccines (117). Even though DNA vaccines can

be constructed using many different promoters, polyadenylation sequences and antigens of interest, the manufacturing process is similar and relatively easy for all DNA vaccines. In contrast, vaccines based on cells, viral vectors or recombinant proteins/peptides all require unique manufacturing processes. Furthermore, DNA vaccines are also more stable, have fewer safety concerns and are less likely to induce anti-vector immunity compared to, for instance, vaccines based on attenuated live viruses (117).

The first evidence demonstrating that naked plasmid DNA could be taken up and processed by cells *in vivo* was provided by Wolff and co-workers in 1990 (118) when they observed protein expression after plasmid injection into mouse skeletal muscle. Since then, DNA vaccines have been successfully used to induce antibody and CTL responses against viruses e.g. influenza (119, 120) and HIV (121) in both mouse and non-human primates.

A major element of DNA vaccines is their ability to deliver proteins for processing and presentation via the MHC I pathway which allows for the induction of CTL responses and thus CTL-mediated antitumor immunity. Induction of CTL responses after DNA immunization can occur by two different means, 1) **direct priming** occurs when the APC is directly transfected by the plasmid, which leads to endogenous production of the protein and peptide presentation through the MHC I pathway (122); 2) **cross-priming** occurs when other cells than APCs, such as myocytes, are transfected and the expressed protein is taken up by APCs which then present the antigen to CTLs via cross-presentation (123) (Fig. 3). The predominating pathway of priming after DNA vaccination is most likely depending on the site of injection and delivery method (124). Part of the DNA vaccines' potency in inducing CTL responses lies in the plasmid backbone which contains CpG motifs that act as an intrinsic adjuvant (125).

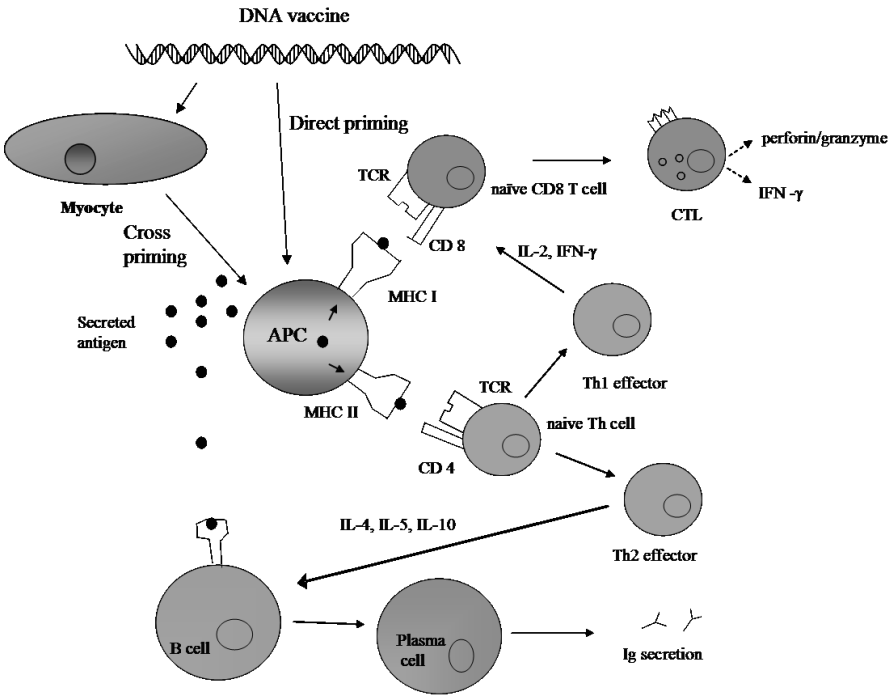

Figure 3. Priming of immune response after DNA vaccine administration

The efficacy of DNA vaccines against cancer antigens has been demonstrated in several mouse models. Immunizations with plasmid DNA encoding antigens such as SV40 large T antigen (126), HPV E7 (127), and human PSA (128) were all protective against a challenge with tumor cells expressing the corresponding antigen. However, in all these studies the antigens used were foreign to the animal in contrast to spontaneously arising tumor antigens, which mainly represent self-antigens. After vaccinations of mice using DNA which encodes mouse homologues of human TAAs or of transgenic mice with DNA encoding the human TAA, results are generally much poorer. For instance, Her-2 DNA vaccination in Her-2 transgenic mice only results in delay of tumor growth and no protection (129). These results also reflect the situation in humans where DNA vaccines against self TAA generally result in poorer immune responses compared to foreign antigens. In one study, a plasmid expressing both a foreign protein, hepatitis B surface antigen (HBsAg), and carcinoembryonic antigen (CEA) was used for immunization. While a robust response against the HBsAg was observed, only limited responses to CEA were detected (130). Nevertheless, DNA

vaccines have proven to be safe, well tolerated and to induce immune responses in patients (131).

Several approaches for enhancing the potency of DNA vaccines by co-administration of adjuvants have been evaluated. For instance, immunization with a plasmid encoding an ovalbumin-tetanus toxin fusion protein broke both B and T cell tolerance in ovalbumin transgenic mice (132) and MUC-1 transgenic mice were protected against a challenge with MUC-1 expressing tumor cells when MUC-1 DNA was co-administered with an IL-18 encoding plasmid (133). Another factor which influences the generation of immune responses after DNA vaccination is the delivery method. The intramuscular and intradermal routes of DNA administration are the most commonly used. Other DNA delivery techniques include needle-free biolistic delivery of DNA coated gold particles *i.e.* gene gun, and biojector which target resident APCs in the skin (Langerhans' cells). The gene gun approach has shown promising results in the TRAMP mouse model of spontaneous prostate cancer (134) and a DNA vaccine encoding human tyrosinase resulted in prolonged survival in dogs with malignant melanoma when delivered by biojector (135). Other methods of DNA vaccine delivery include association of the DNA with microparticles (136), encapsulation of plasmids in bacteriophages (137) and application of electroporation after either intradermal or intramuscular DNA delivery (electrovaccination) (138, 139).

In vivo electroporation has recently gained a lot of attention as a delivery method of gene transfer. From being a technique used to transfect cells *in vitro* it became clear in the 1990s that it was also possible to transfect various tissues *in vivo* (140, 141). Compared to injections with naked DNA alone, the addition of electroporation results in increased and more stable levels of gene expression (142). The application of an electric field is suggested to have two roles in enhancing DNA uptake. First it creates pores by disrupting cellular membranes and then it promotes electrophoresis of the negatively charged DNA (143, 144). Pulse duration, number, amplitude and type of electrode are parameters that need to be optimized for each tissue, not only for gene expression but also for their effect on induction of immune responses (145, 146). Electroporation in combination with DNA injection has been used to induce immune responses against tumor antigens in animal models. In the B16 melanoma model,

electrovaccination using a plasmid encoding the mouse melanocyte antigen tyrosinase-related protein-2 induced antigen-specific CTLs which delayed tumor outgrowth (147). Likewise, the BALB-neuT$^{664V\text{-}E}$ mice which develop mammary tumors at 10 weeks of age remained tumor free for more than a year after electrovaccination using a p185^{neu} plasmid (148). In humans, intramuscular electrovaccination is under clinical evaluation (149) and our lab is planning to perform a phase I study using intradermal electrovaccination against prostate cancer starting fall 2008.

2.3 Limitations of cancer vaccines

In contrast to vaccines against infectious diseases, vaccines against cancer generally have to break tolerance against self antigens (with the exceptions of tumor antigens that are derived from viral oncogenes and mutated self proteins). Nevertheless, some self-reactive T cell clones escape thymic deletion, thus having the potential of being activated to respond to self antigens (150). However, T cells that escape thymic deletion and that are capable of recognizing TAAs are only present in low precursor numbers and are often of low avidity. In order for T cell-mediated eradication of established tumors to occur, large numbers of high avidity CTLs have to be generated (151). If an active vaccination fails to generate sufficient numbers of high avidity antigen-specific T cells, a risk exists that the resulting weak immune response leads to the selection of tumor cells that no longer express the tumor antigen, *i.e.* immunoediting (see section 3.0). Active vaccination may also result in induction of antigen-specific Tregs (152). Besides the limitations discussed, tumors and tumor cells themselves possess multiple mechanisms to avoid immune recognition, a phenomenon referred to as tumor immune evasion.

3.0 Tumor Immune Evasion

In 2000, the six hallmarks of cancer cells were defined: cancer cells are 1) self-sufficient in acquiring growth signals; 2) insensitive to antigrowth signals; 3) resistant to apoptosis; 4) have limitless replicative potential; 5) able to sustain angiogenesis and 6) able to invade surrounding tissues and metastasize (153). While these hallmarks may be sufficient to maintain cancer growth, several steps of tumor progression are supported by altered stromal cells, e.g. fibroblasts, endothelial cells and leukocytes which supply the tumor with growth and angiogenic factors (153).

It was recently suggested that inability of the immune system to eradicate tumors or ***avoidance of immunosurveillance*** (154-157) is the seventh hallmark of cancer. To simplify, the immunosurveillance theory postulates that the immune system continuously eradicates spontaneously arising tumor cells before they develop into detectable tumors (158). It is well documented that mice deficient in various components of the immune system, such as RAG2, IFN-γ receptor and perforin knock-out mice, are more susceptible to develop some types of tumors (159-161). An increase in cancer incidence is also observed in immunosuppressed transplant recipients (162). However, despite immunosurveillance, cancers develop in immunocompetent hosts and this has been proposed to be a consequence of an immunologic pressure that functions to select for tumor variants with a reduced immunogenicity. The term **"cancer immunoediting"** was coined to explain this phenomenon (156). The cancer immunoediting model describes three phases; 1) *elimination* (immunosurveillance) in which the immune system targets and destroys antigen bearing tumor cells; 2) the *equilibrium* phase in which the immune system applies immunologic pressure which controls tumor growth, but not fully eliminates the tumor cells. During this phase, tumor cells acquire mutations which render them less sensitive to immune attack; 3) in the *escape* phase the immune resistant tumor cells expand to a detectable tumor.

In animal models and humans, tumors exhibit several strategies to escape recognition of the immune system and to actively suppress the immune response. Mechanisms of tumor immune evasion include downregulation of MHC molecules, resistance to

apoptosis, secretion of immunosuppressive cytokines, and recruitment of suppressive stroma cells (Fig. 4).

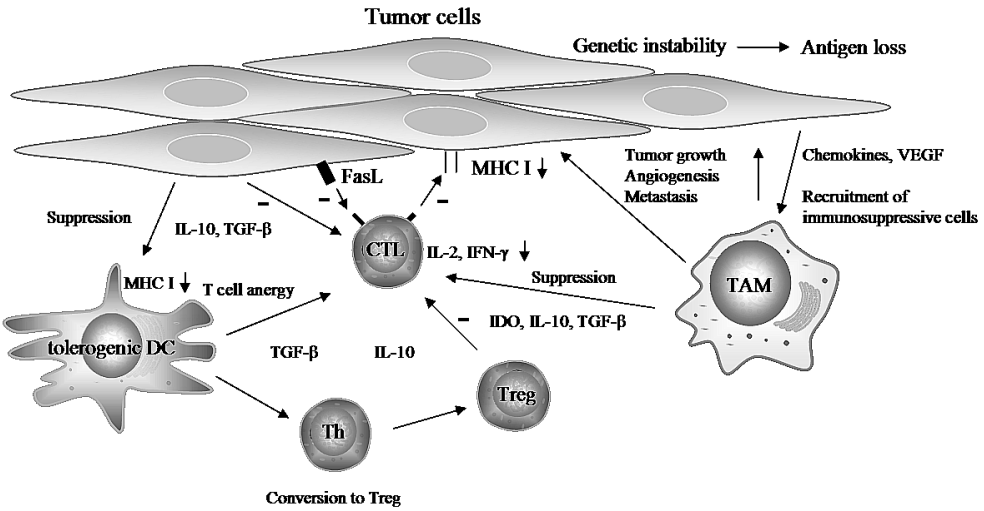

Figure 4. Mechanisms of tumor escape and immunosuppression.

3.1 Loss of antigen presentation and resistance to apoptosis

As a consequence of genetic instability and immunological pressure, tumors acquire mutations which render them invisible for immune targeting and resistant to apoptosis. A common strategy of tumors to avoid recognition by T cells is through downregulation of MHC class I molecules (163). Additionally, it has been shown that tumors often downregulate the expression of molecules that are involved in antigen processing and presentation, such as TAP1/2 (164), tapasin (165) and β2-microglobulin (166). As complete loss of MHC I would result in NK-cell killing (167) tumors can compensate loss of MHC I by upregulation of non-classical MHC I molecules (163) thus avoiding NK cell-mediated killing. Other mechanisms of immune evasion utilized by tumors are expression of apoptosis inhibitory proteins such as the caspase inhibitor survivin (168); secretion of decoy receptors for FasL which inhibits FasL-mediated apoptosis; expression of FasL which kills activated T cells expressing Fas (169); expression of molecules that inhibit lysis through the granzyme B/perforin pathway (170) and of ligands that act inhibitory on T cells such as the programmed cell death 1 ligand (PD-L1/B7-H1) (171).

3.2 Inhibitory cell populations

Tumors also evade immune targeting by actively suppressing immune responses. Tumor cells and cells of the stroma release various cytokines and chemokines that, in addition to having direct immunosuppressive effects, also attract a diverse population of leukocytes. These include DCs, lymphocytes, myeloid-derived suppressor cells (MDSCs) and macrophages, all of which, under certain conditions, are able to subvert antitumor immune responses. The first observation that tumors harbor leukocytic infiltrates was made in 1863 when Rudolf Virchow made a connection between chronic inflammation and cancer (9). The accumulation and actions of inflammatory cells in tumors share several features with tissue repair. However, in tumors these processes are not properly regulated which results in continuous release of factors contributing to angiogenesis, tissue remodeling and leukocyte recruitment (172). Based on the similarities to wound healing, tumors have been described as "wounds that do not heal" (173).

3.2.1 Tolerogenic DCs and regulatory T cells

Tumor-derived IL-10 and TGF-β have several suppressive effects on antitumor immune responses (174, 175). In addition to their direct effect on T cells, e.g. inhibition of IL-2 production and inhibition of cytolytic gene expression (176, 177), secretion of IL-10 and TGF-β was reported to suppress differentiation and function of local DCs (178, 179). The result is the presence of tolerogenic DCs in the tumor microenvironment which in turn leads to ineffective T cell priming and induction of T cell anergy (45). Other factors contributing to the accumulation of tolerogenic DCs in tumors are macrophage-colony stimulating factor (M-CSF) and IL-6 (180). IL-10, TGF-β and tolerogenic DCs have also been shown to promote the conversion of naïve CD4 T cells to Tregs (181-183).

Tregs, which are commonly detected in tumors, can be described as a T cell population which mediate peripheral tolerance by suppressing T cells that react to self-antigens, and thereby they also mediate suppression of a large portion of TAA-specific T cells (184). There are several subpopulations of this cell type including IL-10 producing Tr1 cells (185) and TGF-β producing Th3 cells (186) and the natural $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs. Tumor associated Tregs either originate from the thymus or they can be induced locally in the tumor (187). Some Tregs are antigen-specific

(188) but once activated they can also suppress by an antigen-independent bystander mechanism (189). The mechanisms by which Treg mediate immune suppression are not completely elucidated but are likely to include both cell-cell contact and cytokine secretion (190).

3.2.2 Tumor Associated Macrophages

Along with the factors mentioned above, tumors also secrete monocyte chemoattractant protein-1 (MCP-1/CCL2), CCL5 (RANTES), macrophage migration inhibitory factor (MIF) and vascular endothelial growth factor (VEGF) (191-194) which may recruit and promote survival of tumor associated macrophages (TAMs).

TAMs are a major component of the leukocytic infiltrate in tumors and can comprise more than 50 % of the tumor mass (195). TAMs are derived from circulating monocytes that are attracted to the tumor by chemokines. An alternative source of TAMs could be MDSCs (196) as these seem to differentiate into TAMs in several animal models of cancer (197). Their role in tumor microenvironment is complex and was initially considered to be tumoricidal. However, it is now clear that monocytes can differentiate into "alternatively activated" macrophages which stimulate tumor growth, metastasis and angiogenesis (198). The presence of TAMs correlates with poor prognosis in several cancers such as cervical cancer, melanoma and breast cancer (195, 199, 200). However, TAM function might be cancer dependent since TAM presence was shown to be associated with good prognosis in colorectal cancer (201).

As for Th1 and Th2 cells, macrophages can adopt the polarized M1 and M2 phenotypes which differ in cytokine secretion and effector function. M1 macrophages produce IL-12 and TNF-α (202) and in response to IFN-γ they release tumoricidal products such as ROS and NO (203, 204). Consequently, tumor debris from dead tumor cells can be taken up by APCs (including macrophages) and adaptive antitumor immune responses can be initiated.

Within the tumor microenvironment TAMs are shifted towards the M2 phenotype. In contrast to M1 macrophages, the M2 polarized counterparts are poor producers of IL-12 but produce high levels of IL-10 and TGF-β (205, 206). Examples of other

immunosuppressive functions mediated by M2 polarized macrophages are expression of indoleamine 2,3-dioxygenase (IDO), which suppresses T cell growth through tryptophan depletion (207), and expression of B7-H4 which is a negative regulator of T cell growth (208). Moreover, in M2 polarized TAMs the L-Arginine metabolism is shifted towards the arginase (ARG) pathway, thus making them poor producers of NO and less tumoricidal (209). L-Arginine depletion due to ARG-1 overexpression, has also been shown to cause loss of the CD3 ζ chain, the major signal transducer of the TCR (210) (Fig. 5).

Besides having immunosuppressive effects, TAMs actively support tumor growth and angiogenesis. As early as in 1974 (211), the presence of TAMs was associated with metastatic disease and later also with increase in angiogenesis (212). In the presence of tumor-derived CCL2 and CCL5, macrophages secrete MMPs (213, 214) which degrade the basement membrane and the ECM and consequently support migration of tumor cells. Examples of angiogenic factors released by TAMs are VEGF, FGF-2 and PDGF (215) (Fig. 5).

Generally, the M1 and M2 phenotypes are induced by IFN-γ and IL-4/IL-13 respectively. The exact signals driving the M2 polarization of TAMs are not fully understood, but most likely IL-10 and CSF-1 are involved. For instance, it was shown that IL-10 is responsible for the defective IL-12 production observed in TAMs (205) and CSF-1 was shown to induce the release of VEGF (216), thus contributing to their angiogenic phenotype. Several studies have reported induction of genes encoding angiogenic factors in macrophages exposed to hypoxia (217, 218), suggesting a role for oxygen availability in TAM function.

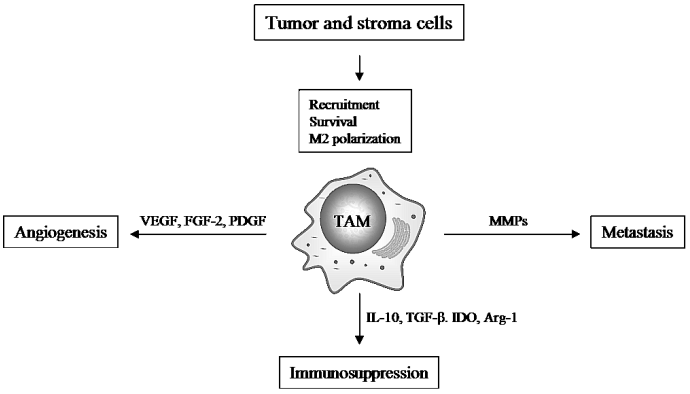

Figure 5. Functions of polarized macrophages in the tumor microenvironment.

In summary, tumors may escape immune recognition by loss of antigen presentation and by acquiring resistance to CTL-mediated apoptosis. Additionally, tumors actively subvert immune responses by secretion of immunosuppressive agents and by recruitment of cells with immunosuppressive functions.

4.0 Counteracting tolerance and tumor immune evasion

The following sections describe some strategies to improve the current protocols of cancer immunotherapy, both in terms of augmenting T cell responses and counteracting tumor-induced immune suppression.

4.1 Which antigen to target?

Tumors consist not only of tumor cells but also of altered stromal cells which play an important role in supporting tumor growth. Analogous to tumor cells, tumor stromal cells differ from their normal counterparts and several **tumor stroma-associated antigens (TSAGs)** with altered expression have been identified.

The most commonly used antigens in vaccination studies against cancer are TAAs, such as the melanoma differentiation antigens tyrosinase and MART-1 (219, 220). The expression of most TAAs is, however, irrelevant for the progression and survival of the tumor and therefore their expression can be downregulated without affecting tumor growth. Such immunoselection results in antigen loss variants which can escape tumor antigen-specific T cells. Therefore, antigens that are directly involved in and necessary for promotion of carcinogenesis (oncoantigens) would represent the ideal targets as using such antigens would reduce the probability of antigen loss variants. The ERBB2 (Her-2) expressed by breast cancer cells represents one such antigen (221) and Her-2 expressing tumors have been successfully treated in both experimental (222) and clinical settings (223). Since Her-2 is expressed on the cell membrane, tumor escape due to MHC class I down regulation can be circumvented by employing vaccination strategies that also induce anti-Her-2 antibody responses. Other approaches based on tumor associated antigens are the use of heteroclitic epitopes (altered self peptides) or homologous antigens derived from other species (xenoantigens). The use of such antigens to overcome tolerance against poorly immunogenic self antigens has demonstrated efficiency in a mouse melanoma model (224), canine malignant melanoma (135) and also disease stabilization in prostate cancer patients vaccinated with rat prostate acid phosphatase (PAP) (225).

In contrast to tumor cells, stroma cells are genetically stable and therefore some of the escape mechanisms associated with tumor cells are less likely to occur. Thus, TSAGs

represent promising targets for CTL- and antibody-mediated cancer immunotherapy. Various vaccination approaches targeting TSAGs have demonstrated promising results in mice. A DNA vaccine targeting VEGF-R induced a CTL response, inhibited angiogenesis and reduced tumor growth (226). In another study, a DC-based vaccine against the pH regulator carbonic anhydrase IX, which is overexpressed in cancer associated fibroblasts, induced antigen-specific CTLs and inhibited tumor growth (227). Moreover, a DNA vaccine targeting legumain, a molecule highly expressed in TAMs, induced a CTL response against TAMs which reduced TAM numbers in tumor stroma leading to suppressed tumor growth and metastasis (228). While active immunization approaches against stromal antigens are still at the evaluation phase a number of antibody-based therapies have reached clinical evaluation (229). For instance, treatment with Bevacizumab, which targets VEGF thus blocking VEGF-R signaling significantly inhibits progression of several tumor types (230, 231).

4.2 Counteracting tumor-induced immune suppression

Despite the major efforts that have been put into developing sufficient numbers of "high quality" T cells and in identifying novel target antigens, the relative lack of tumor eradication by T cells is still a major problem. It has become evident that the failure is not just a result of poorly generated effector cells but also a consequence of active immunosuppression. Accordingly, many approaches of cancer immunotherapy now focus on the counteraction or reversion of these mechanisms.

4.2.1 Treg depletion

One strategy under development is depletion of Tregs. This can be accomplished by systemic delivery of antibodies directed against antigens that are expressed by Tregs such as CD25 (IL-2 receptor α subunit). Systemic depletion of CD25 positive cells was reported to suppress growth of several tumor types either as a monotherapy (232) or in combination with vaccination (233) in mice. Depletion of $CD25^+$ cells using the clinically approved IL-2 diphtheria toxin conjugate (Ontak) has also shown clinical responses when combined with vaccination in mice and humans (234, 235). However, CD25 is also expressed on activated non-regulatory T cells and therefore administration of anti-CD25 antibodies could possibly have a negative impact on antitumor immune responses. The only truly Treg-specific marker defined so far is

Foxp3 (in mice). However, it is not expressed on the surface and thus it can not be targeted by antibodies. It was recently reported that vaccination with DCs loaded with Foxp3 mRNA induced Foxp3 specific T cells that selectively depleted Tregs in the tumor and thereby enhanced the immune response in mice (236). When administered at low doses, the chemotherapeutic agent cyclophosphamide has been shown to deplete Tregs in mice (237) and to improve immune responses in humans (238).

4.2.2 Interference with stroma formation and function

Targeting of molecules involved in recruitment of suppressive cells to tumor stroma and interference with their functions are strategies that could possibly overcome some of the limitations associated with CTL-based therapy.

Several studies have demonstrated the importance of chemokines and cytokines for the recruitment of inflammatory cells and angiogenic processes in tumors. For instance, deficiencies in CCL2, M-CSF and IL-1 signaling in transgenic mice result in reduced TAM infiltrates, delayed angiogenesis and reduced tumor growth (239-241). Accordingly, targeting of chemokine/cytokine pathways may influence both tumor growth and recruitment of inflammatory cells. Indeed, blocking of CCL2 activity by a gene therapy approach where intramuscular injection of a plasmid encoding the dominant negative MCP-1 mutant resulted in reduction of tumor growth, capillary density and TAM recruitment (242). Another possible strategy is targeting of MIF. It was recently reported that vaccination with a DNA vaccine encoding MIF significantly reduced inflammation in mouse models of rheumatoid arthritis (243). As MIF is also secreted by tumors (244), suggested to play a role in TAM recruitment (193) and known to promote angiogenesis (245), it represents a possible target for reducing immunosuppression.

Selective targeting of TAMs, causing polarization to a tumoricidal phenotype is an approach under development. Attempts have been made to skew TAM activity to the M1 phenotype. In one study the combination therapy of CCL16, to accumulate macrophages at the tumor site, the TLR9 agonist CpG and anti-IL-10R to block IL-10 signaling resulted in rapid debulking of the tumor mass. The effect was attributed to TAMs which switched their cytokine repertoire from IL-10 to TNF and IL-12.

Furthermore, the combination therapy restored the function of tumor associated DCs which resulted in an adaptive antitumor immune response (246). Systemic treatment with IL-12 was demonstrated to alter TAM function in favor of antitumor immunity (247).

A number of **chemical agents** have been shown to target stroma cells and to interfere with their immunosuppressive functions. They may therefore be used to augment antitumor immune responses. The drug Trabectedin which is used for mammary carcinoma treatment has a preferential toxicity to macrophages. At sub-cytotoxic levels, Trabectedin was shown to block monocyte differentiation into macrophages and also macrophage secretion of CCL2 and IL-6 (248). Systemic treatment with 1-methyltryptophan (1ME), an inhibitor of IDO demonstrated a delay of tumor growth in mice (249) and treatment with the arginase inhibitor N-hydroxyl-L-Arginine (NOHA) reduced arginase-induced T cell dysfuction (250).

Another group of compounds are nitrogen containing **bisphosphonates** (N-BPs). N-BPs are currently used for prevention and treatment of osteoporosis and skeletal metastases. In addition to reducing bone loss, recent observations suggest that N-BPs also exert direct antitumor, antiangiogenic and immunomodulatory effects (251). The main mechanism by which N-BPs induce apoptosis seems to be by targeting the mevalonate pathway, thereby interfering with the post-translational modification of G-proteins and cellular signaling (252). The antiangiogenic effects of N-BPs include inhibition of TAM-, MDSC- and tumor-produced MMP-9 and MMP-9-mediated VEGF mobilization (253, 254). The reduction of VEGF correlated with a decrease of inflammatory TAMs and improved antitumor immunity after DNA vaccination (254).

Bisphosphonates also have immunomodulatory capacities. It was demonstrated that N-BPs have several effects on the function and differentiation of myeloid cells. Upon treatment with zoledronic acid (ZA), a reduced level of TNF-α, reduction of phagocytic capacity, impaired maturation and allostimulation of DCs and finally downregulation of NF-κβ activation was reported (255). The latter probably reflecting the MMP-9 downregulation observed in TAMs as NF-κβ has been shown to regulate MMP expression in macrophages (256).

Bisphosphonates have been shown to induce expansion of γδ T cells. In contrast to conventional T cells bearing the αβ TCR, the antigen-specificity of γδ T cells is not entirely random and their recognition is skewed towards a pre-defined set of conserved phosphate containing, stress-induced self-structures and therefore they are considered to be innate-like (257). The recognition is not restricted to antigen presentation by classical MHC molecules and their activation does not require DCs (251). Since tumor cells often lose their MHC expression, thus making them resistant to CTL-mediated toxicity, activation of γδ T cells offers potential as cancer immunotherapy. Induction of γδ T cells by N-BP is dependent on cell-cell contact with either tumor cells or monocyte lineage cells e.g. macrophages (258). Internalization of N-BPs causes an intracellular accumulation of isopentenyl pyrophosphate which then serves as a danger signal that activates γδ T cells (258). Effector mechanisms of γδ T cells include secretion of IFN-γ and TNF-α and perforin-mediated cytotoxicity (259, 260).

4.2.3 Recruitment of innate effectors

Several studies have demonstrated the efficacy of innate-mediated tumor destruction. It was demonstrated that tumor cells engineered to secrete various cytokines such as TNF-α and IL-2 create a microenvironment which promotes neutrophil infiltration and subsequent tumor rejection (261). The role of neutrophils is also prominent for effective BCG therapy of bladder cancer where neutrophils not only eradicate tumor cells but also orchestrate subsequent antitumor immune responses by regulating T cell chemotaxis and secretion of inflammatory cytokines and chemokines (262). In addition, a possible role for neutrophils in antibody-mediated tumor killing has been suggested (263). One interesting molecule is the neutrophil-activating protein of *Helicobacter pylori* (HP-NAP), which promotes neutrophil adhesion to endothelial cells. In addition, it was demonstrated that HP-NAP induces IL-12 and IL-23 production in neutrophils, monocytes and DCs. It also induces DC maturation and drives Th1 polarization of immune responses (264). Thus, targeting of HP-NAP to tumor sites represents a possible approach for cancer immunotherapy.

Macrophages and eosinophils have also been reported to eradicate tumors. Treatment with an agonistic anti-CD40 antibody in combination with CpG or CpG alone resulted

in eradication of poorly immunogenic B16 mouse melanoma tumors and the antitumor activity was mediated by cytotoxic macrophages (265, 266). Another interesting observation is that tumor-specific Th2 cells efficiently cleared CTL resistant lung metastases and that the clearance was eosinophil dependent (267).

Collectively these findings suggest that CTL-mediated cancer immunotherapy could be improved by targeting immunosuppressive elements associated with the tumor microenvironment. Furthermore, approaches which specifically aim at recruitment and stimulation of cells of the innate arm represent viable alternatives for CTL-based cancer immunotherapies.

II Aims of the thesis

Effective cancer immunotherapy requires new strategies which not only target the tumor cells but also counteract mechanisms contributing to immunosuppression.

The general aim of this thesis was to develop and evaluate novel approaches for cancer immunotherapy.

The specific aims were:

1. To introduce the tumor-specific phage display particles as a cancer treatment of established tumors.
2. To elucidate the mechanism of action of phage-induced tumor eradication.
3. To dissect the effect bisphosphonates as modulators of prostate cancer-tumor associated macrophage function.
4. To improve an electroporation protocol for intradermal administration of a DNA vaccine.

III Results and Discussion

1.0 Introduction to paper I and II

The two first papers in this thesis describe the use of tumor-specific bacteriophages to treat established mouse melanoma tumors. This chapter intends to give the reader an overview of bacteriophages, the phage display technology and its applications in cancer therapy.

1.1 Bacteriophages

Bacteriophages, or phages, are a family of viruses that has evolved to replicate in bacteria. One group of phages are the filamentous phages and the best characterized of these is the Ff phage (subtypes M13, f1 and fd) which infects *E. coli* harboring the F plasmid. They consist of a single-stranded 6400 base pair long DNA genome encapsulated in a protein cylinder (268). The protein cylinder consists of approximately 2700 copies of the major coat protein pVIII and at the ends of the phage particle there are five copies each of the gene products of genes 3, 6, 7 and 9 (pIII, pVI, pVII and pIX) (Fig. 6). The remaining five genes are necessary for DNA replication and assembly of the phage coat proteins (269).

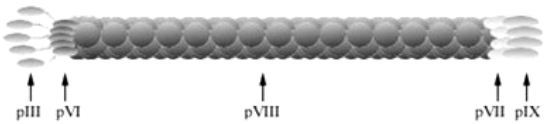

Figure 6. The filamentous bacteriophage particle. The five proteins which make up the protein cylinder are indicated. Picture adapted from: www.helsinki.fi/bioscience/biochemistry/koivunen.htm

Since most phages are able to lyse bacteria, phage therapy represents an alternative strategy to treat antibiotic resistant bacterial infections. Phage therapy was successfully used to cure bacterial infections such as *E. coli* induced diarrhea in calves (270) and antibiotic resistant gastrointestinal infections in mice (271). As reviewed by Sulakvelidze (272), phage therapy has also demonstrated efficiency for numerous bacterial infections in humans and it appears to be safe as no serious side effects were reported in these studies.

1.2 Phage display

The principle of the method is based on insertion of specific DNA sequences encoding peptides or proteins into the genes encoding phage capsid proteins. Fusion proteins are produced which are incorporated into the phage particle during assembly. Since the first description of the technology by Smith in 1985 (273), Ff phages have been widely used to construct peptide/protein (274) and antibody libraries (275) of up to billions of variants. The majority of phage display vectors use the N-terminus of pIII or pVIII to display the foreign sequences (276) allowing display from 1 (pIII) to 2700 (pVIII) copies. Through various selection protocols such as biopanning (Fig. 7), phage display libraries offer the possibility of rapid identification of peptides and proteins with specificity for a huge diversity of targets, including TAAs.

1.3 Phage display applications in cancer therapy

Numerous ligands with specificity for tumors, such as melanoma (277), prostate (278) and breast cancer (279), and tumor vasculature (280, 281) have been identified using phage display technology. Ligands identified through panning of phage libraries, may be used as drugs in cancer therapy by themselves, e.g. through targeting of receptors necessary for tumor formation (282) or progression (283). Tumor-specific ligands have also been used to target cytostatic drugs (284), proapoptotic agents (285, 286), cytokines (287) and superantigens (288) to tumor sites in mice.

More direct effects of phages to inhibit tumor growth have been demonstrated. Phages are generally considered to have no intrinsic tropism for mammalian cells. However it was reported already in 1940 that phages possess antitumor activity in mice and rabbits (289). More recently, it was demonstrated that a substrain of phage T4 could significantly reduce growth of experimental subcutaneous melanoma and lung cancer in mice when administered intraperitoneally (290) and also inhibit the formation of lung metastases (291). The suggested inhibitory mechanism is by binding to β3-integrin, a receptor of importance for tumor metastasis (292).

1.4 Phage immunogenicity

Phages are highly immunogenic and are known to induce immune responses directed to their naïve coat proteins (293). As a result, phages are used to monitor CD4 T cell function by their ability to provide B cell help in anti-phage antibody production in

HIV-patients (294). It was demonstrated that humoral anti-phage immune responses are T cell dependent as nude mice mounted a significantly weaker immune response and were unable to undergo a switch to produce IgG. Furthermore, no adjuvant was needed to induce potent anti-phage immune responses (295), demonstrating that phages are strong immunostimulators. Immune responses can also be induced by phage DNA. Administration of M13 phage DNA induced IFNs and protected mice against a vaccinia virus infection, most likely due to the presence of CpG motifs in the genome (296). Phages have been shown to induce IL-6 expression in splenocytes and macrophages (297) which suggests an ability to trigger inflammation. Furthermore, phages have been used to enhance vaccine potency. It was demonstrated that fusion of the domain I fragment of pIII to a non-immunogenic model antigen induced a Th1 response while no immune response was elicited using the non-fused antigen (293). Additionally, phage T4 enhanced the efficacy of a DC-based vaccine against colon carcinoma, characterized by an increase of DC maturation markers, increased IFN-γ production and delayed tumor growth (298).

1.5 Phage display particles as vaccine vehicles

Phages are taken up by DCs and processed through both MHC class I and II pathways (299) and are therefore also able to trigger CTL responses. When administered intravenously they have been shown to extravasate, and penetrate into tissues (300). Phages are also found in the circulation and internal organs after intraperitoneal, intranasal and intramuscular administration (301-303) where the intranasal route yielded an anti-phage antibody response (304). Phages are also non-toxic to animals (305). These features together with the possibility of displaying foreign structures on their surfaces have made them attractive for vaccine design

Several approaches of using phages as vaccine delivery vehicles have been explored, e.g. phages expressing mimitopes of immunogenic epitopes of, for instance, HIV (306), or phages that express known antigenic determinants derived from parasites (295, 307). Another approach is to use phage particles as carriers of DNA vaccines. This approach was superior compared to naked DNA immunization in generating antibodies against hepatitis B virus (308). Screening of phage display peptide libraries has also identified peptides, which after fusion to antigens, direct immunogenic epitopes to lymph nodes (309) and DCs (310) thus enhancing the immune response.

Phage-derived vaccines have also been explored in animal models of cancer. Promising results were obtained in mice with phages expressing antigen epitopes of mastocytoma (311), breast cancer (HER-2) (312) and melanoma (MAGE-A1) (313). Given these observations it is worthwhile to evaluate tumor-specific phages, which can be selected from phage display libraries, as anticancer agents.

2.0 Paper I and II

In **paper I** and **II** the potential of a novel passive immunotherapeutic approach using tumor-specific phage display particles to treat established tumors in a mouse model was evaluated. The hypothesis that accumulation of highly immunogenic phage-particles at the tumor site would attract an anti-phage inflammatory response and result in the destruction of the tumor by a bystander mechanism was investigated in paper I and a possible mechanism of action was suggested in paper II.

Two tumor-specific phage clones were used in the study. One clone was selected from a phage peptide display library through a combined *in vivo/in vitro* panning on established B16.F10 melanoma tumors and B16.F10 cells in culture (Fig. 7A). The second phage was constructed by inserting an antibody Fab-fragment (Fig. 7B) with known specificity for the model tumor antigen, HLA-A2.

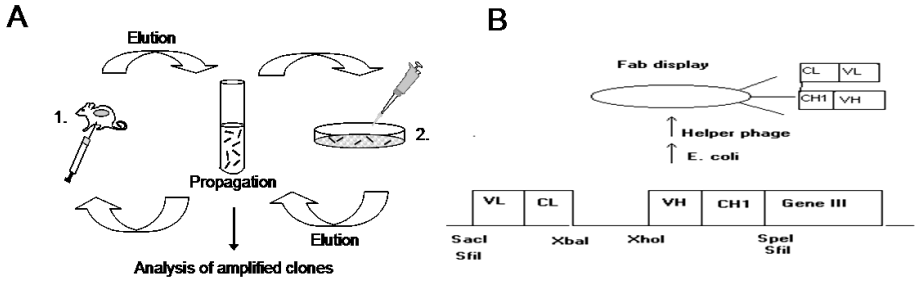

Figure 7. A. Schematic overview of a phage panning protocol**.** The phage library is either injected to tumor bearing mice (1) or added to tumor cells in culture (2). After non-binding phages have been washed off, the binding clones are eluted and propagated in *E. coli*. The phages are then panned for another 3-4 rounds to yield highly specific phage clones and analyzed for their affinity to tumor cells and ability to treat established tumors. B. Construction of Fab expressing phage. The vector containing the Fab-fragments is transformed into bacteria. After superinfection with helper phages, which confer the genes necessary for phage particle assembly, the resulting phages express the Fab-fragment as a fusion protein on pIII.

After panning of the peptide phage library on B16.F10 cells and tumors and construction of the HLA-A2-specific Fab expressing phage, the ability of phages to bind B16.F10 and B16.F10/A2Kb cells (B16.F10 transfected with HLA-A2), respectively, was analyzed using flow cytometry. One clone, selected through the combined *in vivo/in vitro* panning showed high specificity for B16.F10 melanoma cells but not for syngeneic, non-tumorigenic, melan-a cells which confirmed the tumor-specificity of the peptide phage. The Fab-phage showed specificity to HLA-A2

expressing cells but not to HLA-A2 negative cells, which verified that the Fab-fragment still exhibited antigen-specificity when fused to the phage tail.

The tumor-specific phages were used to treat mice bearing established B16-F10 or B16/A2Kb tumors. Phages were administered subcutaneously adjacent to palpable tumors every 3-4 days for two weeks. Treatment with tumor-specific phages was superior to wild type phage treatment and led to complete regression of B16/A2Kb tumors and long-term survival in 40-50 % of the mice (Table 1). Treatment of the less immunogenic parental B16.F10 tumor however, did not lead to complete regression but tumor growth was significantly delayed and a minor increase in survival was observed compared to controls.

Table 1. Outcome of phage treatment of established tumors

Treatment	Tumor free	% Survival
PBS	0/20	0
Wild type phage	1/18	5.5
Peptide phage (WDC-2)	8/19	42
Fab-phage	9/17	53

Morphological and histological analysis of tumors undergoing treatment revealed a massive infiltration of neutrophils, suggesting a significant role for the innate arm of the immune response in the initial phases of phage treatment. The infiltration of neutrophils was accompanied with local ulceration of the skin and necrosis of tumor tissue. The long-term survival suggested that the cellular arm of the immune system also might be involved. Phage administration induced secretion of proinflammatory cytokines (IL-12 and IFN-γ) both *in vivo* as measured in serum after phage administration and *in vitro* when administered to cultured splenocytes. These results demonstrate that accumulation of tumor-specific phages to the tumor site triggers an inflammatory response which recruits inflammatory cells to the tumor, and that these cells have the ability to kill tumor cells by a non-specific mechanism.

In **paper II** (manuscript) the mechanism behind the phage-induced tumor regression observed in paper I was investigated. Given the observation described above, that phages induce secretion of proinflammatory cytokines and also have CpG in their

DNA, we hypothesized a role for TLRs in the induction of the anti-phage immune response.

The contribution of TLR signaling was tested in MyD88$^{-/-}$ mice, in which TLR signal transduction is impaired (314). Upon phage stimulation of splenocytes isolated from MyD88$^{-/-}$ no IFN-γ could be detected in tissue supernatants and no tumor infiltration of neutrophils or tumor regression was observed after phage treatment of tumor bearing MyD88$^{-/-}$. The data indicate that TLR signaling is necessary for phage-mediated tumor regression to occur. As signaling through TLRs is a distinct characteristic of APCs and since macrophages comprise the majority of APCs in tumor stroma, the effect of bacteriophages on macrophages was investigated.

To induce the M2 polarized phenotype associated with TAMs, primary mouse peritoneal macrophages were cultured in B16-conditioned medium (CM). After conditioning, the gene expression was analyzed using real-time quantitative PCR. Exposure to tumor-CM resulted in a M2-like phenotype characterized by an upregulation of IL-10 and TGF-β and downregulation of IL-12 and IFN-γ. Phage stimulation of TAMs resulted in an upregulation of IFN-γ and IL-12 and thus switched the gene expression profile to a M1-like phenotype. Expression of molecules involved in antigen presentation (MHC I/II) and co-stimulation (CD80) was also increased. The latter was also true for bone marrow-derived DCs which also increased their CD86 expression after phage exposure. To establish a link between TAM activation and neutrophil accumulation at the tumor site a series of *in vitro* migration assays were performed. Medium from phage-stimulated TAMs was shown to recruit a substantial fraction of CD11b/Gr-1 double positive cells from mouse splenocyte preparations. Compared to medium from non-stimulated TAMs and normal macrophages the number of migrated CD11b/Gr-1 cells was significantly higher.

Next we investigated the contribution of soluble and cellular compartments to induce B16 tumor cell death. Medium from phage stimulated TAMs caused an increase in apoptosis of B16 cells compared to medium from non-stimulated TAMs. Although addition of neutrophils to tumor cell cultures resulted in increased tumor cell apoptosis the effect was markedly increased when neutrophils were added to tumor cells in the presence of medium from phage stimulated TAMs. The data suggest that

the observed tumor eradication is mainly a consequence of neutrophil-mediated cell death rather than a result of soluble factors such as TNF-α and IFN-γ secreted by stimulated macrophages. In addition, only low amounts of ROS and NO were released by TAMs after phage stimulation, which speaks for the neutrophils as the main effectors in this model. The mode of action of neutrophil-mediated tumor cell killing is not completely understood. Most likely it involves several effector functions such as secretion of toxic oxidative metabolites and defensins (263). A role for granzymes and perforin has also been reported (315, 316) but conflicting data exist (317).

The two studies demonstrate that tumor-specific phage particles are able to promote tumor eradication. They describe a close interaction of the innate immune system in which bacteriophages, directed to the tumor microenvironment due to their specificity, trigger an acute inflammatory response. The phage-induced tumor regression appears to be mediated by TLR signaling in TAMs, which skew their activity from a "tumor friendly" immunosuppressive M2 phenotype to the M1 activation state. The proinflammatory environment created by phage-stimulated TAMs attracts neutrophils to the tumor site. In the presence of TAM-secreted factors, the neutrophils become activated and gain an increased tumoricidal activity.The proposed mechanism of phage-induced tumor regression is illustrated in figure 8.

Although promising, these studies have limitations. One limitation is that the tumor-specific phage approach has only been evaluated when phages are administered peritumorally. Therefore we do not know whether the strategy works for tumors that can be targeted only via the intravenous route and if targeting actually occurs, there may not be enough phage particles localized to the tumor. The consequence may be a weaker immune response since the therapy seems to depend on a high number of phages accumulating at the tumor site. Another limitation is that the effects of phages on TAMs have been performed in an *in vitro* system. To study the effect on TAMs *in vivo* or on TAMs isolated from tumors would be more biologically relevant.

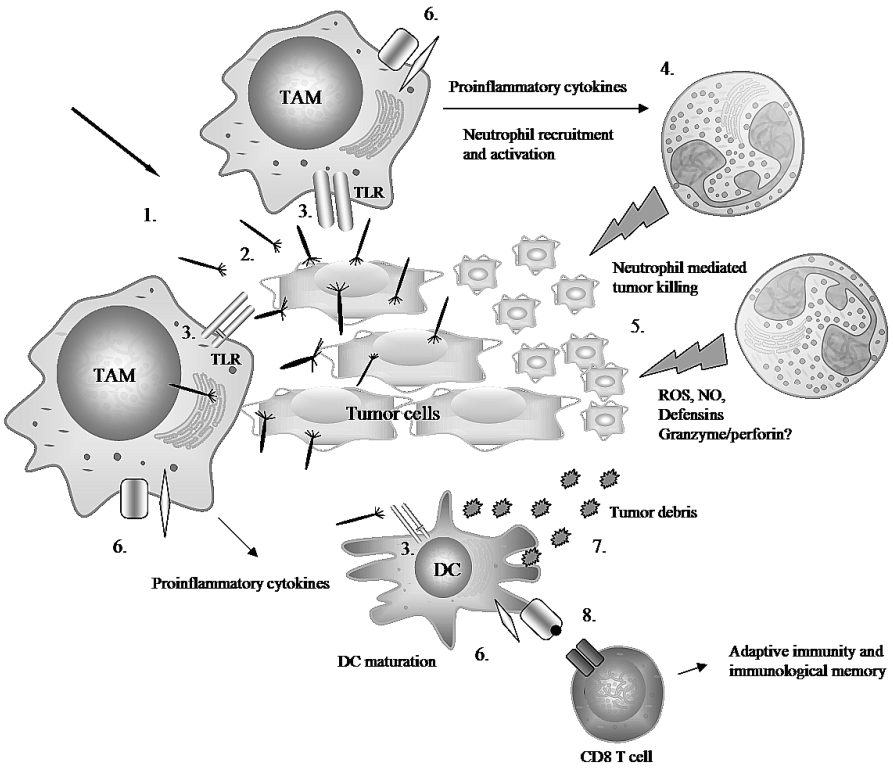

Figure 8. A proposed mechanism of tumor-specific phage-mediated tumor eradication. After administration of tumor-specific phage particles (1), these localize to tumor cells (2).Through TLR-mediated signaling (3), TAMs switch their phenotype and secrete proinflammatory cytokines (4) which recruit neutrophils to the tumor site. The proinflammatory environment activates the neutrophils to release tumoricidal factors (5). In addition, phage treatment results in upregulation of maturation markers on TAMs and DCs (6). Cell debris from dead tumor cells is taken up by DCs (7) which initiate an adaptive immune response (8).

2.1 Related but yet unpublished observations

The long-term survival of mice in which phage treatment resulted in complete tumor remission indicates that the adaptive arm might be involved at a later phase after phage treatment. The observation that both DCs and macrophages increased their expression of MHC and costimulatory molecules also pointed in that direction. Indeed, mice that were tumor free after phage treatment rejected a re-challenge with B16/A2Kb cells but not with syngeneic control tumor cells. The protective effect appeared to be mediated by CD8 T cells since adoptive transfer of CD8 T cells but not CD4 T cells from tumor free mice conferred protective immunity to naïve recipients. Moreover, phage treatment in $RAG^{-/-}$mice did not result in complete remission or

long-term survival even though the initial effects of the therapy were similar to that in wild type mice.

3.0 Introduction to paper III and IV

The common subject of **paper III** and **IV** is immunotherapy of prostate cancer (PC). In paper III we investigated the effect of the bisphosphonate ZA on the immunosuppressive and pro-tumorigenic function of prostate cancer TAMs. In paper IV we optimized a previously published protocol for intradermal administration of a DNA vaccine against PC in combination with electroporation (145).

3.1 Prostate cancer immunotherapy

Prostate cancer is currently the most commonly diagnosed form of cancer and the fourth leading cause of cancer related deaths among men in the Western world (318). Since there are no curative treatments for the advanced stages of the disease, *i.e.* androgen independent metastases, novel treatment strategies are needed. Several TAAs preferentially expressed by PC have been described, e.g. PSA (319), prostate-specific membrane antigen (PSMA) (320) and PAP (321).

Several approaches for vaccination against PC such as: protein/peptide (322), DC-based (323) and DNA encoded antigen delivery (128) have been evaluated and demonstrated promising results in animal models. Passive immunotherapeutic approaches include GM-CSF treatment, anti-CTLA-4 and anti-PSMA antibody administration (324). However, clinical responses after PC vaccination have been modest (325-327), which could partly be explained by PC immune evasion. As for other cancers several mechanisms of tumor escape and immunosuppression have been described in PC patients. These include loss of MHC I, expression of immunosuppressive substances and increased levels of Tregs (328).

3.1.1 Prostate specific antigen (PSA)

PSA is a secreted, 237 amino acids androgen regulated serine protease belonging to the kallikrein family (329). The primary function of PSA is cleavage of semenogelin and fibronectin to inhibit coagulation of semen (330). PSA levels are elevated in serum of men with PC and PSA is therefore a commonly used biomarker to diagnose

and monitor PC. Due to its high restriction to prostate epithelia (331) PSA represents a good target for PC immunotherapy. In addition, PSA-specific T cells (332) and antibodies (333) were detected in healthy individuals as well as in cancer patients showing that tolerance to PSA can be broken. PSA has been used as the target antigen in numerous clinical trials where PSA-specific immunity was induced, however clinical outcomes have been limited (131, 334, 335).

4.0 Paper III

In addition to having direct cytotoxic effects on tumor cells, bisphosphonates have been reported to suppress the angiogenic function of TAMs and to have immunomodulatory effects on myeloid cells. The aim of this study was to investigate the impact of ZA treatment on tumor associated macrophages in prostate cancer.

First we investigated the effect of two PC cell lines (LNCaP and PC3) on monocyte migration and macrophage proliferation. Monocytes migrated towards LNCaP-CM but not towards factors secreted by PC3 cells. The opposite relationship was demonstrated for macrophage proliferation where macrophages readily expanded upon exposure to PC3 whereas proliferation was completely absent after exposure to LNCaP-CM. The data suggest that the recruitment to, and the proliferation of leukocytes in the tumor depend on the grade of tumor cell differentiation. Next, we characterized the phenotypic changes induced in macrophages after exposure to PC-CM. A significant overexpression of several genes described in immunosuppression and cancer progression was observed. These included IDO, VEGF, IL-10 and MMP-9. The pattern of upregulated genes was to a large extent similar in response to LNCaP- and PC3-CM.

After confirming that macrophages adopt a M2 phenotype after when exposed to PC-CM, the effects on TAMs after ZA treatment were studied. TAMs were exposed to increasing concentrations of ZA and gene expression was evaluated. ZA treatment did not result in any significant change of expression of the majority of the analyzed genes. However, a significant decrease of MMP-9 expression and an increase of IDO were shown after treatment with high concentrations of ZA.

Since ZA treatment alone did not result in reversion of the immunosuppressive phenotype of TAMs, we investigated whether a combination of ZA with known immunomodulatory agents could shift the balance towards the M1 phenotype. The combination of ZA treatment with either IL-12 or the TLR3 ligand poly:IC significantly increased the expression of IFN-γ compared to either alone, both in terms of gene expression and at the protein level. A minor increase of TNF-α and IL-12 was also observed. Importantly the effect was also observed in the lower range of ZA concentration, contrasting to the high ZA concentration needed to achieve a significant decrease in MMP-9 expression. The data indicate that ZA has the ability to constrain the expression of genes involved in PC-TAM-mediated tumor progression. Although ZA treatment alone does not cause a shift in PC-TAM polarization, ZA can act synergistically with other immunomodulatory agents to skew PC-TAMs towards a M1 phenotype.

It is well established that bisphosphonates can trigger activation of γδ T cells (336) and that the activation requires cell-cell contact with either tumor or myeloid cells (258). We therefore investigated the potential of ZA treated PC-TAMs to drive activation and proliferation of this innate lymphocyte subset. ZA treated PC TAMs were able to induce a significant expansion of γδ T cells from peripheral blood mononuclear cells. ZA expanded γδ T cells were also activated as demonstrated by upregulation of CD3 and intracellular IFN-γ. Furthermore, cytotoxicity assays revealed that the activated γδ T cells had a significantly higher capacity to lyse ZA treated PC cells compared to non-treated target cells.

The study shows that ZA treatment of PC-TAMs results in downregulation of MMP-9 which is implicated to have a role in tumor metastasis. On its own ZA does not seem to possess the ability to affect the immunosuppressive phenotype of PC-TAMs. However, in combination with other immunomodulatory agents it acts to shift the cytokine profile towards a tumor-eliminating phenotype. ZA treated PC-TAMs expand and activate γδ T cells, which exhibit an increased ability to lyse ZA-treated target cells. Due to its effects on tumor progression, TAM activation, and the ability to evoke innate antitumor responses, a potential use of ZA as an immunomodulating agent in immunotherapeutic protocols is suggested. However, the approach needs to be tested *in vivo* to be validated.

5.0 Paper IV (Manuscript)

DNA vaccination in combination with electroporation is a promising approach of active immunization and several studies have demonstrated high levels of gene expression and induction of tumor-reactive T cells after electrovaccination. Thus far, the intramuscular route of DNA electrovaccination has been the most commonly used technique. However, intramuscular electroporation is invasive and associated with pain and discomfort. The present study aimed at optimizing a previously described protocol for intradermal DNA electroporation to enhance tolerability. The focus was on reducing total pulse duration without affecting the amplitude of the induced immune response.

Mice were injected intradermally with the PSA expressing pVax-PSA plasmid and subjected to electroporation. The pulses, which were optimized to generate a potent PSA-specific CTL response in a previous study (145), were a combination of two "high amplitude short duration" and eight "low amplitude, long duration" pulses. The rationale is that the pulses of high voltage cause pore formation in the cellular membranes and the long pulses transfer the DNA into the cell via electrophoresis. Six different protocols, only varying in intervals between pulses, were applied. The total pulse duration ranged between 0.24-2.98 seconds. Application of the longest pulse protocol resulted in 10 visible muscle contractions as opposed to the shorter pulse protocols where only one contraction was observed. The frequency of PSA-specific CD8 T cells was not affected by the pulse length as there were no significant differences in levels of IFN-γ producing CD8 T cells (2.1-3.8 %) in response to *in vitro* stimulation with a PSA-derived peptide between mice vaccinated with any of the electroporation protocols. A protocol of 0.27 seconds was selected for comparison with the previously published slow protocol (2.98 seconds) in subsequent experiments. The result shows that the total pulse duration can be reduced more than 10-fold, thus limiting discomfort without affecting the immune response.

Using a luciferase reporter system we investigated whether pulse length had any impact on gene expression. The results demonstrated that using higher pulse frequency does not affect either the time of induction of gene expression or expression over time. Luciferase expression was stable over 2 months regardless of

the protocol used. The long-term expression of the luciferase gene is probably associated with its lack of immunogenicity. When the luciferase encoding plasmid was co-administered with pVax-PSA the luciferase expression dropped below the limit of detection within 15 days (unpublished data) suggesting that cells transfected with an immunogenic antigen are eliminated. A potential danger of using DNA electroporation is that increased cellular uptake of DNA may lead to an increased integration frequency. Although a study showing that plasmid integration frequency is below the spontaneous rate of gene inactivating mutations after intramuscular DNA electroporation (337), studies examining integration after skin electroporation need to be undertaken.

To further reduce the sensation of pain during electroporation we evaluated if administration of an anesthetic cream containing lidocaine and prilocaine (EMLA®) would influence the immune response. Even though it has been demonstrated that the cream reduces pain associated with immunization and does not affect antibody responses (338, 339) there are no studies describing the effect on the induction of vaccine-specific CTLs. We show that application of local anesthesia had no negative effects on either PSA-specific CD8 T cell number or function, as demonstrated by their ability to produce IFN-γ and degranulate upon PSA peptide stimulation.

In summary, this study describes that DNA electrovaccination protocols can be considerably shortened to reduce discomfort without affecting the induction of immune responses or gene expression. Application of local anesthesia, to further reduce pain sensation has no impact on the immune response. We suggest that this protocol for DNA electroporation is suitable for clinical evaluation.

IV General Conclusions

The data presented in this thesis describe three novel immunotherapeutic approaches which could possibly overcome some of the mechanisms of tumor escape and immunosuppression.

We demonstrate that tumor-specific bacteriophages efficiently target an innate immune response to tumors and that the tumor eradication can be attributed to neutrophils. Interestingly, phages seem to reverse the immunosuppressive phenotype of TAMs to a phenotype that favors cellular immunity. The observation that phage treatment initiates adaptive immune responses suggests that employing strategies that allow for innate-mediated tumor destruction provide an antigen source for DCs and thereby the need for active immunization may be circumvented.

DNA vaccination in combination with electroporation has been demonstrated to break immunological tolerance and to induce potent antitumor CTL responses. We show that DNA electroporation protocols can be made considerably more tolerable by using the intradermal route of DNA administration in combination with short pulse protocols and local anesthesia. The same protocol, using xenogenic PSA to enhance immunogenicity, is expected to enter a phase I trial by the fall of 2008. The observed effects of ZA treatment on the pro-tumorigenic and immunosuppressive functions of TAMs and on the induction of γδ T cells suggest that ZA may serve as an adjuvant in immunotherapeutic protocols. However, possible adjuvant effects of ZA on cancer vaccination need to be evaluated *in vivo*.

In conclusion, I believe that cancer immunotherapy can be made successful. A combination of an efficient immunization modality and targeting of immunosuppressive cells would most likely improve clinical outcomes. Although the efficacy of immunotherapies aiming at CTL-mediated tumor eradication may be improved by reversal of immunosuppression, strategies aiming at the induction of innate antitumor responses represent possible alternatives. Treatment with tumor-specific phages offers a novel approach for treatment of established tumors which merits further investigation.

V Acknowledgements

The work presented in this thesis would never have become a reality without the support and encouragement of many people. I wish to express my sincere gratitude to all who contributed to making these years such a memorable time, especially:

Pavel Pisa, my main supervisor. Thank you for letting me become a member of your group. For teaching me how to think and work independently, and to trust my own ability. For creating a group with such fantastic members, which made this time a true pleasure. Thank you for inspiration and encouragement during hard periods, and above all, for believing in me.

Mats Persson, my co-supervisor. Thank you for introducing me to the world of bacteriophages, for fruitful discussions, support, and your never-ending enthusiasm.

Kajsa, thank you for your contagious optimism. Thank you for helping me with experiments during the thesis-writing period. For persuading me to exercise, without you I would never have managed to finish the Göteborgsvarvet alive. Sharing the "aquarium" with you is a genuine delight.

The former members of the group; **Anki**, for always being kind and helpful. For all the good times in the lab and in the "real world". I wish you, Aaron and Theo all the best; my homie **David**, for sharing my perspective of the important matters of life. Thanks for all the fun we have had, your generosity, and for being such a good friend. I really look forward to come and visit you and your family soon; **Panos**, for your never-ending pool of ideas, for helping me with experiments and putting my last papers together; **Ashley**, **Maxim**, **Lasse** and **Andreas** for contributing to the fantastic spirit of the group; **Tina** and **Eveline**, for being great students; **Bob**, for helping me overcome my fears of the *Mus musculus*; and all the other people that I have had the opportunity to work with.

All my **co-authors**, especially **Lars Egevad** and **Bob Harris**.

Rolf Kiessling and all **past and present members of his group**, especially; **Anna**, for being a good listener and friend; **Micke**, **Simona**, **Chiara** and **Alvaro**, for nice company at conferences; **Christian J**, for always being helpful; **Kalle L**, for answering questions regarding everything and; **Isabel**, for updating me with the latest review papers.

Tina Dalianis and all her **past and present group members**, especially; **Kalle**, for fruitful discussions and for scrutinizing my thesis more thoroughly than anyone else; **Karin**, for encouragement in the lab and for making the social events at the department more fun and; **Torbjörn**, for always having an answer.

All members of Håkan Mellstedts' group, especially; **Eva**, **Karin**, **Amir**, **Ingrid** and **Barbro** for always being helpful.

Past and present PhD-colleagues and staff at CCK for help in any way, in particular; **Micke L**, **Jacob**, **Linn**, **Mira**, **Janna**, **Christina**, **Åsa**, **Maggan**, **Liss**, **Joe**, **Elisabeth**, **Sören**, **Lars H**, **Dan G**, **Eva-Lena**, **Anita**, **Evi** and **Juan**.

The MTC **animal facility staff** for always helping me out and keeping track of my fury little friends.

I would also like to thank **my friends** for making me feel smarter than I actually am and for always being there when needed.

My closest **family** for all your help, encouragement and generosity. I am sorry for always talking about cancer during the family dinners.

My son **Olle**, you make me happy.

Ulrika, thank you for your limitless patience, support, encouragement and most importantly love. I am very lucky that we met and I am really looking forward to what is coming. You are the best and I love you.

VI References

1. Rus, H., Cudrici, C., and Niculescu, F. The role of the complement system in innate immunity. Immunol Res, *33:* 103-112, 2005.
2. Fearon, D. T. and Locksley, R. M. The instructive role of innate immunity in the acquired immune response. Science, *272:* 50-53, 1996.
3. Rather, L. J. Disturbance of function (functio laesa): the legendary fifth cardinal sign of inflammation, added by Galen to the four cardinal signs of Celsus. Bull N Y Acad Med, *47:* 303-322, 1971.
4. Nathan, C. Points of control in inflammation. Nature, *420:* 846-852, 2002.
5. Janeway, C. A., Jr. and Medzhitov, R. Innate immune recognition. Annu Rev Immunol, *20:* 197-216, 2002.
6. Underhill, D. M. and Ozinsky, A. Phagocytosis of microbes: complexity in action. Annu Rev Immunol, *20:* 825-852, 2002.
7. Dempsey, P. W., Vaidya, S. A., and Cheng, G. The art of war: Innate and adaptive immune responses. Cell Mol Life Sci, *60:* 2604-2621, 2003.
8. Borish, L. C. and Steinke, J. W. 2. Cytokines and chemokines. J Allergy Clin Immunol, *111:* S460-475, 2003.
9. Coussens, L. M. and Werb, Z. Inflammation and cancer. Nature, *420:* 860-867, 2002.
10. van Buul, J. D. and Hordijk, P. L. Signaling in leukocyte transendothelial migration. Arterioscler Thromb Vasc Biol, *24:* 824-833, 2004.
11. Borregaard, N. and Cowland, J. B. Granules of the human neutrophilic polymorphonuclear leukocyte. Blood, *89:* 3503-3521, 1997.
12. Parker, L. C., Whyte, M. K., Dower, S. K., and Sabroe, I. The expression and roles of Toll-like receptors in the biology of the human neutrophil. J Leukoc Biol, *77:* 886-892, 2005.
13. Kumar, S. and Jack, R. Origin of monocytes and their differentiation to macrophages and dendritic cells. J Endotoxin Res, *12:* 278-284, 2006.
14. Greenberg, S. and Grinstein, S. Phagocytosis and innate immunity. Curr Opin Immunol, *14:* 136-145, 2002.
15. Desjardins, M., Huber, L. A., Parton, R. G., and Griffiths, G. Biogenesis of phagolysosomes proceeds through a sequential series of interactions with the endocytic apparatus. J Cell Biol, *124:* 677-688, 1994.
16. Dahlgren, C. and Karlsson, A. Respiratory burst in human neutrophils. J Immunol Methods, *232:* 3-14, 1999.
17. Bogdan, C., Rollinghoff, M., and Diefenbach, A. Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. Curr Opin Immunol, *12:* 64-76, 2000.
18. Robbiani, D. F., Finch, R. A., Jager, D., Muller, W. A., Sartorelli, A. C., and Randolph, G. J. The leukotriene C(4) transporter MRP1 regulates CCL19 (MIP-3beta, ELC)-dependent mobilization of dendritic cells to lymph nodes. Cell, *103:* 757-768, 2000.
19. Yang, D., Chertov, O., Bykovskaia, S. N., Chen, Q., Buffo, M. J., Shogan, J., Anderson, M., Schroder, J. M., Wang, J. M., Howard, O. M., and Oppenheim, J. J. Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. Science, *286:* 525-528, 1999.

20. Guo, R. F. and Ward, P. A. Mediators and regulation of neutrophil accumulation in inflammatory responses in lung: insights from the IgG immune complex model. Free Radic Biol Med, *33:* 303-310, 2002.
21. Shapiro, S. D. Proteinases in chronic obstructive pulmonary disease. Biochem Soc Trans, *30:* 98-102, 2002.
22. Fitch, P. M., Roghanian, A., Howie, S. E., and Sallenave, J. M. Human neutrophil elastase inhibitors in innate and adaptive immunity. Biochem Soc Trans, *34:* 279-282, 2006.
23. Grobmyer, S. R., Barie, P. S., Nathan, C. F., Fuortes, M., Lin, E., Lowry, S. F., Wright, C. D., Weyant, M. J., Hydo, L., Reeves, F., Shiloh, M. U., and Ding, A. Secretory leukocyte protease inhibitor, an inhibitor of neutrophil activation, is elevated in serum in human sepsis and experimental endotoxemia. Crit Care Med, *28:* 1276-1282, 2000.
24. Ashcroft, G. S., Lei, K., Jin, W., Longenecker, G., Kulkarni, A. B., Greenwell-Wild, T., Hale-Donze, H., McGrady, G., Song, X. Y., and Wahl, S. M. Secretory leukocyte protease inhibitor mediates non-redundant functions necessary for normal wound healing. Nat Med, *6:* 1147-1153, 2000.
25. Serhan, C. N., Brain, S. D., Buckley, C. D., Gilroy, D. W., Haslett, C., O'Neill, L. A., Perretti, M., Rossi, A. G., and Wallace, J. L. Resolution of inflammation: state of the art, definitions and terms. Faseb J, *21:* 325-332, 2007.
26. Tonegawa, S. Somatic generation of antibody diversity. Nature, *302:* 575-581, 1983.
27. Hedrick, S. M., Nielsen, E. A., Kavaler, J., Cohen, D. I., and Davis, M. M. Sequence relationships between putative T-cell receptor polypeptides and immunoglobulins. Nature, *308:* 153-158, 1984.
28. Zinkernagel, R. M. and Doherty, P. C. Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. Nature, *248:* 701-702, 1974.
29. Borst, J., Jacobs, H., and Brouns, G. Composition and function of T-cell receptor and B-cell receptor complexes on precursor lymphocytes. Curr Opin Immunol, *8:* 181-190, 1996.
30. Goldberg, A. L., Cascio, P., Saric, T., and Rock, K. L. The importance of the proteasome and subsequent proteolytic steps in the generation of antigenic peptides. Mol Immunol, *39:* 147-164, 2002.
31. Howard, J. C. Supply and transport of peptides presented by class I MHC molecules. Curr Opin Immunol, *7:* 69-76, 1995.
32. Ortmann, B., Copeman, J., Lehner, P. J., Sadasivan, B., Herberg, J. A., Grandea, A. G., Riddell, S. R., Tampe, R., Spies, T., Trowsdale, J., and Cresswell, P. A critical role for tapasin in the assembly and function of multimeric MHC class I-TAP complexes. Science, *277:* 1306-1309, 1997.
33. Villadangos, J. A. Presentation of antigens by MHC class II molecules: getting the most out of them. Mol Immunol, *38:* 329-346, 2001.
34. Germain, R. N. MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. Cell, *76:* 287-299, 1994.
35. Hiltbold, E. M. and Roche, P. A. Trafficking of MHC class II molecules in the late secretory pathway. Curr Opin Immunol, *14:* 30-35, 2002.
36. Fink, P. J. and Bevan, M. J. H-2 antigens of the thymus determine lymphocyte specificity. J Exp Med, *148:* 766-775, 1978.

37. Kisielow, P., Bluthmann, H., Staerz, U. D., Steinmetz, M., and von Boehmer, H. Tolerance in T-cell-receptor transgenic mice involves deletion of nonmature CD4+8+ thymocytes. Nature, *333:* 742-746, 1988.
38. Steinman, R. M. and Cohn, Z. A. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. J Exp Med, *137:* 1142-1162, 1973.
39. Edwards, A. D., Diebold, S. S., Slack, E. M., Tomizawa, H., Hemmi, H., Kaisho, T., Akira, S., and Reis e Sousa, C. Toll-like receptor expression in murine DC subsets: lack of TLR7 expression by CD8 alpha+ DC correlates with unresponsiveness to imidazoquinolines. Eur J Immunol, *33:* 827-833, 2003.
40. Cella, M., Engering, A., Pinet, V., Pieters, J., and Lanzavecchia, A. Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. Nature, *388:* 782-787, 1997.
41. Cella, M., Salio, M., Sakakibara, Y., Langen, H., Julkunen, I., and Lanzavecchia, A. Maturation, activation, and protection of dendritic cells induced by double-stranded RNA. J Exp Med, *189:* 821-829, 1999.
42. Sallusto, F. and Lanzavecchia, A. The instructive role of dendritic cells on T-cell responses. Arthritis Res, *4 Suppl 3:* S127-132, 2002.
43. Dustin, M. L. and Shaw, A. S. Costimulation: building an immunological synapse. Science, *283:* 649-650, 1999.
44. Larsen, C. P., Ritchie, S. C., Hendrix, R., Linsley, P. S., Hathcock, K. S., Hodes, R. J., Lowry, R. P., and Pearson, T. C. Regulation of immunostimulatory function and costimulatory molecule (B7-1 and B7-2) expression on murine dendritic cells. J Immunol, *152:* 5208-5219, 1994.
45. Steinbrink, K., Jonuleit, H., Muller, G., Schuler, G., Knop, J., and Enk, A. H. Interleukin-10-treated human dendritic cells induce a melanoma-antigen-specific anergy in CD8(+) T cells resulting in a failure to lyse tumor cells. Blood, *93:* 1634-1642, 1999.
46. Smith, K. A. Interleukin-2: inception, impact, and implications. Science, *240:* 1169-1176, 1988.
47. Mosmann, T. R. and Coffman, R. L. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol, *7:* 145-173, 1989.
48. Kalinski, P., Hilkens, C. M., Wierenga, E. A., and Kapsenberg, M. L. T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. Immunol Today, *20:* 561-567, 1999.
49. Medzhitov, R. Recognition of microorganisms and activation of the immune response. Nature, *449:* 819-826, 2007.
50. Seder, R. A., Paul, W. E., Davis, M. M., and Fazekas de St Groth, B. The presence of interleukin 4 during in vitro priming determines the lymphokine-producing potential of CD4+ T cells from T cell receptor transgenic mice. J Exp Med, *176:* 1091-1098, 1992.
51. Buller, R. M., Holmes, K. L., Hugin, A., Frederickson, T. N., and Morse, H. C., 3rd Induction of cytotoxic T-cell responses in vivo in the absence of CD4 helper cells. Nature, *328:* 77-79, 1987.
52. Ridge, J. P., Di Rosa, F., and Matzinger, P. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. Nature, *393:* 474-478, 1998.

53. Caux, C., Massacrier, C., Vanbervliet, B., Dubois, B., Van Kooten, C., Durand, I., and Banchereau, J. Activation of human dendritic cells through CD40 cross-linking. J Exp Med, *180:* 1263-1272, 1994.
54. Lane, P., Traunecker, A., Hubele, S., Inui, S., Lanzavecchia, A., and Gray, D. Activated human T cells express a ligand for the human B cell-associated antigen CD40 which participates in T cell-dependent activation of B lymphocytes. Eur J Immunol, *22:* 2573-2578, 1992.
55. Wu, Y. and Liu, Y. Viral induction of co-stimulatory activity on antigen-presenting cells bypasses the need for CD4+ T-cell help in CD8+ T-cell responses. Curr Biol, *4:* 499-505, 1994.
56. Meurs, E., Chong, K., Galabru, J., Thomas, N. S., Kerr, I. M., Williams, B. R., and Hovanessian, A. G. Molecular cloning and characterization of the human double-stranded RNA-activated protein kinase induced by interferon. Cell, *62:* 379-390, 1990.
57. Schroder, K., Hertzog, P. J., Ravasi, T., and Hume, D. A. Interferon-gamma: an overview of signals, mechanisms and functions. J Leukoc Biol, *75:* 163-189, 2004.
58. Donze, O., Dostie, J., and Sonenberg, N. Regulatable expression of the interferon-induced double-stranded RNA dependent protein kinase PKR induces apoptosis and fas receptor expression. Virology, *256:* 322-329, 1999.
59. Varfolomeev, E. E., Schuchmann, M., Luria, V., Chiannilkulchai, N., Beckmann, J. S., Mett, I. L., Rebrikov, D., Brodianski, V. M., Kemper, O. C., Kollet, O., Lapidot, T., Soffer, D., Sobe, T., Avraham, K. B., Goncharov, T., Holtmann, H., Lonai, P., and Wallach, D. Targeted disruption of the mouse Caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. Immunity, *9:* 267-276, 1998.
60. Lowin, B., Hahne, M., Mattmann, C., and Tschopp, J. Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways. Nature, *370:* 650-652, 1994.
61. Smyth, M. J. and Trapani, J. A. Granzymes: exogenous proteinases that induce target cell apoptosis. Immunol Today, *16:* 202-206, 1995.
62. Kaspar, A. A., Okada, S., Kumar, J., Poulain, F. R., Drouvalakis, K. A., Kelekar, A., Hanson, D. A., Kluck, R. M., Hitoshi, Y., Johnson, D. E., Froelich, C. J., Thompson, C. B., Newmeyer, D. D., Anel, A., Clayberger, C., and Krensky, A. M. A distinct pathway of cell-mediated apoptosis initiated by granulysin. J Immunol, *167:* 350-356, 2001.
63. Hildeman, D. A., Zhu, Y., Mitchell, T. C., Kappler, J., and Marrack, P. Molecular mechanisms of activated T cell death in vivo. Curr Opin Immunol, *14:* 354-359, 2002.
64. Seder, R. A. and Ahmed, R. Similarities and differences in CD4+ and CD8+ effector and memory T cell generation. Nat Immunol, *4:* 835-842, 2003.
65. Krammer, P. H., Arnold, R., and Lavrik, I. N. Life and death in peripheral T cells. Nat Rev Immunol, *7:* 532-542, 2007.
66. Sytwu, H. K., Liblau, R. S., and McDevitt, H. O. The roles of Fas/APO-1 (CD95) and TNF in antigen-induced programmed cell death in T cell receptor transgenic mice. Immunity, *5:* 17-30, 1996.
67. Algeciras-Schimnich, A., Griffith, T. S., Lynch, D. H., and Paya, C. V. Cell cycle-dependent regulation of FLIP levels and susceptibility to Fas-mediated apoptosis. J Immunol, *162:* 5205-5211, 1999.

68. Lenardo, M., Chan, K. M., Hornung, F., McFarland, H., Siegel, R., Wang, J., and Zheng, L. Mature T lymphocyte apoptosis--immune regulation in a dynamic and unpredictable antigenic environment. Annu Rev Immunol, *17:* 221-253, 1999.
69. Strasser, A. and Pellegrini, M. T-lymphocyte death during shutdown of an immune response. Trends Immunol, *25:* 610-615, 2004.
70. Old, L. J. and Boyse, E. A. Immunology of Experimental Tumors. Annu Rev Med, *15:* 167-186, 1964.
71. Klein, G. Tumor antigens. Annu Rev Microbiol, *20:* 223-252, 1966.
72. McCarthy, E. F. The toxins of William B. Coley and the treatment of bone and soft-tissue sarcomas. Iowa Orthop J, *26:* 154-158, 2006.
73. Novellino, L., Castelli, C., and Parmiani, G. A listing of human tumor antigens recognized by T cells: March 2004 update. Cancer Immunol Immunother, *54:* 187-207, 2005.
74. Goldenberg, M. M. Trastuzumab, a recombinant DNA-derived humanized monoclonal antibody, a novel agent for the treatment of metastatic breast cancer. Clin Ther, *21:* 309-318, 1999.
75. Anderson, D. R., Grillo-Lopez, A., Varns, C., Chambers, K. S., and Hanna, N. Targeted anti-cancer therapy using rituximab, a chimaeric anti-CD20 antibody (IDEC-C2B8) in the treatment of non-Hodgkin's B-cell lymphoma. Biochem Soc Trans, *25:* 705-708, 1997.
76. Mackensen, A., Meidenbauer, N., Vogl, S., Laumer, M., Berger, J., and Andreesen, R. Phase I study of adoptive T-cell therapy using antigen-specific CD8+ T cells for the treatment of patients with metastatic melanoma. J Clin Oncol, *24:* 5060-5069, 2006.
77. Herr, H. W. and Morales, A. History of bacillus Calmette-Guerin and bladder cancer: an immunotherapy success story. J Urol, *179:* 53-56, 2008.
78. Kempf, R. A., Grunberg, S. M., Daniels, J. R., Skinner, D. G., Venturi, C. L., Spiegel, R., Neri, R., Greiner, J. M., Rudnick, S., and Mitchell, M. S. Recombinant interferon alpha-2 (INTRON A) in a phase II study of renal cell carcinoma. J Biol Response Mod, *5:* 27-35, 1986.
79. Lotem, M., Shiloni, E., Pappo, I., Drize, O., Hamburger, T., Weitzen, R., Isacson, R., Kaduri, L., Merims, S., Frankenburg, S., and Peretz, T. Interleukin-2 improves tumour response to DNP-modified autologous vaccine for the treatment of metastatic malignant melanoma. British Journal of Cancer, *90:* 773-780, 2004.
80. Kochenderfer, J. N., Chien, C. D., Simpson, J. L., and Gress, R. E. Synergism between CpG-containing oligodeoxynucleotides and IL-2 causes dramatic enhancement of vaccine-elicited CD8(+) T cell responses. Journal of Immunology, *177:* 8860-8873, 2006.
81. Rosenberg, S. A., Yang, J. C., and Restifo, N. P. Cancer immunotherapy: moving beyond current vaccines. Nat Med, *10:* 909-915, 2004.
82. Nakano, O., Sato, M., Naito, Y., Suzuki, K., Orikasa, S., Aizawa, M., Suzuki, Y., Shintaku, I., Nagura, H., and Ohtani, H. Proliferative activity of intratumoral CD8(+) T-lymphocytes as a prognostic factor in human renal cell carcinoma: clinicopathologic demonstration of antitumor immunity. Cancer Res, *61:* 5132-5136, 2001.
83. Ossendorp, F., Mengede, E., Camps, M., Filius, R., and Melief, C. J. M. Specific T helper cell requirement for optimal induction of cytotoxic T

lymphocytes against major histocompatibility complex class II negative tumors. Journal of Experimental Medicine, *187:* 693-702, 1998.
84. Rodolfo, M., Melani, C., Zilocchi, C., Cappetti, B., Luison, E., Arioli, I., Parenza, M., Canevari, S., and Colombo, M. P. IgG2a induced by interleukin (IL) 12-producing tumor cell vaccines but not IgG1 induced by IL-4 vaccine is associated with the eradication of experimental metastases. Cancer Research, *58:* 5812-5817, 1998.
85. Nishimura, T., Iwakabe, K., Sekimoto, M., Ohmi, Y., Yahata, T., Nakui, M., Sato, T., Habu, S., Tashiro, H., Sato, M., and Ohta, A. Distinct role of antigen-specific T helper type 1 (Th1) and Th2 cells in tumor eradication in vivo. Journal of Experimental Medicine, *190:* 617-627, 1999.
86. European approval for HPV vaccine. Eur J Cancer, *42:* 3079, 2006.
87. Parmiani, G., De Filippo, A., Novellino, L., and Castelli, C. Unique human tumor antigens: immunobiology and use in clinical trials. J Immunol, *178:* 1975-1979, 2007.
88. Gjertsen, M. K. and Gaudernack, G. Mutated RAS peptides as vaccines in immunotherapy of cancer. Vox Sanguinis, *74:* 489-495, 1998.
89. Yanuck, M., Carbone, D. P., Pendleton, C. D., Tsukui, T., Winter, S. F., Minna, J. D., and Berzofsky, J. A. A Mutant P53 Tumor-Suppressor Protein Is a Target for Peptide-Induced Cd8+ Cytotoxic T-Cells. Cancer Research, *53:* 3257-3261, 1993.
90. Stewart, B. W. and Kleihues, P. World Cancer Report, p. 351. Lyon: IARC Press, 2003.
91. Eiben, G. L., da Silva, D. M., Fausch, S. C., Le Poole, I. C., Nishimura, M. I., and Kast, W. M. Cervical cancer vaccines: recent advances in HPV research. Viral Immunol, *16:* 111-121, 2003.
92. Taylor, G. S. T cell-based therapies for EBV-associated malignancies. Expert Opin Biol Ther, *4:* 11-21, 2004.
93. Coulie, P. G., Brichard, V., Vanpel, A., Wolfel, T., Schneider, J., Traversari, C., Mattei, S., Deplaen, E., Lurquin, C., Szikora, J. P., Renauld, J. C., and Boon, T. A New Gene Coding for a Differentiation Antigen Recognized by Autologous Cytolytic T-Lymphocytes on Hla-A2 Melanomas. Journal of Experimental Medicine, *180:* 35-42, 1994.
94. Brichard, V., Van Pel, A., Wolfel, T., Wolfel, C., De Plaen, E., Lethe, B., Coulie, P., and Boon, T. The tyrosinase gene codes for an antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. J Exp Med, *178:* 489-495, 1993.
95. Corman, J. M., Sercarz, E. E., and Nanda, N. K. Recognition of prostate-specific antigenic peptide determinants by human CD4 and CD8 T cells. Clin Exp Immunol, *114:* 166-172, 1998.
96. Fisk, B., Blevins, T. L., Wharton, J. T., and Ioannides, C. G. Identification of an immunodominant peptide of HER-2/neu protooncogene recognized by ovarian tumor-specific cytotoxic T lymphocyte lines. J Exp Med, *181:* 2109-2117, 1995.
97. Minev, B., Hipp, J., Firat, H., Schmidt, J. D., Langlade-Demoyen, P., and Zanetti, M. Cytotoxic T cell immunity against telomerase reverse transcriptase in humans. Proc Natl Acad Sci U S A, *97:* 4796-4801, 2000.
98. Andersen, M. H., Pedersen, L. O., Becker, J. C., and Straten, P. T. Identification of a cytotoxic T lymphocyte response to the apoptosis inhibitor protein survivin in cancer patients. Cancer Res, *61:* 869-872, 2001.

99. Wu, T. Y. and Fleischmann, W. R., Jr. Efficacy of B16 melanoma cells exposed in vitro to long-term IFN-alpha treatment (B16alpha cells) as a tumor vaccine in mice. J Interferon Cytokine Res, *18:* 829-839, 1998.
100. Trefzer, U., Herberth, G., Wohlan, K., Milling, A., Thiemann, M., Sherev, T., Sparbier, K., Sterry, W., and Walden, P. Vaccination with hybrids of tumor and dendritic cells induces tumor-specific T-cell and clinical responses in melanoma stage III and IV patients. Int J Cancer, *110:* 730-740, 2004.
101. Bernhardt, S. L., Gjertsen, M. K., Trachsel, S., Moller, M., Eriksen, J. A., Meo, M., Buanes, T., and Gaudernack, G. Telomerase peptide vaccination of patients with non-resectable pancreatic cancer: A dose escalating phase I/II study. Br J Cancer, *95:* 1474-1482, 2006.
102. Zhang, H. M., Zhang, L. W., Liu, W. C., Cheng, J., Si, X. M., and Ren, J. Comparative analysis of DC fused with tumor cells or transfected with tumor total RNA as potential cancer vaccines against hepatocellular carcinoma. Cytotherapy, *8:* 580-588, 2006.
103. Yamano, T., Kaneda, Y., Hiramatsu, S. H., Huang, S., Tran, A. N., Giuliano, A. E., and Hoon, D. S. Immunity against breast cancer by TERT DNA vaccine primed with chemokine CCL21. Cancer Gene Ther, 2007.
104. Zhang, L., Tang, Y., Akbulut, H., Zelterman, D., Linton, P. J., and Deisseroth, A. B. An adenoviral vector cancer vaccine that delivers a tumor-associated antigen/CD40-ligand fusion protein to dendritic cells. Proc Natl Acad Sci U S A, *100:* 15101-15106, 2003.
105. Dredge, K., Marriott, J. B., Todryk, S. M., and Dalgleish, A. G. Adjuvants and the promotion of Th1-type cytokines in tumour immunotherapy. Cancer Immunol Immunother, *51:* 521-531, 2002.
106. Kim, J. J., Yang, J. S., Dang, K., Manson, K. H., and Weiner, D. B. Engineering enhancement of immune responses to DNA-based vaccines in a prostate cancer model in rhesus macaques through the use of cytokine gene adjuvants. Clin Cancer Res, *7:* 882s-889s, 2001.
107. Baines, J. and Celis, E. Immune-mediated tumor regression induced by CpG-containing oligodeoxynucleotides. Clin Cancer Res, *9:* 2693-2700, 2003.
108. Jenkins, M. K., Ashwell, J. D., and Schwartz, R. H. Allogeneic non-T spleen cells restore the responsiveness of normal T cell clones stimulated with antigen and chemically modified antigen-presenting cells. J Immunol, *140:* 3324-3330, 1988.
109. Gallucci, S. and Matzinger, P. Danger signals: SOS to the immune system. Curr Opin Immunol, *13:* 114-119, 2001.
110. Alexandroff, A. B., Jackson, A. M., O'Donnell, M. A., and James, K. BCG immunotherapy of bladder cancer: 20 years on. Lancet, *353:* 1689-1694, 1999.
111. Duda, R. B., Yang, H., Dooley, D. D., and Abu-Jawdeh, G. Recombinant BCG therapy suppresses melanoma tumor growth. Ann Surg Oncol, *2:* 542-549, 1995.
112. Brunner, C., Seiderer, J., Schlamp, A., Bidlingmaier, M., Eigler, A., Haimerl, W., Lehr, H. A., Krieg, A. M., Hartmann, G., and Endres, S. Enhanced dendritic cell maturation by TNF-alpha or cytidine-phosphate-guanosine DNA drives T cell activation in vitro and therapeutic anti-tumor immune responses in vivo. J Immunol, *165:* 6278-6286, 2000.
113. Leonard, J. P., Link, B. K., Emmanouilides, C., Gregory, S. A., Weisdorf, D., Andrey, J., Hainsworth, J., Sparano, J. A., Tsai, D. E., Horning, S., Krieg, A. M., and Weiner, G. J. Phase I trial of toll-like receptor 9 agonist PF-3512676

with and following rituximab in patients with recurrent indolent and aggressive non Hodgkin's lymphoma. Clin Cancer Res, *13:* 6168-6174, 2007.
114. Biragyn, A., Tani, K., Grimm, M. C., Weeks, S., and Kwak, L. W. Genetic fusion of chemokines to a self tumor antigen induces protective, T-cell dependent antitumor immunity. Nat Biotechnol, *17:* 253-258, 1999.
115. Colaco, C. A. Towards a unified theory of immunity: dendritic cells, stress proteins and antigen capture. Cell Mol Biol (Noisy-le-grand), *44:* 883-890, 1998.
116. Hoos, A. and Levey, D. L. Vaccination with heat shock protein-peptide complexes: from basic science to clinical applications. Expert Rev Vaccines, *2:* 369-379, 2003.
117. Srivastava, I. K. and Liu, M. A. Gene vaccines. Ann Intern Med, *138:* 550-559, 2003.
118. Wolff, J. A., Malone, R. W., Williams, P., Chong, W., Acsadi, G., Jani, A., and Felgner, P. L. Direct gene transfer into mouse muscle in vivo. Science, *247:* 1465-1468, 1990.
119. Ulmer, J. B., Donnelly, J. J., Parker, S. E., Rhodes, G. H., Felgner, P. L., Dwarki, V. J., Gromkowski, S. H., Deck, R. R., DeWitt, C. M., Friedman, A., and et al. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science, *259:* 1745-1749, 1993.
120. Donnelly, J. J., Friedman, A., Ulmer, J. B., and Liu, M. A. Further protection against antigenic drift of influenza virus in a ferret model by DNA vaccination. Vaccine, *15:* 865-868, 1997.
121. Shiver, J. W., Davies, M. E., Yasutomi, Y., Perry, H. C., Freed, D. C., Letvin, N. L., and Liu, M. A. Anti-HIV env immunities elicited by nucleic acid vaccines. Vaccine, *15:* 884-887, 1997.
122. Chattergoon, M. A., Robinson, T. M., Boyer, J. D., and Weiner, D. B. Specific immune induction following DNA-based immunization through in vivo transfection and activation of macrophages/antigen-presenting cells. J Immunol, *160:* 5707-5718, 1998.
123. Corr, M., von Damm, A., Lee, D. J., and Tighe, H. In vivo priming by DNA injection occurs predominantly by antigen transfer. J Immunol, *163:* 4721-4727, 1999.
124. Donnelly, J. J., Liu, M. A., and Ulmer, J. B. Antigen presentation and DNA vaccines. Am J Respir Crit Care Med, *162:* S190-193, 2000.
125. Sato, Y., Roman, M., Tighe, H., Lee, D., Corr, M., Nguyen, M. D., Silverman, G. J., Lotz, M., Carson, D. A., and Raz, E. Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. Science, *273:* 352-354, 1996.
126. Bright, R. K., Beames, B., Shearer, M. H., and Kennedy, R. C. Protection against a lethal challenge with SV40-transformed cells by the direct injection of DNA-encoding SV40 large tumor antigen. Cancer Res, *56:* 1126-1130, 1996.
127. Tuting, T., Gambotto, A., DeLeo, A., Lotze, M. T., Robbins, P. D., and Storkus, W. J. Induction of tumor antigen-specific immunity using plasmid DNA immunization in mice. Cancer Gene Ther, *6:* 73-80, 1999.
128. Roos, A. K., Pavlenko, M., Charo, J., Egevad, L., and Pisa, P. Induction of PSA-specific CTLs and anti-tumor immunity by a genetic prostate cancer vaccine. Prostate, *62:* 217-223, 2005.

129. Jacob, J., Radkevich, O., Forni, G., Zielinski, J., Shim, D., Jones, R. F., and Wei, W. Z. Activity of DNA vaccines encoding self or heterologous Her-2/neu in Her-2 or neu transgenic mice. Cell Immunol, *240:* 96-106, 2006.
130. Conry, R. M., Curiel, D. T., Strong, T. V., Moore, S. E., Allen, K. O., Barlow, D. L., Shaw, D. R., and LoBuglio, A. F. Safety and immunogenicity of a DNA vaccine encoding carcinoembryonic antigen and hepatitis B surface antigen in colorectal carcinoma patients. Clin Cancer Res, *8:* 2782-2787, 2002.
131. Pavlenko, M., Roos, A. K., Lundqvist, A., Palmborg, A., Miller, A. M., Ozenci, V., Bergman, B., Egevad, L., Hellstrom, M., Kiessling, R., Masucci, G., Wersall, P., Nilsson, S., and Pisa, P. A phase I trial of DNA vaccination with a plasmid expressing prostate-specific antigen in patients with hormone-refractory prostate cancer. British Journal of Cancer, *91:* 688-694, 2004.
132. Steinaa, L., Rasmussen, P. B., Gautam, A., and Mouritsen, S. Breaking B-cell tolerance and CTL tolerance in three OVA-transgenic mouse strains expressing different levels of OVA. Scand J Immunol, *67:* 113-120, 2008.
133. Snyder, L. A., Goletz, T. J., Gunn, G. R., Shi, F. F., Harris, M. C., Cochlin, K., McCauley, C., McCarthy, S. G., Branigan, P. J., and Knight, D. M. A MUC1/IL-18 DNA vaccine induces anti-tumor immunity and increased survival in MUC1 transgenic mice. Vaccine, *24:* 3340-3352, 2006.
134. Garcia-Hernandez Mde, L., Gray, A., Hubby, B., Klinger, O. J., and Kast, W. M. Prostate stem cell antigen vaccination induces a long-term protective immune response against prostate cancer in the absence of autoimmunity. Cancer Res, *68:* 861-869, 2008.
135. Bergman, P. J., McKnight, J., Novosad, A., Charney, S., Farrelly, J., Craft, D., Wulderk, M., Jeffers, Y., Sadelain, M., Hohenhaus, A. E., Segal, N., Gregor, P., Engelhorn, M., Riviere, I., Houghton, A. N., and Wolchok, J. D. Long-term survival of dogs with advanced malignant melanoma after DNA vaccination with xenogeneic human tyrosinase: a phase I trial. Clin Cancer Res, *9:* 1284-1290, 2003.
136. Denis-Mize, K. S., Dupuis, M., MacKichan, M. L., Singh, M., Doe, B., O'Hagan, D., Ulmer, J. B., Donnelly, J. J., McDonald, D. M., and Ott, G. Plasmid DNA adsorbed onto cationic microparticles mediates target gene expression and antigen presentation by dendritic cells. Gene Ther, *7:* 2105-2112, 2000.
137. Clark, J. R. and March, J. B. Bacteriophage-mediated nucleic acid immunisation. FEMS Immunol Med Microbiol, *40:* 21-26, 2004.
138. Drabick, J. J., Glasspool-Malone, J., King, A., and Malone, R. W. Cutaneous transfection and immune responses to intradermal nucleic acid vaccination are significantly enhanced by in vivo electropermeabilization. Mol Ther, *3:* 249-255, 2001.
139. Quaglino, E., Iezzi, M., Mastini, C., Amici, A., Pericle, F., Di Carlo, E., Pupa, S. M., De Giovanni, C., Spadaro, M., Curcio, C., Lollini, P. L., Musiani, P., Forni, G., and Cavallo, F. Electroporated DNA vaccine clears away multifocal mammary carcinomas in her-2/neu transgenic mice. Cancer Res, *64:* 2858-2864, 2004.
140. Titomirov, A. V., Sukharev, S., and Kistanova, E. In vivo electroporation and stable transformation of skin cells of newborn mice by plasmid DNA. Biochim Biophys Acta, *1088:* 131-134, 1991.

141. Suzuki, T., Shin, B. C., Fujikura, K., Matsuzaki, T., and Takata, K. Direct gene transfer into rat liver cells by in vivo electroporation. FEBS Lett, *425:* 436-440, 1998.
142. Mir, L. M., Bureau, M. F., Gehl, J., Rangara, R., Rouy, D., Caillaud, J. M., Delaere, P., Branellec, D., Schwartz, B., and Scherman, D. High-efficiency gene transfer into skeletal muscle mediated by electric pulses. Proc Natl Acad Sci U S A, *96:* 4262-4267, 1999.
143. Bureau, M. F., Gehl, J., Deleuze, V., Mir, L. M., and Scherman, D. Importance of association between permeabilization and electrophoretic forces for intramuscular DNA electrotransfer. Biochim Biophys Acta, *1474:* 353-359, 2000.
144. Satkauskas, S., Andre, F., Bureau, M. F., Scherman, D., Miklavcic, D., and Mir, L. M. Electrophoretic component of electric pulses determines the efficacy of in vivo DNA electrotransfer. Hum Gene Ther, *16:* 1194-1201, 2005.
145. Roos, A. K., Moreno, S., Leder, C., Pavlenko, M., King, A., and Pisa, P. Enhancement of cellular immune response to a prostate cancer DNA vaccine by intradermal electroporation. Mol Ther, *13:* 320-327, 2006.
146. Vandermeulen, G., Staes, E., Vanderhaeghen, M. L., Bureau, M. F., Scherman, D., and Preat, V. Optimisation of intradermal DNA electrotransfer for immunisation. J Control Release, *124:* 81-87, 2007.
147. Kalat, M., Kupcu, Z., Schuller, S., Zalusky, D., Zehetner, M., Paster, W., and Schweighoffer, T. In vivo plasmid electroporation induces tumor antigen-specific CD8+ T-cell responses and delays tumor growth in a syngeneic mouse melanoma model. Cancer Res, *62:* 5489-5494, 2002.
148. Curcio, C., Khan, A. S., Amici, A., Spadaro, M., Quaglino, E., Cavallo, F., Forni, G., and Draghia-Akli, R. DNA immunization using constant-current electroporation affords long-term protection from autochthonous mammary carcinomas in cancer-prone transgenic mice. Cancer Gene Ther, *15:* 108-114, 2008.
149. Stevenson, F. K. and Rice, J. Optimizing cancer immunotherapy trials: back to basics. Eur J Immunol, *36:* 1070-1073, 2006.
150. van den Boorn, J. G., Le Poole, I. C., and Luiten, R. M. T-cell avidity and tuning: the flexible connection between tolerance and autoimmunity. Int Rev Immunol, *25:* 235-258, 2006.
151. Melief, C. J., Toes, R. E., Medema, J. P., van der Burg, S. H., Ossendorp, F., and Offringa, R. Strategies for immunotherapy of cancer. Adv Immunol, *75:* 235-282, 2000.
152. Zhou, G., Drake, C. G., and Levitsky, H. I. Amplification of tumor-specific regulatory T cells following therapeutic cancer vaccines. Blood, *107:* 628-636, 2006.
153. Hanahan, D. and Weinberg, R. A. The hallmarks of cancer. Cell, *100:* 57-70, 2000.
154. Smyth, M. J., Dunn, G. P., and Schreiber, R. D. Cancer immunosurveillance and immunoediting: the roles of immunity in suppressing tumor development and shaping tumor immunogenicity. Adv Immunol, *90:* 1-50, 2006.
155. Dunn, G. P., Old, L. J., and Schreiber, R. D. The immunobiology of cancer immunosurveillance and immunoediting. Immunity, *21:* 137-148, 2004.

156. Dunn, G. P., Bruce, A. T., Ikeda, H., Old, L. J., and Schreiber, R. D. Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol, *3:* 991-998, 2002.
157. Zitvogel, L., Tesniere, A., and Kroemer, G. Cancer despite immunosurveillance: immunoselection and immunosubversion. Nat Rev Immunol, *6:* 715-727, 2006.
158. Burnet, F. M. The concept of immunological surveillance. Prog Exp Tumor Res, *13:* 1-27, 1970.
159. Shankaran, V., Ikeda, H., Bruce, A. T., White, J. M., Swanson, P. E., Old, L. J., and Schreiber, R. D. IFN gamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature, *410:* 1107-1111, 2001.
160. Kaplan, D. H., Shankaran, V., Dighe, A. S., Stockert, E., Aguet, M., Old, L. J., and Schreiber, R. D. Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. Proceedings of the National Academy of Sciences of the United States of America, *95:* 7556-7561, 1998.
161. Smyth, M. J., Thia, K. Y., Street, S. E., MacGregor, D., Godfrey, D. I., and Trapani, J. A. Perforin-mediated cytotoxicity is critical for surveillance of spontaneous lymphoma. J Exp Med, *192:* 755-760, 2000.
162. Birkeland, S. A., Storm, H. H., Lamm, L. U., Barlow, L., Blohme, I., Forsberg, B., Eklund, B., Fjeldborg, O., Friedberg, M., Frodin, L., and et al. Cancer risk after renal transplantation in the Nordic countries, 1964-1986. Int J Cancer, *60:* 183-189, 1995.
163. Algarra, I., Garcia-Lora, A., Cabrera, T., Ruiz-Cabello, F., and Garrido, F. The selection of tumor variants with altered expression of classical and nonclassical MHC class I molecules: implications for tumor immune escape. Cancer Immunol Immunother, *53:* 904-910, 2004.
164. Restifo, N. P., Esquivel, F., Kawakami, Y., Yewdell, J. W., Mule, J. J., Rosenberg, S. A., and Bennink, J. R. Identification of human cancers deficient in antigen processing. J Exp Med, *177:* 265-272, 1993.
165. Delp, K., Momburg, F., Hilmes, C., Huber, C., and Seliger, B. Functional deficiencies of components of the MHC class I antigen pathway in human tumors of epithelial origin. Bone Marrow Transplant, *25 Suppl 2:* S88-95, 2000.
166. Restifo, N. P., Marincola, F. M., Kawakami, Y., Taubenberger, J., Yannelli, J. R., and Rosenberg, S. A. Loss of functional beta 2-microglobulin in metastatic melanomas from five patients receiving immunotherapy. J Natl Cancer Inst, *88:* 100-108, 1996.
167. Karre, K. NK cells, MHC class I molecules and the missing self. Scand J Immunol, *55:* 221-228, 2002.
168. Hinnis, A. R., Luckett, J. C., and Walker, R. A. Survivin is an independent predictor of short-term survival in poor prognostic breast cancer patients. Br J Cancer, *96:* 639-645, 2007.
169. Igney, F. H., Behrens, C. K., and Krammer, P. H. CD95L mediates tumor counterattack in vitro but induces neutrophil-independent tumor rejection in vivo. Int J Cancer, *113:* 78-87, 2005.
170. Medema, J. P., de Jong, J., Peltenburg, L. T., Verdegaal, E. M., Gorter, A., Bres, S. A., Franken, K. L., Hahne, M., Albar, J. P., Melief, C. J., and Offringa, R. Blockade of the granzyme B/perforin pathway through overexpression of the serine protease inhibitor PI-9/SPI-6 constitutes a

mechanism for immune escape by tumors. Proc Natl Acad Sci U S A, *98:* 11515-11520, 2001.
171. Dong, H. D., Strome, S. E., Salomao, D. R., Tamura, H., Hirano, F., Flies, D. B., Roche, P. C., Lu, J., Zhu, G. F., Tamada, K., Lennon, V. A., Celis, E., and Chen, L. P. Tumor-associated B7-H1 promotes T-cell apoptosis: A potential mechanism of immune evasion. Nature Medicine, *8:* 793-800, 2002.
172. Lin, E. Y. and Pollard, J. W. Role of infiltrated leucocytes in tumour growth and spread. Br J Cancer, *90:* 2053-2058, 2004.
173. Dvorak, H. F. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. N Engl J Med, *315:* 1650-1659, 1986.
174. Mocellin, S., Marincola, F., Rossi, C. R., Nitti, D., and Lise, M. The multifaceted relationship between IL-10 and adaptive immunity: putting together the pieces of a puzzle. Cytokine Growth Factor Rev, *15:* 61-76, 2004.
175. Teicher, B. A. Transforming growth factor-beta and the immune response to malignant disease. Clin Cancer Res, *13:* 6247-6251, 2007.
176. Thomas, D. A. and Massague, J. TGF-beta directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. Cancer Cell, *8:* 369-380, 2005.
177. de Waal Malefyt, R., Yssel, H., and de Vries, J. E. Direct effects of IL-10 on subsets of human CD4+ T cell clones and resting T cells. Specific inhibition of IL-2 production and proliferation. J Immunol, *150:* 4754-4765, 1993.
178. Moore, K. W., de Waal Malefyt, R., Coffman, R. L., and O'Garra, A. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol, *19:* 683-765, 2001.
179. Mou, H. B., Lin, M. F., Cen, H., Yu, J., and Meng, X. J. TGF-beta1 treated murine dendritic cells are maturation resistant and down-regulate Toll-like receptor 4 expression. J Zhejiang Univ Sci, *5:* 1239-1244, 2004.
180. Gabrilovich, D. Mechanisms and functional significance of tumour-induced dendritic-cell defects. Nat Rev Immunol, *4:* 941-952, 2004.
181. Chen, W., Jin, W., Hardegen, N., Lei, K. J., Li, L., Marinos, N., McGrady, G., and Wahl, S. M. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. J Exp Med, *198:* 1875-1886, 2003.
182. Seo, N., Hayakawa, S., Takigawa, M., and Tokura, Y. Interleukin-10 expressed at early tumour sites induces subsequent generation of CD4(+) T-regulatory cells and systemic collapse of antitumour immunity. Immunology, *103:* 449-457, 2001.
183. Ghiringhelli, F., Puig, P. E., Roux, S., Parcellier, A., Schmitt, E., Solary, E., Kroemer, G., Martin, F., Chauffert, B., and Zitvogel, L. Tumor cells convert immature myeloid dendritic cells into TGF-beta-secreting cells inducing CD4+CD25+ regulatory T cell proliferation. J Exp Med, *202:* 919-929, 2005.
184. Curiel, T. J., Coukos, G., Zou, L., Alvarez, X., Cheng, P., Mottram, P., Evdemon-Hogan, M., Conejo-Garcia, J. R., Zhang, L., Burow, M., Zhu, Y., Wei, S., Kryczek, I., Daniel, B., Gordon, A., Myers, L., Lackner, A., Disis, M. L., Knutson, K. L., Chen, L., and Zou, W. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med, *10:* 942-949, 2004.
185. Roncarolo, M. G., Gregori, S., Battaglia, M., Bacchetta, R., Fleischhauer, K., and Levings, M. K. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. Immunol Rev, *212:* 28-50, 2006.

186. Faria, A. M. and Weiner, H. L. Oral tolerance and TGF-beta-producing cells. Inflamm Allergy Drug Targets, *5:* 179-190, 2006.
187. Bluestone, J. A. and Abbas, A. K. Natural versus adaptive regulatory T cells. Nat Rev Immunol, *3:* 253-257, 2003.
188. Wang, H. Y., Lee, D. A., Peng, G., Guo, Z., Li, Y., Kiniwa, Y., Shevach, E. M., and Wang, R. F. Tumor-specific human CD4+ regulatory T cells and their ligands: implications for immunotherapy. Immunity, *20:* 107-118, 2004.
189. Tang, Q. and Bluestone, J. A. The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. Nat Immunol, *9:* 239-244, 2008.
190. Zou, W. Regulatory T cells, tumour immunity and immunotherapy. Nat Rev Immunol, *6:* 295-307, 2006.
191. Goede, V., Brogelli, L., Ziche, M., and Augustin, H. G. Induction of inflammatory angiogenesis by monocyte chemoattractant protein-1. Int J Cancer, *82:* 765-770, 1999.
192. Luboshits, G., Shina, S., Kaplan, O., Engelberg, S., Nass, D., Lifshitz-Mercer, B., Chaitchik, S., Keydar, I., and Ben-Baruch, A. Elevated expression of the CC chemokine regulated on activation, normal T cell expressed and secreted (RANTES) in advanced breast carcinoma. Cancer Res, *59:* 4681-4687, 1999.
193. Murdoch, C., Giannoudis, A., and Lewis, C. E. Mechanisms regulating the recruitment of macrophages into hypoxic areas of tumors and other ischemic tissues. Blood, *104:* 2224-2234, 2004.
194. Duyndam, M. C. A., Hilhorst, M. C. G. W., Schluper, H. M. M., Verheul, H. M. W., van Diest, P. J., Kraal, G., Pinedo, H. M., and Boven, E. Vascular endothelial growth factor-165 overexpression stimulates angiogenesis and induces cyst formation and macrophage infiltration in human ovarian cancer xenografts. American Journal of Pathology, *160:* 537-548, 2002.
195. Leek, R. D., Lewis, C. E., Whitehouse, R., Greenall, M., Clarke, J., and Harris, A. L. Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. Cancer Res, *56:* 4625-4629, 1996.
196. Kusmartsev, S. and Gabrilovich, D. I. STAT1 signaling regulates tumor-associated macrophage-mediated T cell deletion. J Immunol, *174:* 4880-4891, 2005.
197. Sica, A. and Bronte, V. Altered macrophage differentiation and immune dysfunction in tumor development. J Clin Invest, *117:* 1155-1166, 2007.
198. Mantovani, A. Tumor-associated macrophages in neoplastic progression: a paradigm for the in vivo function of chemokines. Lab Invest, *71:* 5-16, 1994.
199. Salvesen, H. B. and Akslen, L. A. Significance of tumour-associated macrophages, vascular endothelial growth factor and thrombospondin-1 expression for tumour angiogenesis and prognosis in endometrial carcinomas. Int J Cancer, *84:* 538-543, 1999.
200. Ono, M., Torisu, H., Fukushi, J., Nishie, A., and Kuwano, M. Biological implications of macrophage infiltration in human tumor angiogenesis. Cancer Chemother Pharmacol, *43 Suppl:* S69-71, 1999.
201. Nakayama, Y., Nagashima, N., Minagawa, N., Inoue, Y., Katsuki, T., Onitsuka, K., Sako, T., Hirata, K., Nagata, N., and Itoh, H. Relationships between tumor-associated macrophages and clinicopathological factors in patients with colorectal cancer. Anticancer Res, *22:* 4291-4296, 2002.
202. Mantovani, A., Romero, P., Palucka, A. K., and Marincola, F. M. Tumour immunity: effector response to tumour and role of the microenvironment. Lancet, *371:* 771-783, 2008.

203. Nathan, C. F. Secretory products of macrophages. J Clin Invest, *79:* 319-326, 1987.
204. MacMicking, J., Xie, Q. W., and Nathan, C. Nitric oxide and macrophage function. Annu Rev Immunol, *15:* 323-350, 1997.
205. Sica, A., Saccani, A., Bottazzi, B., Polentarutti, N., Vecchi, A., Van Damme, J., and Mantovani, A. Autocrine production of IL-10 mediates defective IL-12 production and NF-kappa B activation in tumor-associated macrophages. Journal of Immunology, *164:* 762-767, 2000.
206. Mantovani, A., Sozzani, S., Locati, M., Allavena, P., and Sica, A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol, *23:* 549-555, 2002.
207. Lee, J. R., Dalton, R. R., Messina, J. L., Sharma, M. D., Smith, D. M., Burgess, R. E., Mazzella, F., Antonia, S. J., Mellor, A. L., and Munn, D. H. Pattern of recruitment of immunoregulatory antigen-presenting cells in malignant melanoma. Laboratory Investigation, *83:* 1457-1466, 2003.
208. Kryczek, I., Zou, L., Rodriguez, P., Zhu, G., Wei, S., Mottram, P., Brumlik, M., Cheng, P., Curiel, T., Myers, L., Lackner, A., Alvarez, X., Ochoa, A., Chen, L., and Zou, W. B7-H4 expression identifies a novel suppressive macrophage population in human ovarian carcinoma. J Exp Med, *203:* 871-881, 2006.
209. Klimp, A. H., Hollema, H., Kempinga, C., van der Zee, A. G., de Vries, E. G., and Daemen, T. Expression of cyclooxygenase-2 and inducible nitric oxide synthase in human ovarian tumors and tumor-associated macrophages. Cancer Res, *61:* 7305-7309, 2001.
210. Zea, A. H., Rodriguez, P. C., Atkins, M. B., Hernandez, C., Signoretti, S., Zabaleta, J., McDermott, D., Quiceno, D., Youmans, A., O'Neill, A., Mier, J., and Ochoa, A. C. Arginase-producing myeloid suppressor cells in renal cell carcinoma patients: a mechanism of tumor evasion. Cancer Res, *65:* 3044-3048, 2005.
211. Eccles, S. A. and Alexander, P. Macrophage content of tumours in relation to metastatic spread and host immune reaction. Nature, *250:* 667-669, 1974.
212. Torisu, H., Ono, M., Kiryu, H., Furue, M., Ohmoto, Y., Nakayama, J., Nishioka, Y., Sone, S., and Kuwano, M. Macrophage infiltration correlates with tumor stage and angiogenesis in human malignant melanoma: possible involvement of TNFalpha and IL-1alpha. Int J Cancer, *85:* 182-188, 2000.
213. Locati, M., Deuschle, U., Massardi, M. L., Martinez, F. O., Sironi, M., Sozzani, S., Bartfai, T., and Mantovani, A. Analysis of the gene expression profile activated by the CC chemokine ligand 5/RANTES and by lipopolysaccharide in human monocytes. J Immunol, *168:* 3557-3562, 2002.
214. Robinson, S. C., Scott, K. A., and Balkwill, F. R. Chemokine stimulation of monocyte matrix metalloproteinase-9 requires endogenous TNF-alpha. Eur J Immunol, *32:* 404-412, 2002.
215. Pollard, J. W. Tumour-educated macrophages promote tumour progression and metastasis. Nature Reviews Cancer, *4:* 71-78, 2004.
216. Eubank, T. D., Galloway, M., Montague, C. M., Waldman, W. J., and Marsh, C. B. M-CSF induces vascular endothelial growth factor production and angiogenic activity from human monocytes. J Immunol, *171:* 2637-2643, 2003.
217. Harmey, J. H., Dimitriadis, E., Kay, E., Redmond, H. P., and Bouchier-Hayes, D. Regulation of macrophage production of vascular endothelial growth factor

(VEGF) by hypoxia and transforming growth factor beta-1. Ann Surg Oncol, *5:* 271-278, 1998.
218. Kuwabara, K., Ogawa, S., Matsumoto, M., Koga, S., Clauss, M., Pinsky, D. J., Lyn, P., Leavy, J., Witte, L., Joseph-Silverstein, J., and et al. Hypoxia-mediated induction of acidic/basic fibroblast growth factor and platelet-derived growth factor in mononuclear phagocytes stimulates growth of hypoxic endothelial cells. Proc Natl Acad Sci U S A, *92:* 4606-4610, 1995.
219. Andersen, M. H., Keikavoussi, P., Brocker, E. B., Schuler-Thurner, B., Jonassen, M., Sondergaard, I., Straten, P. T., Becker, J. C., and Kampgen, E. Induction of systemic CTL responses in melanoma patients by dendritic cell vaccination: cessation of CTL responses is associated with disease progression. Int J Cancer, *94:* 820-824, 2001.
220. Slingluff, C. L., Jr., Petroni, G. R., Yamshchikov, G. V., Barnd, D. L., Eastham, S., Galavotti, H., Patterson, J. W., Deacon, D. H., Hibbitts, S., Teates, D., Neese, P. Y., Grosh, W. W., Chianese-Bullock, K. A., Woodson, E. M., Wiernasz, C. J., Merrill, P., Gibson, J., Ross, M., and Engelhard, V. H. Clinical and immunologic results of a randomized phase II trial of vaccination using four melanoma peptides either administered in granulocyte-macrophage colony-stimulating factor in adjuvant or pulsed on dendritic cells. J Clin Oncol, *21:* 4016-4026, 2003.
221. Yarden, Y. and Sliwkowski, M. X. Untangling the ErbB signalling network. Nat Rev Mol Cell Biol, *2:* 127-137, 2001.
222. Reilly, R. T., Machiels, J. P., Emens, L. A., Ercolini, A. M., Okoye, F. I., Lei, R. Y., Weintraub, D., and Jaffee, E. M. The collaboration of both humoral and cellular HER-2/neu-targeted immune responses is required for the complete eradication of HER-2/neu-expressing tumors. Cancer Res, *61:* 880-883, 2001.
223. Vogel, C. L., Cobleigh, M. A., Tripathy, D., Gutheil, J. C., Harris, L. N., Fehrenbacher, L., Slamon, D. J., Murphy, M., Novotny, W. F., Burchmore, M., Shak, S., Stewart, S. J., and Press, M. Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. J Clin Oncol, *20:* 719-726, 2002.
224. Engelhorn, M. E., Guevara-Patino, J. A., Noffz, G., Hooper, A. T., Lou, O., Gold, J. S., Kappel, B. J., and Houghton, A. N. Autoimmunity and tumor immunity induced by immune responses to mutations in self. Nat Med, *12:* 198-206, 2006.
225. Fong, L., Brockstedt, D., Benike, C., Breen, J. K., Strang, G., Ruegg, C. L., and Engleman, E. G. Dendritic cell-based xenoantigen vaccination for prostate cancer immunotherapy. J Immunol, *167:* 7150-7156, 2001.
226. Rafii, S. Vaccination against tumor neovascularization: Promise and reality. Cancer Cell, *2:* 429-431, 2002.
227. Hernandez, J. M., Bui, M. H., Han, K. R., Mukouyama, H., Freitas, D. G., Nguyen, D., Caliliw, R., Shintaku, P. I., Paik, S. H., Tso, C. L., Figlin, R. A., and Belldegrun, A. S. Novel kidney cancer immunotherapy based on the granulocyte-macrophage colony-stimulating factor and carbonic anhydrase IX fusion gene. Clin Cancer Res, *9:* 1906-1916, 2003.
228. Luo, Y., Zhou, H., Krueger, J., Kaplan, C., Lee, S. H., Dolman, C., Markowitz, D., Wu, W., Liu, C., Reisfeld, R. A., and Xiang, R. Targeting tumor-associated macrophages as a novel strategy against breast cancer. J Clin Invest, *116:* 2132-2141, 2006.

229. Hofmeister, V., Schrama, D., and Becker, J. C. Anti-cancer therapies targeting the tumor stroma. Cancer Immunol Immunother, *57:* 1-17, 2008.
230. Hurwitz, H., Fehrenbacher, L., Novotny, W., Cartwright, T., Hainsworth, J., Heim, W., Berlin, J., Baron, A., Griffing, S., Holmgren, E., Ferrara, N., Fyfe, G., Rogers, B., Ross, R., and Kabbinavar, F. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med, *350:* 2335-2342, 2004.
231. Zhu, A. X., Blaszkowsky, L. S., Ryan, D. P., Clark, J. W., Muzikansky, A., Horgan, K., Sheehan, S., Hale, K. E., Enzinger, P. C., Bhargava, P., and Stuart, K. Phase II study of gemcitabine and oxaliplatin in combination with bevacizumab in patients with advanced hepatocellular carcinoma. J Clin Oncol, *24:* 1898-1903, 2006.
232. Shimizu, J., Yamazaki, S., and Sakaguchi, S. Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity. J Immunol, *163:* 5211-5218, 1999.
233. Van Meirvenne, S., Dullaers, M., Heirman, C., Straetman, L., Michiels, A., and Thielemans, K. In vivo depletion of CD4+CD25+ regulatory T cells enhances the antigen-specific primary and memory CTL response elicited by mature mRNA-electroporated dendritic cells. Mol Ther, *12:* 922-932, 2005.
234. Knutson, K. L., Dang, Y., Lu, H., Lukas, J., Almand, B., Gad, E., Azeke, E., and Disis, M. L. IL-2 immunotoxin therapy modulates tumor-associated regulatory T cells and leads to lasting immune-mediated rejection of breast cancers in neu-transgenic mice. J Immunol, *177:* 84-91, 2006.
235. Dannull, J., Su, Z., Rizzieri, D., Yang, B. K., Coleman, D., Yancey, D., Zhang, A., Dahm, P., Chao, N., Gilboa, E., and Vieweg, J. Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells. J Clin Invest, *115:* 3623-3633, 2005.
236. Nair, S., Boczkowski, D., Fassnacht, M., Pisetsky, D., and Gilboa, E. Vaccination against the forkhead family transcription factor Foxp3 enhances tumor immunity. Cancer Res, *67:* 371-380, 2007.
237. Ghiringhelli, F., Larmonier, N., Schmitt, E., Parcellier, A., Cathelin, D., Garrido, C., Chauffert, B., Solary, E., Bonnotte, B., and Martin, F. CD4+CD25+ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. Eur J Immunol, *34:* 336-344, 2004.
238. Berd, D. and Mastrangelo, M. J. Effect of low dose cyclophosphamide on the immune system of cancer patients: reduction of T-suppressor function without depletion of the CD8+ subset. Cancer Res, *47:* 3317-3321, 1987.
239. Nakao, S., Kuwano, T., Tsutsumi-Miyahara, C., Ueda, S., Kimura, Y. N., Hamano, S., Sonoda, K. H., Saijo, Y., Nukiwa, T., Strieter, R. M., Ishibashi, T., Kuwano, M., and Ono, M. Infiltration of COX-2-expressing macrophages is a prerequisite for IL-1 beta-induced neovascularization and tumor growth. J Clin Invest, *115:* 2979-2991, 2005.
240. Lin, E. Y., Nguyen, A. V., Russell, R. G., and Pollard, J. W. Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. J Exp Med, *193:* 727-740, 2001.
241. Bunt, S. K., Yang, L., Sinha, P., Clements, V. K., Leips, J., and Ostrand-Rosenberg, S. Reduced inflammation in the tumor microenvironment delays the accumulation of myeloid-derived suppressor cells and limits tumor progression. Cancer Res, *67:* 10019-10026, 2007.

242. Koga, M., Kai, H., Egami, K., Murohara, T., Ikeda, A., Yasuoka, S., Egashira, K., Matsuishi, T., Kai, M., Kataoka, Y., Kuwano, M., and Imaizumi, T. Mutant MCP-1 therapy inhibits tumor angiogenesis and growth of malignant melanoma in mice. Biochem Biophys Res Commun, *365:* 279-284, 2008.
243. Onodera, S., Ohshima, S., Tohyama, H., Yasuda, K., Nishihira, J., Iwakura, Y., Matsuda, I., Minami, A., and Koyama, Y. A novel DNA vaccine targeting macrophage migration inhibitory factor protects joints from inflammation and destruction in murine models of arthritis. Arthritis Rheum, *56:* 521-530, 2007.
244. Shimizu, T., Abe, R., Nakamura, H., Ohkawara, A., Suzuki, M., and Nishihira, J. High expression of macrophage migration inhibitory factor in human melanoma cells and its role in tumor cell growth and angiogenesis. Biochem Biophys Res Commun, *264:* 751-758, 1999.
245. Amin, M. A., Volpert, O. V., Woods, J. M., Kumar, P., Harlow, L. A., and Koch, A. E. Migration inhibitory factor mediates angiogenesis via mitogen-activated protein kinase and phosphatidylinositol kinase. Circ Res, *93:* 321-329, 2003.
246. Guiducci, C., Vicari, A. P., Sangaletti, S., Trinchieri, G., and Colombo, M. P. Redirecting in vivo elicited tumor infiltrating macrophages and dendritic cells towards tumor rejection. Cancer Res, *65:* 3437-3446, 2005.
247. Watkins, S. K., Egilmez, N. K., Suttles, J., and Stout, R. D. IL-12 rapidly alters the functional profile of tumor-associated and tumor-infiltrating macrophages in vitro and in vivo. J Immunol, *178:* 1357-1362, 2007.
248. Allavena, P., Signorelli, M., Chieppa, M., Erba, E., Bianchi, G., Marchesi, F., Olimpio, C. O., Bonardi, C., Garbi, A., Lissoni, A., de Braud, F., Jimeno, J., and D'Incalci, M. Anti-inflammatory properties of the novel antitumor agent yondelis (trabectedin): inhibition of macrophage differentiation and cytokine production. Cancer Res, *65:* 2964-2971, 2005.
249. Uyttenhove, C., Pilotte, L., Theate, I., Stroobant, V., Colau, D., Parmentier, N., Boon, T., and Van den Eynde, B. J. Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. Nat Med, *9:* 1269-1274, 2003.
250. Bronte, V., Serafini, P., Mazzoni, A., Segal, D. M., and Zanovello, P. L-arginine metabolism in myeloid cells controls T-lymphocyte functions. Trends Immunol, *24:* 302-306, 2003.
251. Stresing, V., Daubine, F., Benzaid, I., Monkkonen, H., and Clezardin, P. Bisphosphonates in cancer therapy. Cancer Lett, *257:* 16-35, 2007.
252. Green, J. R. Antitumor effects of bisphosphonates. Cancer, *97:* 840-847, 2003.
253. Giraudo, E., Inoue, M., and Hanahan, D. An amino-bisphosphonate targets MMP-9-expressing macrophages and angiogenesis to impair cervical carcinogenesis. J Clin Invest, *114:* 623-633, 2004.
254. Melani, C., Sangaletti, S., Barazzetta, F. M., Werb, Z., and Colombo, M. P. Amino-biphosphonate-mediated MMP-9 inhibition breaks the tumor-bone marrow axis responsible for myeloid-derived suppressor cell expansion and macrophage infiltration in tumor stroma. Cancer Res, *67:* 11438-11446, 2007.
255. Wolf, A. M., Rumpold, H., Tilg, H., Gastl, G., Gunsilius, E., and Wolf, D. The effect of zoledronic acid on the function and differentiation of myeloid cells. Haematologica, *91:* 1165-1171, 2006.
256. Yoshida, M., Korfhagen, T. R., and Whitsett, J. A. Surfactant protein D regulates NF-kappa B and matrix metalloproteinase production in alveolar

macrophages via oxidant-sensitive pathways. J Immunol, *166:* 7514-7519, 2001.
257. Bendelac, A., Bonneville, M., and Kearney, J. F. Autoreactivity by design: innate B and T lymphocytes. Nat Rev Immunol, *1:* 177-186, 2001.
258. Green, A. E., Lissina, A., Hutchinson, S. L., Hewitt, R. E., Temple, B., James, D., Boulter, J. M., Price, D. A., and Sewell, A. K. Recognition of nonpeptide antigens by human V gamma 9V delta 2 T cells requires contact with cells of human origin. Clin Exp Immunol, *136:* 472-482, 2004.
259. Kunzmann, V., Bauer, E., Feurle, J., Weissinger, F., Tony, H. P., and Wilhelm, M. Stimulation of gammadelta T cells by aminobisphosphonates and induction of antiplasma cell activity in multiple myeloma. Blood, *96:* 384-392, 2000.
260. Spada, F. M., Grant, E. P., Peters, P. J., Sugita, M., Melian, A., Leslie, D. S., Lee, H. K., van Donselaar, E., Hanson, D. A., Krensky, A. M., Majdic, O., Porcelli, S. A., Morita, C. T., and Brenner, M. B. Self-recognition of CD1 by gamma/delta T cells: implications for innate immunity. J Exp Med, *191:* 937-948, 2000.
261. Musiani, P., Allione, A., Modica, A., Lollini, P. L., Giovarelli, M., Cavallo, F., Belardelli, F., Forni, G., and Modesti, A. Role of neutrophils and lymphocytes in inhibition of a mouse mammary adenocarcinoma engineered to release IL-2, IL-4, IL-7, IL-10, IFN-alpha, IFN-gamma, and TNF-alpha. Lab Invest, *74:* 146-157, 1996.
262. Suttmann, H., Riemensberger, J., Bentien, G., Schmaltz, D., Stockle, M., Jocham, D., Bohle, A., and Brandau, S. Neutrophil granulocytes are required for effective Bacillus Calmette-Guerin immunotherapy of bladder cancer and orchestrate local immune responses. Cancer Res, *66:* 8250-8257, 2006.
263. van Egmond, M. Neutrophils in antibody-based immunotherapy of cancer. Expert Opin Biol Ther, *8:* 83-94, 2008.
264. D'Elios, M. M., Amedei, A., Cappon, A., Del Prete, G., and de Bernard, M. The neutrophil-activating protein of Helicobacter pylori (HP-NAP) as an immune modulating agent. FEMS Immunol Med Microbiol, *50:* 157-164, 2007.
265. Buhtoiarov, I. N., Sondel, P. M., Eickhoff, J. C., and Rakhmilevich, A. L. Macrophages are essential for antitumour effects against weakly immunogenic murine tumours induced by class B CpG-oligodeoxynucleotides. Immunology, *120:* 412-423, 2007.
266. Buhtoiarov, I. N., Lum, H. D., Berke, G., Sondel, P. M., and Rakhmilevich, A. L. Synergistic activation of macrophages via CD40 and TLR9 results in T cell independent antitumor effects. J Immunol, *176:* 309-318, 2006.
267. Mattes, J., Hulett, M., Xie, W., Hogan, S., Rothenberg, M. E., Foster, P., and Parish, C. Immunotherapy of cytotoxic T cell-resistant tumors by T helper 2 cells: an eotaxin and STAT6-dependent process. J Exp Med, *197:* 387-393, 2003.
268. Kay, B. K., Winter, J., and McCafferty, J. Phage Display of Peptides and Proteins: a laboratory manual. San Diego: Academic Press, Inc., 1996.
269. Rasched, I. and Oberer, E. Ff Coliphages - Structural and Functional-Relationships. Microbiological Reviews, *50:* 401-427, 1986.
270. Smith, H. W., Huggins, M. B., and Shaw, K. M. The control of experimental Escherichia coli diarrhoea in calves by means of bacteriophages. J Gen Microbiol, *133:* 1111-1126, 1987.

271. Biswas, B., Adhya, S., Washart, P., Paul, B., Trostel, A. N., Powell, B., Carlton, R., and Merril, C. R. Bacteriophage therapy rescues mice bacteremic from a clinical isolate of vancomycin-resistant Enterococcus faecium. Infect Immun, *70:* 204-210, 2002.
272. Sulakvelidze, A., Alavidze, Z., and Morris, J. G., Jr. Bacteriophage therapy. Antimicrob Agents Chemother, *45:* 649-659, 2001.
273. Smith, G. P. Filamentous Fusion Phage - Novel Expression Vectors That Display Cloned Antigens on the Virion Surface. Science, *228:* 1315-1317, 1985.
274. Smith, G. P. Surface presentation of protein epitopes using bacteriophage expression systems. Curr Opin Biotechnol, *2:* 668-673, 1991.
275. Barbas, C. F., 3rd, Kang, A. S., Lerner, R. A., and Benkovic, S. J. Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. Proc Natl Acad Sci U S A, *88:* 7978-7982, 1991.
276. Smith, G. P. and Scott, J. K. Libraries of Peptides and Proteins Displayed on Filamentous Phage. Methods in Enzymology, *217:* 228-257, 1993.
277. Kupsch, J. M., Tidman, N. H., Kang, N. V., Truman, H., Hamilton, S., Patel, N., Newton Bishop, J. A., Leigh, I. M., and Crowe, J. S. Isolation of human tumor-specific antibodies by selection of an antibody phage library on melanoma cells. Clin Cancer Res, *5:* 925-931, 1999.
278. Popkov, M., Rader, C., and Barbas, C. F., 3rd Isolation of human prostate cancer cell reactive antibodies using phage display technology. J Immunol Methods, *291:* 137-151, 2004.
279. Essler, M. and Ruoslahti, E. Molecular specialization of breast vasculature: a breast-homing phage-displayed peptide binds to aminopeptidase P in breast vasculature. Proc Natl Acad Sci U S A, *99:* 2252-2257, 2002.
280. Mutuberria, R., Satijn, S., Huijbers, A., Van Der Linden, E., Lichtenbeld, H., Chames, P., Arends, J. W., and Hoogenboom, H. R. Isolation of human antibodies to tumor-associated endothelial cell markers by in vitro human endothelial cell selection with phage display libraries. J Immunol Methods, *287:* 31-47, 2004.
281. Kolonin, M., Pasqualini, R., and Arap, W. Molecular addresses in blood vessels as targets for therapy. Curr Opin Chem Biol, *5:* 308-313, 2001.
282. Romanov, V. I., Durand, D. B., and Petrenko, V. A. Phage display selection of peptides that affect prostate carcinoma cells attachment and invasion. Prostate, *47:* 239-251, 2001.
283. Zurita, A. J., Troncoso, P., Cardo-Vila, M., Logothetis, C. J., Pasqualini, R., and Arap, W. Combinatorial screenings in patients: the interleukin-11 receptor alpha as a candidate target in the progression of human prostate cancer. Cancer Res, *64:* 435-439, 2004.
284. Arap, W., Pasqualini, R., and Ruoslahti, E. Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. Science, *279:* 377-380, 1998.
285. Arap, W., Haedicke, W., Bernasconi, M., Kain, R., Rajotte, D., Krajewski, S., Ellerby, H. M., Bredesen, D. E., Pasqualini, R., and Ruoslahti, E. Targeting the prostate for destruction through a vascular address. Proc Natl Acad Sci U S A, *99:* 1527-1531, 2002.
286. Ellerby, H. M., Arap, W., Ellerby, L. M., Kain, R., Andrusiak, R., Rio, G. D., Krajewski, S., Lombardo, C. R., Rao, R., Ruoslahti, E., Bredesen, D. E., and Pasqualini, R. Anti-cancer activity of targeted pro-apoptotic peptides. Nat Med, *5:* 1032-1038, 1999.

287. Curnis, F., Sacchi, A., Borgna, L., Magni, F., Gasparri, A., and Corti, A. Enhancement of tumor necrosis factor alpha antitumor immunotherapeutic properties by targeted delivery to aminopeptidase N (CD13). Nat Biotechnol, *18:* 1185-1190, 2000.
288. Tordsson, J. M., Ohlsson, L. G., Abrahmsen, L. B., Karlstrom, P. J., Lando, P. A., and Brodin, T. N. Phage-selected primate antibodies fused to superantigens for immunotherapy of malignant melanoma. Cancer Immunol Immunother, *48:* 691-702, 2000.
289. Bloch, H. Experimental investigation of the relationship between bacteriophage and malignant tumors. Archiv für die gesamte virusforschung 481-496, 1940.
290. Dabrowska, K., Opolski, A., Wietrzyk, J., Switala-Jelen, K., Godlewska, J., Boratynski, J., Syper, D., Weber-Dabrowska, B., and Gorski, A. Anticancer activity of bacteriophage T4 and its mutant HAP1 in mouse experimental tumour models. Anticancer Res, *24:* 3991-3995, 2004.
291. Dabrowska, K., Opolski, A., Wietrzyk, J., Switala-Jelen, K., Boratynski, J., Nasulewicz, A., Lipinska, L., Chybicka, A., Kujawa, M., Zabel, M., Dolinska-Krajewska, B., Piasecki, E., Weber-Dabrowska, B., Rybka, J., Salwa, J., Wojdat, E., Nowaczyk, M., and Gorski, A. Antitumor activity of bacteriophages in murine experimental cancer models caused possibly by inhibition of beta3 integrin signaling pathway. Acta Virol, *48:* 241-248, 2004.
292. Hosotani, R., Kawaguchi, M., Masui, T., Koshiba, T., Ida, J., Fujimoto, K., Wada, M., Doi, R., and Imamura, M. Expression of integrin alphaVbeta3 in pancreatic carcinoma: relation to MMP-2 activation and lymph node metastasis. Pancreas, *25:* e30-35, 2002.
293. Cuesta, A. M., Suarez, E., Larsen, M., Jensen, K. B., Sanz, L., Compte, M., Kristensen, P., and Alvarez-Vallina, L. Enhancement of DNA vaccine potency through linkage of antigen to filamentous bacteriophage coat protein III domain I. Immunology, *117:* 502-506, 2006.
294. Fogelman, I., Davey, V., Ochs, H. D., Elashoff, M., Feinberg, M. B., Mican, J., Siegel, J. P., Sneller, M., and Lane, H. C. Evaluation of CD4+ T cell function In vivo in HIV-infected patients as measured by bacteriophage phiX174 immunization. J Infect Dis, *182:* 435-441, 2000.
295. Willis, A. E., Perham, R. N., and Wraith, D. Immunological properties of foreign peptides in multiple display on a filamentous bacteriophage. Gene, *128:* 79-83, 1993.
296. Mori, K., Kubo, T., Kibayashi, Y., Ohkuma, T., and Kaji, A. Anti-vaccinia virus effect of M13 bacteriophage DNA. Antiviral Res, *31:* 79-86, 1996.
297. Zimecki, M., Weber-Dabrowska, B., Lusiak-Szelachowska, M., Mulczyk, M., Boratynski, J., Pozniak, G., Syper, D., and Gorski, A. Bacteriophages provide regulatory signals in mitogen-induced murine splenocyte proliferation. Cell Mol Biol Lett, *8:* 699-711, 2003.
298. Pajtasz-Piasecka, E., Rossowska, J., Dus, D., Weber-Dabrowska, B., Zablocka, A., and Gorski, A. Bacteriophages support anti-tumor response initiated by DC-based vaccine against murine transplantable colon carcinoma. Immunol Lett, *116:* 24-32, 2008.
299. Gaubin, M., Fanutti, C., Mishal, Z., Durrbach, A., De Berardinis, P., Sartorius, R., Del Pozzo, G., Guardiola, J., Perham, R. N., and Piatier-Tonneau, D. Processing of filamentous bacteriophage virions in antigen-presenting cells

targets both HLA class I and class II peptide loading compartments. DNA Cell Biol, *22:* 11-18, 2003.
300. Yip, Y. L., Hawkins, N. J., Smith, G., and Ward, R. L. Biodistribution of filamentous phage-Fab in nude mice. J Immunol Methods, *225:* 171-178, 1999.
301. Barrow, P., Lovell, M., and Berchieri, A., Jr. Use of lytic bacteriophage for control of experimental Escherichia coli septicemia and meningitis in chickens and calves. Clin Diagn Lab Immunol, *5:* 294-298, 1998.
302. Carrera, M. R., Kaufmann, G. F., Mee, J. M., Meijler, M. M., Koob, G. F., and Janda, K. D. Treating cocaine addiction with viruses. Proc Natl Acad Sci U S A, *101:* 10416-10421, 2004.
303. Merril, C. R., Biswas, B., Carlton, R., Jensen, N. C., Creed, G. J., Zullo, S., and Adhya, S. Long-circulating bacteriophage as antibacterial agents. Proc Natl Acad Sci U S A, *93:* 3188-3192, 1996.
304. Delmastro, P., Meola, A., Monaci, P., Cortese, R., and Galfre, G. Immunogenicity of filamentous phage displaying peptide mimotopes after oral administration. Vaccine, *15:* 1276-1285, 1997.
305. Krag, D. N., Fuller, S. P., Oligino, L., Pero, S. C., Weaver, D. L., Soden, A. L., Hebert, C., Mills, S., Liu, C., and Peterson, D. Phage-displayed random peptide libraries in mice: toxicity after serial panning. Cancer Chemother Pharmacol, *50:* 325-332, 2002.
306. Lundin, K., Samuelsson, A., Jansson, M., Hinkula, J., Wahren, B., Wigzell, H., and Persson, M. A. Peptides isolated from random peptide libraries on phage elicit a neutralizing anti-HIV-1 response: analysis of immunological mimicry. Immunology, *89:* 579-586, 1996.
307. Manoutcharian, K., Diaz-Orea, A., Gevorkian, G., Fragoso, G., Acero, G., Gonzalez, E., De Aluja, A., Villalobos, N., Gomez-Conde, E., and Sciutto, E. Recombinant bacteriophage-based multiepitope vaccine against Taenia solium pig cysticercosis. Vet Immunol Immunopathol, *99:* 11-24, 2004.
308. March, J. B., Clark, J. R., and Jepson, C. D. Genetic immunisation against hepatitis B using whole bacteriophage lambda particles. Vaccine, *22:* 1666-1671, 2004.
309. Trepel, M., Arap, W., and Pasqualini, R. Modulation of the immune response by systemic targeting of antigens to lymph nodes. Cancer Res, *61:* 8110-8112, 2001.
310. Curiel, T. J., Morris, C., Brumlik, M., Landry, S. J., Finstad, K., Nelson, A., Joshi, V., Hawkins, C., Alarez, X., Lackner, A., and Mohamadzadeh, M. Peptides identified through phage display direct immunogenic antigen to dendritic cells. J Immunol, *172:* 7425-7431, 2004.
311. Wu, Y., Wan, Y., Bian, J., Zhao, J., Jia, Z., Zhou, L., Zhou, W., and Tan, Y. Phage display particles expressing tumor-specific antigens induce preventive and therapeutic anti-tumor immunity in murine p815 model. Int J Cancer, *98:* 748-753, 2002.
312. Coelho, M., Gauthier, P., Pugniere, M., Roquet, F., Pelegrin, A., and Navarro-Teulon, I. Isolation and characterisation of a human anti-idiotypic scFv used as a surrogate tumour antigen to elicit an anti-HER-2/neu humoral response in mice. Br J Cancer, *90:* 2032-2041, 2004.
313. Fang, J., Wang, G., Yang, Q., Song, J., Wang, Y., and Wang, L. The potential of phage display virions expressing malignant tumor specific antigen MAGE-A1 epitope in murine model. Vaccine, *23:* 4860-4866, 2005.

314. Adachi, O., Kawai, T., Takeda, K., Matsumoto, M., Tsutsui, H., Sakagami, M., Nakanishi, K., and Akira, S. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. Immunity, *9:* 143-150, 1998.
315. Wagner, C., Iking-Konert, C., Denefleh, B., Stegmaier, S., Hug, F., and Hansch, G. M. Granzyme B and perforin: constitutive expression in human polymorphonuclear neutrophils. Blood, *103:* 1099-1104, 2004.
316. Hochegger, K., Eller, P., Huber, J. M., Bernhard, D., Mayer, G., Zlabinger, G. J., and Rosenkranz, A. R. Expression of granzyme A in human polymorphonuclear neutrophils. Immunology, *121:* 166-173, 2007.
317. Grossman, W. J. and Ley, T. J. Granzymes A and B are not expressed in human neutrophils. Blood, *104:* 906-907; author reply 907-908, 2004.
318. Jemal, A., Siegel, R., Ward, E., Murray, T., Xu, J. Q., Smigal, C., and Thun, M. J. Cancer statistics, 2006. Ca-a Cancer Journal for Clinicians, *56:* 106-130, 2006.
319. Balk, S. P., Ko, Y. J., and Bubley, G. J. Biology of prostate-specific antigen. J Clin Oncol, *21:* 383-391, 2003.
320. Rajasekaran, A. K., Anilkumar, G., and Christiansen, J. J. Is prostate-specific membrane antigen a multifunctional protein? Am J Physiol Cell Physiol, *288:* C975-981, 2005.
321. Peshwa, M. V., Shi, J. D., Ruegg, C., Laus, R., and van Schooten, W. C. Induction of prostate tumor-specific CD8+ cytotoxic T-lymphocytes in vitro using antigen-presenting cells pulsed with prostatic acid phosphatase peptide. Prostate, *36:* 129-138, 1998.
322. Kohlgraf, K. G., Gawron, A. J., Higashi, M., VanLith, M. L., Shen, X., Caffrey, T. C., Anderson, J. M., and Hollingsworth, M. A. Tumor-specific immunity in MUC1.Tg mice induced by immunization with peptide vaccines from the cytoplasmic tail of CD227 (MUC1). Cancer Immunol Immunother, *53:* 1068-1084, 2004.
323. Degl'Innocenti, E., Grioni, M., Boni, A., Camporeale, A., Bertilaccio, M. T. S., Freschi, M., Monno, A., Arcelloni, C., Greenberg, N. M., and Bellone, M. Peripheral T cell tolerance occurs early during spontaneous prostate cancer development and can be rescued by dendritic cell immunization. European Journal of Immunology, *35:* 66-75, 2005.
324. McNeel, D. G. Prostate cancer immunotherapy. Curr Opin Urol, *17:* 175-181, 2007.
325. Murphy, G. P., Tjoa, B. A., Simmons, S. J., Rogers, M. K., Kenny, G. M., and Jarisch, J. Higher-dose and less frequent dendritic cell infusions with PSMA peptides in hormone-refractory metastatic prostate cancer patients. Prostate, *43:* 59-62, 2000.
326. Michael, A., Ball, G., Quatan, N., Wushishi, F., Russell, N., Whelan, J., Chakraborty, P., Leader, D., Whelan, M., and Pandha, H. Delayed disease progression after allogeneic cell vaccination in hormone-resistant prostate cancer and correlation with immunologic variables. Clin Cancer Res, *11:* 4469-4478, 2005.
327. Su, Z., Dannull, J., Yang, B. K., Dahm, P., Coleman, D., Yancey, D., Sichi, S., Niedzwiecki, D., Boczkowski, D., Gilboa, E., and Vieweg, J. Telomerase mRNA-transfected dendritic cells stimulate antigen-specific CD8+ and CD4+ T cell responses in patients with metastatic prostate cancer. J Immunol, *174:* 3798-3807, 2005.

328. Miller, A. M. and Pisa, P. Tumor escape mechanisms in prostate cancer. Cancer Immunol Immunother 1-7, 2005.
329. Watt, K. W. K., Lee, P. J., Mtimkulu, T., Chan, W. P., and Loor, R. Human Prostate-Specific Antigen - Structural and Functional Similarity with Serine Proteases. Proceedings of the National Academy of Sciences of the United States of America, *83:* 3166-3170, 1986.
330. Lilja, H., Oldbring, J., Rannevik, G., and Laurell, C. B. Seminal vesicle-secreted proteins and their reactions during gelation and liquefaction of human semen. J Clin Invest, *80:* 281-285, 1987.
331. Young, C. Y., Andrews, P. E., Montgomery, B. T., and Tindall, D. J. Tissue-specific and hormonal regulation of human prostate-specific glandular kallikrein. Biochemistry, *31:* 818-824, 1992.
332. McNeel, D. G., Nguyen, L. D., Ellis, W. J., Higano, C. S., Lange, P. H., and Disis, M. L. Naturally occurring prostate cancer antigen-specific T cell responses of a Th1 phenotype can be detected in patients with prostate cancer. Prostate, *47:* 222-229, 2001.
333. McNeel, D. G., Nguyen, L. D., Storer, B. E., Vessella, R., Lange, P. H., and Disis, M. L. Antibody immunity to prostate cancer associated antigens can be detected in the serum of patients with prostate cancer. Journal of Urology, *164:* 1825-1829, 2000.
334. Arlen, P. M., Gulley, J. L., Parker, C., Skarupa, L., Pazdur, M., Panicali, D., Beetham, P., Tsang, K. Y., Grosenbach, D. W., Feldman, J., Steinberg, S. M., Jones, E., Chen, C., Marte, J., Schlom, J., and Dahut, W. A randomized phase II study of concurrent docetaxel plus vaccine versus vaccine alone in metastatic androgen-independent prostate cancer. Clin Cancer Res, *12:* 1260-1269, 2006.
335. Barrou, B., Benoit, G., Ouldkaci, M., Cussenot, O., Salcedo, M., Agrawal, S., Massicard, S., Bercovici, N., Ericson, M. L., and Thiounn, N. Vaccination of prostatectomized prostate cancer patients in biochemical relapse, with autologous dendritic cells pulsed with recombinant human PSA. Cancer Immunol Immunother, *53:* 453-460, 2004.
336. Roelofs, A. J., Thompson, K., Gordon, S., and Rogers, M. J. Molecular mechanisms of action of bisphosphonates: current status. Clin Cancer Res, *12:* 6222s-6230s, 2006.
337. Wang, Z., Troilo, P. J., Wang, X., Griffiths, T. G., Pacchione, S. J., Barnum, A. B., Harper, L. B., Pauley, C. J., Niu, Z., Denisova, L., Follmer, T. T., Rizzuto, G., Ciliberto, G., Fattori, E., Monica, N. L., Manam, S., and Ledwith, B. J. Detection of integration of plasmid DNA into host genomic DNA following intramuscular injection and electroporation. Gene Ther, *11:* 711-721, 2004.
338. Halperin, S. A., McGrath, P., Smith, B., and Houston, T. Lidocaine-prilocaine patch decreases the pain associated with the subcutaneous administration of measles-mumps-rubella vaccine but does not adversely affect the antibody response. J Pediatr, *136:* 789-794, 2000.
339. Halperin, B. A., Halperin, S. A., McGrath, P., Smith, B., and Houston, T. Use of lidocaine-prilocaine patch to decrease intramuscular injection pain does not adversely affect the antibody response to diphtheria-tetanus-acellular pertussis-inactivated poliovirus-Haemophilus influenzae type b conjugate and hepatitis B vaccines in infants from birth to six months of age. Pediatr Infect Dis J, *21:* 399-405, 2002.