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Immunotherapy of Renal Cell Carcinoma – From Antigen Identification to Patient Treatment

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1. Introduction

Renal cell carcinoma (RCC) ist the 3rd most common urologic cancer leading to an estimated 271 000 new cancer cases worldwide each year (Ferlay *et al.* 2010). RCC is generally associated with poor prognosis because of late diagnosis and bad responsiveness to classical radio- and chemotherapy. For a long period of time immunotherapy with IL-2 or Interferon alpha (IFNα) have been the only therapeutic options to treat advanced stage RCCs. In recent years targeted therapy with tyrosine kinase inhibitors (TKIs; Sunitinib, Sorafenib) or mTor inhibitors (Everolimus, Temsirolimus) has replaced cytokine based immunotherapy as a first line treatment in the management of metastatic RCCs (mRCC). IFNα is nevertheless still playing an important role in the treatment of mRCC not as a monotherapy, but rather in combination with the antibody Bevacizumab which is directed against proangiogenic vascular endothelial growth factor (VEGF).

Despite these recent improvements resistance to this new class of drugs frequently occurs and curative treatment of RCC is still only possible by surgical resection of the tumor mass at an early non-metastatic stage (Rini & Atkins 2009). A lot of effort has therefore been put into the development of a targeted immunotherapeutic approach that aims at the *in vivo* induction or reinforcement of an anti tumor immune response.

2. Cancer immunotherapy in the course of history

The underlying idea that the immune system is able to recognise and kill transformed cells is actually not new (Parish 2003). As early as the 19th century William Coley and others noticed that cancer patients suffering from a bacterial infection sometimes experienced tumor regression. William Coley was also the first to translate this observation into clinical practice by treating cancer patients with a bacterial preparation of Streptococcus pyogenes known as Coley's toxin (Coley 1893). In 1909 Paul Ehrlich evaluated the role of the immune system in tumor control and suggested that without the immune system cancers would occur in much higher frequency (Ehrlich 1909). Unfortunately it took more than half a century before Ehrlich's idea regained attention. First experimental evidence during the 1950s could show that syngenic animals could be immunized against transplantable tumors. In the early 1960s Lewis Thomas argued that long-lived organisms must have developed mechanisms that resemble homograft rejection in order to counter neoplastic diseases

(Lawrence 1959). Based on Thomas' views Frank M. Burnet formulated in 1967 his revolutionary „Immunosurveillance Theory" in which he stated that immune cells were constantly surveying host tissues for the presence of transformed cells that could be recognized by neo-antigens acquired during the transformation process (Burnet 1967). However Burnet's idea remained controversial simply because many people could not believe that the immune system could properly differentiate between healthy tissue and transformed cancer cells. Experiments with athymic nude mice also argued against Burnets idea (Stutman 1979). Despite their supposed lack of mature T cells (which was later shown to be not completely true (Maleckar & Sherman 1987)) these mice had no higher incidence in tumor development as originally predicted by Ehrlich. Since the late 80s, more and more evidence accumulated that revived the immunosurveillance theory. The discovery of tumor associated antigens (TAA) in mice and humans as well as the observation that truly immunodeficient knockout mice (*e.g.* RAG2$^{-/-}$, STAT1$^{-/-}$) really showed a higher incidence in cancer development finally proved Burnets idea.

3. Immunotherapeutical studies in RCC

RCC, along with melanoma, are considered to be the most immunogenic tumor in humans. This is based on the occurence of spontaneous regressions even in metastatic disease (Lokich 1997), the high amount of lymphocytic infiltrates found in tumor tissue (Van den Hove *et al.* 1997) and the comparably good response to nonspecific immunotherapy with cytokines like IL-2 or IFNα, at least in some part of the patients (10-20%) (Negrier *et al.* 1998). First clinical trials using a specific immunotherapy for RCC were therefore conducted during the mid - 90s already. Table 1 provides a comprehensive summary of published results regarding anti-tumor vaccination approaches from that time until today. Many different strategies involving lysate from autologous tumor tissue, allogenic tumor cell lines, whole tumor cell RNA as well as defined antigens (*e.g.* peptides derived from TAAs CAIX or MUC1) have been used. In most cases these antigens were applied in combination with an adjuvant (*e.g.* GM-CSF, BCG or incomplete Freunds adjuvant) or after loading onto dendritic cells (DCs) usually from the same patient. Dendritic cells are professional antigen presenting cells that have the capacity to take up tumor derived products spontaneously (in some trials also inforced by electrofusion with tumor cells) and to present antigen derived peptides *via* HLA class I and II molecules to cytotoxic- and also T helper cells. In most trials dendritic cells were generated from autologous peripheral blood derived monocytes or CD34^{+} bone marrow cells that can both develop *ex vivo* into immature dendritic cells (iDCs). More recent studies almost uniformly apply a cytokine maturation step to generate mature dendritic cells (mDCs) as iDCs have been shown to posess tolerogenic rather than immune stimulatory functionality (Figdor *et al.* 2004). Keyhole limpet hemocyanin has been also applied in many trials as an immunostimulant because it was previously shown to improve dendritic cell based vaccinations by inducing a potent CD4^{+} T helper cell response at least in mouse models (Shimizu *et al.* 2001). Other approaches to enhance the immunogenicity of the vaccine have been tried by virally transducing autologous tumor cells and allogenic cell lines with cytokines (GM-CSF, IL2) or T-cell costimulatory molecule CD80 (B7.1).

Vaccination with all these different approaches has proven to be well tolerated and side effects have been rare and were usually limited to allergic reactions at the site of injection as well as induration or erythema. In trials in which additional cytokine treatment (IL-2, IFNα) was employed, further side effects like fatigue, fever, vomiting and hypotension could be observed.

In some cases systemic application of high dose cytokines can however also lead to serious and life-threatening side effects. These symptoms have been known for a long time to also occur under cytokine monotherapy and are therefore not directly associated with the vaccine.

Inactivated tumor cells and gene modified tumor vaccines:					
Author	**Setting**	**Antigen**	**Adjuvant**	**N***	**Results**
(Galligioni *et al.* 1996)	Adjuvant after nephrectomy	Autol. Irradiated tumor cells	BCG	60 (60)	5-year DFS 63% vs. control 72%; 5-year OS 69% vs. control 78%
(Simons & Mikhak 1998)	mRCC	Autol. Irradiated tumor cells transduced with GM-CSF	None	16	1 PR
(Schwaab *et al.* 2000)	metastatic RCC	Autol. Irradiated tumor cells	BCG IFNg+ IFNa	14	3 MR, 5 SD, 1 PD
Dillman(Dillman *et al.* 2004)	8 primary RCC, 17 mRCC	Autol. Irradiated tumor cells	BCG, GM-CSF, IFNa, IFNg, IL2, CP	25	median PFS 2.4 months, median OS 10,2 months
(Jocham *et al.* 2004)	Adjuvant after nephrectomy	Autol. tumor lysate vaccine (Reniale)	None	177 (202)	5 year PFS 77.4% vs control 67.8%
(Dudek *et al.* 2008)	stage IV RCC	Autol. large multivalent immunogen vaccine	none, CP, IL-2 + CP	31	median PFS 12.2, 1 PR, 12 SD
(May *et al.* 2009)	Adjuvant after nephrectomy	Autol. tumor lysate vaccine (Reniale)	None	495 (495)	5-year OS 80.6% vs. control 79,2%, 10-year OS 79,2% vs. control 62,1%
(Antonia *et al.* 2002)	mRCC	Autol. Irradiated tumor cells transduced with B7-1	IL-2	15	2 PR, 2SD
(Tani *et al.* 2004)	stage IV RCC	Autol. Irradiated tumor cells transduced with GM-CSF	none, low dose IL-2	4	1 SD, 1MR
(Pizza *et al.* 2004)	mRCC	Autol. fixed tumor cells + allog. ACHN cell line transduced with IL-2	None	30 (131)	1 CR, 4 PR, 9 SD
(Moiseyenko *et al.* 2005)	mRCC after cytoreductive surgery	Autol. Irradiated tumor cells transfected with tag7/PGRP-S	None	4	1 SD
(Fishman *et al.* 2008)	stage IV RCC	Autol. Irradiated tumor cells transduced with B7-1	IL-2	39	1 CR, 24 SD
(Buchner *et al.* 2010)	mRCC	Allog.RCC-26 tumor cell line transduced with CD80 and IL 2	None	15	median PFS 5.3 months, median OS 15.6 months
Peptide based vaccines					
Authors	**Setting**	**Antigen**	**Adjuvant**	**N***	**Results**
(Uemura *et al.* 2006)	mRCC, HLA-A*24 pos.	CA9 derived peptides (p219-227, p288-296, p323-331)	IFA	23	3 PR, 6SD, median OS 21 months
(Iiyama *et al.* 2007)	mRCC, HLA-A*24 pos.	WT1 anchor modified peptide	IFA	3	2 SD
(Suekane *et al.* 2007)	mRCC, HLA-A*24 pos or HLA-A*2 pos.	Personalized cocktail of 4 peptides based on presence of Anti Peptide IgG or CTLs	IFA	10	6 SD
(Wood *et al.* 2008)	Adjuvant after nephrectomy	Autol. tumor derived heat-shock protein-peptide complex (Vitespen)	None	367 (361)	No difference in RFS
(Jonasch *et al.* 2008)	mRCC	Autol. tumor derived heat-shock protein-peptide complex (Vitespen)	None, IL2	60	2 CR, 2PR, 7SD
(Reinhardt *et al.* 2010)	mRCC, HLA-A*02 pos.	IMA901, 9 HLA-A*02 + 1 panDR restricted peptides	GMCSF, single dose CP before vaccination	64	OS after 18 months 63% and 80% with CP pretreament

Dendritic cell based vaccines					
Authors	**Setting**	**DC + Antigen**	**Adjuvant/ Additional Treatment**	**N***	**Results**
(Oosterwijk-Wakka *et al.* 2002)	mRCC	Autol. iDCs loaded with autol. tumor cell lysate	IL-2;/ DC pulsed with KLH,none	12	8 SD
(Marten *et al.* 2002)	mRCC	Autol. mDCs loaded with autol. tumor cell lysate	none;/ DC pulsed with KLH, none	15	1 PR, 7 SD
(Holtl *et al.* 2002)	mRCC	Autol. mDCs loaded with autol. tumor cell lysate or allog. A498 cell line lysate	none / DC pulsed with KLH	35	2 CR, 1 PR, 7 SD
(Azuma *et al.* 2002)	mRCC	Autol. iDCs loaded with autol. tumor cell lysate	none / DC pulsed with KLH	3	1 SD
(Marten *et al.* 2003)	mRCC	Allog. mDCs fused with autol. tumor cells	None	12	4 SD
(Marten *et al.* 2006)	mRCC, HLA matched	Autol. mDCs loaded with a telomerase derived peptide	none; DC pulsed with KLH	10	1 MR, 1 SD
(Gitlitz *et al.* 2003)	mRCC	Autol. iDCs loaded with autol. tumor cell lysate	None	12	1 PR, 3 SD
(Barbuto *et al.* 2004)	mRCC	Allog. mDCs fused with autol. tumor derived cells	None	19	3 OR, 14 SD
(Avigan *et al.* 2007)	stage IV RCC	Allog. mDCs fused with autol. tumor derived cells	None	20	2 PR, 8 SD
(Avigan *et al.* 2004)	mRCC	Allog. iDCs fused with autol. tumor derived cells	DC pulsed with KLH	13	5 SD
(Pandha *et al.* 2004)	mRCC	Autol. iDCs loaded with allog. lysate of tumor cell line JM-RCC	KLH	5	2 SD
(Arroyo *et al.* 2004)	mRCC	Autol. mDCs loaded with autol. tumor cell lysate	DC pulsed with KLH	5	3 SD
(Dannull *et al.* 2005)	mRCC	Autol. mDCs transfected with whole tumor RNA	Pretreatment with ONTAK (Treg depletion)	11	not evaluated
(Holtl *et al.* 2005)	mRCC	Allog. mDCs laded with autol. tumor cell lysate	none/ Cyclophosphamide; DC pulsed with KLH	20	2 MR, 3 SD
(Wierecky *et al.* 2006)	mRCC HLA-A*02	Autol. mDCs loaded with two HLA-A*02 MUC1 peptides	pan DR-peptide PADRE, IL-2 (post vaccination period)	20	1 CR, 2 PR, 2 MR, 5 SD
(Bleumer *et al.* 2007)	metastatic ccRCC HLA-A*02	Autol. mDCs loaded with an HLA-A*02 and HLA-DR restricted CA9 derived peptide	DC pulsed with KLH	6	all PD
(Wei *et al.* 2007)	mRCC	Autol. mDCs fused with autol. tumor derived cells (Dendritomas)	IL-2	10	1 PR, 3 SD
(Matsumoto *et al.* 2007)	mRCC	Autol. mDCs loaded with autol. Primary tumor derived cell lysate	DC pulsed with KLH	3	1 SD
(Kim *et al.* 2007)	mRCC	Autol. mDCs loaded with autol. Primary tumor derived cell lysate	DC pulsed with KLH	9	1 PR, 5 SD
(Berntsen *et al.* 2008)	mRCC, HLA-A*02 pos or neg	Autol. mDCs loaded with allog. cell line lysate (A*02 neg) or a mix of 9 different hTERT and 11 Survivin derived peptides (A*02 pos)	IL-2, pan DR-peptide PADRE, DC pulsed with KLH (only A*02 neg)	27	13 SD
(Tatsugami *et al.* 2008)	mRCC	Autol. mDCs loaded with autol. Primary tumor derived cell lysate	IFNa	7	5 SD
(Schwaab *et al.* 2009)	mRCC	Autol. mDCs loaded with autol. Primary tumor derived cell lysate	IL-2 and IFNa	18	3 CR, 6 PR, 6 SD

*Number of patients treated versus number of patients in control group in brackets. mRCC: metastatic renal cell carcinoma, ccRCC: clear cell renal cell carcinoma, autol.: autologous, allog.: allogenic, mDC: mature dendritic cells, iDC: immature dendritic cells, BCG: Bacille Calmette Guerin, IFA: Incomplete Freunds adjuvant CP: Cyclophosphamide, CR: complete response, PR: partial response, MR: mixed response, OR: objective response, SD: stable disease, PD: progressive disease, OS.: overall survival RFS.: regression free survival, PFS: progression free survival, DFS: disease free survival.

Table 1. Immunotherapeutical studies in RCC

Apart from the tumor vaccination studies mentioned above other approaches involving the transfer of leukocytes such as adoptive T-cell transfer and even stem cell transplantation have been applied in some cases. Allogenic stem cell transplantation has been used succesfully for several decades in hematological cancers. Lymphocytes, in particular T-cells of HLA identical donors, are thought to exert a graft-vs-leukaemia effect mainly by recognition of minor histocompatibility antigens on recipient tissues (Bleakley & Riddell 2004) but probably also tumor cell specific antigens (Tykodi *et al.* 2004). The curative or graft-vs-leukaemia effect (GvL) therefore often directly correlates with the appearance of graft vs host disease (GvHD). The use of stem cell transplantation in solid tumors has been shown to provide a similiar graft-vs-tumor (GvT) effect in renal cell carcinoma, but also in ovarian (Bay *et al.* 2002) and breast cancer patients (Bishop *et al.* 2004). In the context of solid tumors non-myeloablative regimens have been preferred to avoid the substantial side effects of myeloablation with high dose chemotherapy and irradiation. Engraftment thus does not lead to complete substitution of the host's immune system but rather to a chimeric state incorperationg both the recipient's and the donor's hematological system. First studies of non-myeloablative allogenic stem cell transplantation (NST) in 17 mRCC patients showed quite promising results (Childs *et al.* 2000) with partial regression in over 50% including three patients with prolonged complete response. Further studies have been conducted (for a comprehensive overview see (Demirer *et al.* 2008)) with small patient numbers and varying response rates ranging from 0% (Rini *et al.* 2006) to more than 50% (Bregni *et al.* 2002) . However, NST is also facing several other difficulties. Besides the need for an HLA matched donor, severe and sometimes fatal complications from transplantation and subsequent GvHD can occur. Furthermore the time from treatment to response can take several months and in order to avoid graft rejection, patients have to be kept in an immunosuppressive state which allows for and might even accelerate rapid disease progression.

Adoptive T cell transfer in RCC has played a rather minor role compared to trials in other immunogenic tumors, especially in melanoma. Initial studies with isolated and *ex vivo* expanded tumor infiltrating lymphocytes (TILs) that have been reinfused into the patient usually in combination with IL-2, showed only modest response (Topalian *et al.* 1988; Kradin *et al.* 1989). In a larger phase III trial involving 160 patients with metastatic RCC, treatment with *ex vivo* expanded TILs and IL-2 showed no benefit compared to IL-2 alone (Figlin *et al.* 1999). Because of these disappointing results and because of the lack of tumor specific T-cell epitopes further use of (antigen specific) adoptive T-cell transfer has been limited although recently the usage of gammadelta T cells in RCC patients has moved into the focus of ongoing research (Bennouna *et al.* 2008; Kobayashi *et al.* 2010). In order to complete the picture of current immunotherapeutic approaches, WX-G250 (Girentuximab) has to be mentioned. This is an antibody developed by Wilex that is specifically intended for adjuvant use in non-metastatic RCC patients and is currently undergoing Phase III clinical trials (Reichert 2011). The chimeric IgG1 antibody is directed against Carbonic anhydrase 9, which is a tumor associated antigen expressed by more than 90% of clear cell renal cell carcinoma. Data from a recent Phase I/II trial of Girentuximab in combination with IFNα for metastatic RCC patients have shown good tolerability, safety and also clinical benefit (Siebels *et al.* 2011).

Considering the diversity of different immunotherapeutic approaches, especially in tumor vaccination, the question remains why many of these studies failed or showed rather limited

clinical success, with only a few trials progressing to clinical phase III. In order to answer this question we will first look at the main problem of all immunotherapeutic approaches, namely the ability of a tumor to escape a directed immune response by inducing an immunosuppresive environment. In this context we will also discuss methodological deficiencies and contradictions that have contributed to clinical failure and describe more promising directions for future immunotherapy.

4. Immunosuppression and tumor escape

As mentioned above, renal cell carcinomas, like other tumors, are highly infiltrated by leukocytes and especially T cells (> 60%) mainly of the $CD8^+$ rather than the $CD4^+$ phenotype. Natural killer cells have also been found to be enriched within the TIL population in some studies at least whereas B cells make up only a minor subset. (Van den Hove, Van Gool et al. 1997). Infiltrating T cells are predominantly of the antigen experienced effector memory type (T_{EM}), and $CD8^+$ cells also encompass highly differentiated T_{EMRA} effector cells (Attig *et al.* 2009). Expression of several lymphocyte activation markers such as CD69 or HLA-DR on TILs has been confirmed and oligoclonal expansion of certain TCR-Vβ regions indicates that a selection of potentially tumor specific T cells has taken place (Angevin *et al.* 1997). Furthermore, after isolation and ex vivo culture T cells are able to express cytokines and show normal cytotoxic activity. However if these TILs show functionality *ex vivo* then why are they not reactive within the tumor microenvironment? Indeed, freshly isolated uncultured TILs often show a reduced capacity of their cytotoxic function (Van den Hove, Van Gool et al. 1997) and also an impaired cytokine production or altered cytokine profile, demonstrating that some kind of immunosuppressive milieu must be present within the tumor microenvironment (Gouttefangeas *et al.* 2007). Defects in T-cell signalling and downregulation of the CD3 ζ-chain, which is necessary for TCR signal transduction into the cell, can be frequently found in T cells isolated from RCC patients (Frey & Monu 2008). Several mechanisms responsible for this locoregional immunosuppression have been discovered within the last decades and only the most important findings will be described here. Some of these mechanisms, such as the activation of T regulatory cells (T_{regs}), have been intentionally developed by evolution to counteract deleterious long term activation of an immune response in order to avoid autoimmune diseases. Others, such as the generation of inhibitory signals have been developed or rather selected within the heterogeneity of tumor cells to escape an existing immune response and thereby provide a selection advantage. Among the latter are immunosuppressive cytokines IL-10 or TGF-β, which are known to inhibit T-cell activation as well as proliferation and can also lead to downregulation of MHC class I molecules (Khong & Restifo 2002; Li *et al.* 2006). In renal cell carcinoma, proangiogenic vascular endothelial growth factor (VEGF), which acts also immunosuppressively by inhibiting DC maturation (Ohm & Carbone 2001) plays a major role because it is usually found highly overexpressed in clear cell RCCs (Rini 2005). Apart from the expression of immunosuppressive factors, tumor cells can also downregulate costimulatory molecules from their surface. Despite the fact that tumor cells are not professional APCs and therefore not supposed to prime T cells, the complete lack of costimulatory molecules such as CD80 (B7.1) or CD86 (B7.2) can lead to a decreased T-cell activation or even induction of T-cell anergy (Jung *et al.* 1999; Lang *et al.* 2000). In this context another B7 family member should be mentioned which, in contrast to the aforementioned, is often found highly upregulated in different types of cancer. B7-H1

(PDL1) is usually expressed on macrophages and provides costimulatiory function for T cells. However, due to its high abundance on tumor cells it has a predominantly negative regulatory activity. After binding to its receptor PD1 on activated T cells B7-H1 can downmodulate T cell activation and even induce apoptosis (Dong *et al.* 2002). B7-H1, and more recently another associate of the B7 family with a similiar function, B7-H4, have been shown to be expressed in RCC patients and their expression correlated with adverse clinical prognosis (Thompson *et al.* 2005).

In order to escape an already existing specific immune response, cancer cells can either escape by downregulating the antigen or even parts of the HLA presentation machinery. Indeed downregulation of HLA expression can be observed in several cancer types preferentially in late metastasized stages (Marincola *et al.* 2000; Campoli *et al.* 2002). Different mechanisms underlying this process of downregulation or even complete loss of HLA expression have been elucidated from several tumor cell lines and seem to affect nearly all parts of the antigen processing machinery (Seliger *et al.* 2002). To which extent HLA downregulation or loss plays a role in RCC is still controversial. Whereas some publications suggest that HLA downregulation is a frequent event (Romero *et al.* 2006) other more recent data could clearly show that HLA-expression is not diminished but rather upregulated in comparison to benign kidney tissue (Saenz-Lopez *et al.* 2010; Stickel *et al.* 2011).

Tumor cells can also indirectly inhibit an immune response by depriving proliferating immune cells of essential nutrients. Indoleamine-2,3-dioxygenase (IDO) is an enzyme that catalyzes the first and also rate-limiting step in the degradation of the essential amino acid tryptophane. IDO has been found to be nearly ubiquitously expressed in human tumors (Uyttenhove *et al.* 2003). By locally depleting tryptophane, IDO can inhibit the proliferation of TILs and also induce or recruit T_{regs} (Prendergast *et al.* 2009).

$CD4^+$ $CD25^{high}$ $FoxP3^+$ regulatory T cells have become a major field of investigation in immunotherapy because of their potential to induce tolerance by suppressing (tumor) antigen specific priming of T cells and also T cell effector functions (Zou 2006). T_{regs} are known to accumulate within the microenvironment of different tumors (Woo *et al.* 2001) and higher levels of T_{regs} in the peripheral blood of cancer patients have been detected. In RCC patients a higher frequency of T_{regs} within the tumor and periphery have been described and correlate with an adverse clinical outcome (Liotta *et al.* 2011). A plethora of different mechanisms have been described on how T_{regs} exert their immunosuppressive functions that range from the expression of immunosuppressive cytokines (IL 10, TGFβ)(Taylor *et al.* 2006), induction of IDO and B7-H4 in APCs (Fallarino *et al.* 2003; Sica *et al.* 2003), and consumption of IL-2 (von Boehmer 2005) to direct cell mediated cytotoxicity (Grossman *et al.* 2004). Antigen specific T_{regs} have been described (Wang *et al.* 2004) but after activation, the suppressive activity of CD4+ T_{regs} seems to be antigen non-specific affecting T cells of varying specificity (Thornton & Shevach 2000). Another type of immunosuppresive cells are the myeloid derived suppressor cells (MDSCs) which have recently acquired increasing attention (Kusmartsev & Vieweg 2009). MDSCs are a heterogeneous population of progenitor cells of the myeloid lineage. Under healthy conditions these cells rapidly differentiate into mature granulocytes, macrophages and also dendritic cells. However, in patients suffering from different types of cancer that include RCC (Rodriguez *et al.* 2009), these immature cells have been shown to strongly accumulate in peripheral blood and also

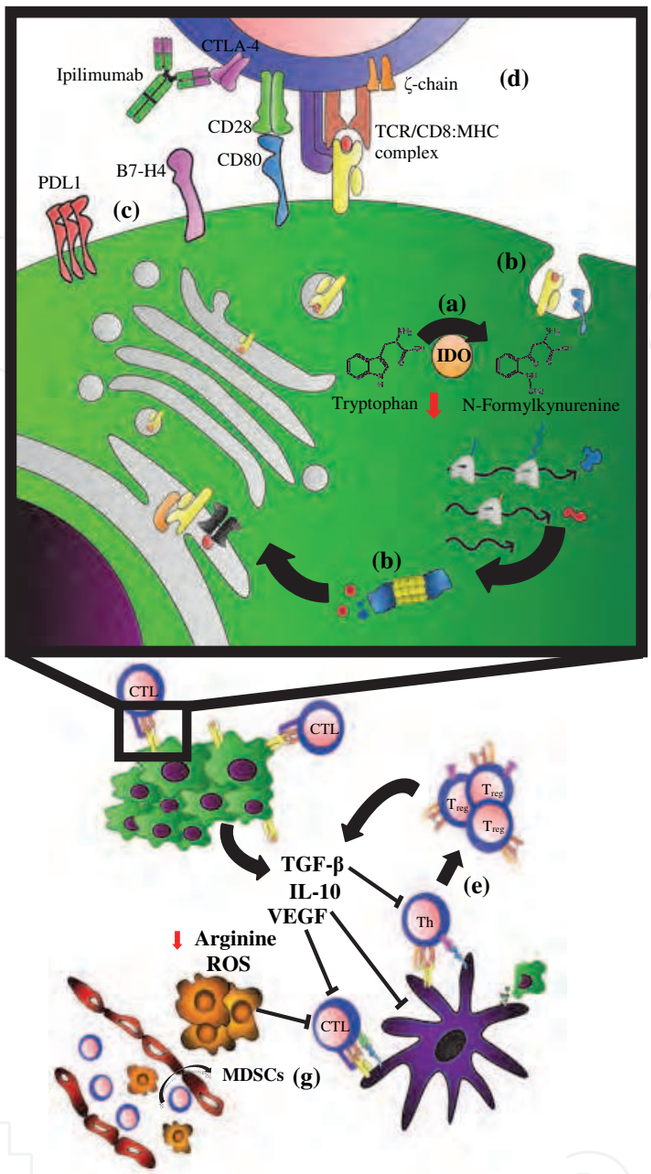

Fig. 1. Immunosuppressive Mechanisms: (a) Overexpression of Indoleamin-2.3-dioxyngenase (IDO) leads to reduced extracellular tryptophan levels thereby inhibiting T cell proliferation. (b) Defects in antigen presentation machinery or downregulation of MHC molecules prevent recognition by cytotoxic lymphocytes (CTLs). (c) Aberrant expression of PDL1 or upregulation of coinhibitory molecules B7-H4 decrease costimulation. (d) Downregulation of T-cell receptor (TCR) ζ-chain inhibits intracellular signalling. (e) Secretion of immunosuppressive cytokines by tumor cells or regulatory T cells (T_{regs}) inhibits activation of T cells (IL-10, TGF-β) and dendritic cells (VEGF). In addition induction of Tregs (f) from the CD4+ T helper cell (Th) population is inceased. (g) Infiltration of myeloid derived suppressor cells (MDSCs) from the blood lead to production of reactive oxygen species (ROS)) and enhanced arginine catabolism through arginase and iNOS thereby further hampering T cell proliferation.

within the tumor microenvironment (Young *et al.* 1997; Nagaraj & Gabrilovich 2010) where they exert potent immunosuppressive effects. Myeloid suppressor cells seem to highly express two enzymes involved in the arginine metabolism: Arginase and inducible nitric oxid synthase (iNOS). These enzymes drive the enhanced catabolism of arginine which subsequently leads to an arginine depletion from the tumor microenvironment thereby strongly inhibiting T-cell proliferation (Ochoa *et al.* 2007; Gabrilovich & Nagaraj 2009). Higher arginase activity in PBMCs from RCC patients could indeed be demonstrated (Zea *et al.* 2005). Other mechanisms of MDSC mediated immunosuppression, such as the production of reactive oxygen species (ROS) *e.g.* peroxynitrite (Kusmartsev *et al.* 2004), the secretion of immunosuppressive cytokine TGF-β (Filipazzi *et al.* 2007) and the induction of other immune suppressive cells such as T_{regs} (Serafini *et al.* 2008) could also be observed.

Despite all these different immunosuppressive cells and the mechanisms discussed (see Figure 1), the infiltration of cancer tissue by leukocytes still remains a reliable marker for patient outcome and correlates positively with clinical prognosis in RCC patients (Pages *et al.* 2010). Galon et. al have shown that the presence and composition of the immunological infiltrate is highly predictive for clinical outcome in colon cancer patients and can even be superior to classical TNM staging (Galon *et al.* 2006).

5. Counteracting immunosuppression

In view of all these sophisticated immunosuppresive mechanisms there are doubts that we will ever be able to overcome immunosuppression and tumor escape. In order to generate a long lasting immune response which keeps the immune system up and running, tumor associated antigens ant T-cell epitopes represent only a part of the whole picture. Adjuvants are therefore urgently needed to specifically address this difficult task. Adjuvants or„Immunologist's dirty little secrets" as Janeway termed them (Janeway 1989) are an active component of most protective vaccines against infectious diseases particularly intended to enhance or modulate a specific immune response. Most of the adjuvants approved for human treatment (Alumn, MPL) were actually developed for passive vaccination of healthy patients against infectious diseases - a setting in which an enormously high safety profile of the adjuvant is clearly necessary. However, considering the highly immunosuppressive environment of late stage tumors in particular, the need for stronger immunostimulatory adjuvants has emerged even if they reduce safety and bear the risk of provoking autoimmunity. In the majority of trials involving RCC patients (Table 1) cytokines such as GM-CSF, IL-2 or IFNα have been used to ensure effective priming of T cells and enhance the immune response. Regardless of toxicity problems considering the systemic application of IL-2 and Interferon α, new studies could show that IL-2 also leads to the induction and stimulation of regulatory T cells (Brandenburg *et al.* 2008). Similarly GM-CSF has been shown to stimulate the recruitment of myeloid derived suppressor cells (Serafini *et al.* 2004) and its beneficial effect in cancer vaccination has been challenged by two randomized vaccination trials in melanoma in which patients that additionally received GM-CSF had an inferior immunologic and patient outcome (Faries *et al.* 2009; Slingluff *et al.* 2009). The use of incomplete Freund's adjuvant and BCG as adjuvants in cancer immunotherapy appears rather outdated. Nevertheless, BCG is still approved for the treatment of superficial bladder cancer. Metabolizable squalene based emulsions (MF59, AF03) and saponins (Quil-A, ISCOM, QS-2) have replaced mineral oil based formulations like Freunds adjuvant. The use of live-attenuated bacteria like BCG has been discontinued in favor of molecularly defined

TLR agonists. The discovery that toll like receptors (TLRs) constitute part of the innate immune system and can act as pattern recognition receptors (PRRs) for different pathogen associated molecular patterns (PAMPs) has boosted the search for agonistic TLR ligands. Synthetic TLR ligands can, as their natural counterparts, bind to TLRs on different cell types and subsequently lead to the activation of the cell (*e.g.* maturation, release of proinflammatory cytokines, enhanced antigen presentation or effector function). In theory a combination of different TLR agonists can be used to tailor an immune response to fit individual needs (Adams 2009). For example, TLRs 3, 7/8 and 9 have been shown to lead to the preferential activation of a Th1 response which has been known for a long time to be a prognostic factor in cancer immunotherapy correlating with favourable outcome. Several of these synthetic TLR agonists such as PolyI:C (TLR3 agonist), Resimiquimod (TLR7/8 agonist) and CpG (TLR9 agonist) are currently being evaluated in clinical trials (Adams *et al.* 2008; Cheever 2008).

Only very few studies in RCC patients have attempted to address the specific requirements needed to overcome immune escape. Some groups have used cyclophosphamide prior to or during vaccination because it is known that low doses of this chemotherapeutic drug lead to improved immune responses potentially by depleting T_{regs}. (Ghiringhelli *et al.* 2004; Lutsiak *et al.* 2005). Denileukin (Diftitox, Ontak), a recombinant fusion protein of IL-2 and diphtheria toxin has been also used successfully for the same purpose (Atchison *et al.* 2010). However some studies have described severe side effects in conjunction with Denileukin like vision loss and vascular leak syndrome (Park *et al.* 2007; Avarbock *et al.* 2008), treatment failure has also been reported (Welters *et al.* 2009). Recent studies are suggesting that pretreatment with less toxic Sunitinib might have a similar effect on regulatory T cells (Finke *et al.* 2008).

The most promising agents that are currently being developed to overcome tumor induced immunosuppression are immune modulating antibodies that inhibit immune checkpoint controls (Fife & Bluestone 2008). The first antibody with this mode of action (Ipilimumab) has just recently been approved by regulatory authorities in the US and Europe as a monotherapy for the treatment of late stage melanoma. Ipilimumab is directed against cytotoxic T-lymphocyte antigen 4 (CTLA-4), a negative immunoregulatory receptor expressed by activated T cells, in particular T_{regs}. CTLA-4 binds to costimulatory molecules (*e.g.* CD80, CD86) on APCs or tumor cells and thereby directly competes with T cell coactivating molecule CD28. However whereas signalling through CD28 is leads to T cell activation CTLA-4 signalling is rather inhibitory and promotes T cell tolerization and anergy. Blocking of CTLA-4 by Ipilimumab consequently leads to an increase in T-cell activating signals resulting in a greatly sustained activation of an immune repsonse. Considering its unspecific mechanism of action it is not surprising that among the different side effects experienced after treatment with Ipilimumab, the induction of autoimmune phenomena was also quite frequent. Nevertheless, in most cases the treatment with corticosteroids was effective and did not interfere with clinical benefit.

Based on this very recent breakthrough, several trials are currently evaluating combinations of Ipilimumab with targeted immunotherapeutic approaches in order to highlight the direction to activated T cells. Another immunomodulating antibody directed against PD1 (MDX-1106) is currently being applied in late phase clinical trials. Interaction of PD1 with its ligand B7-H1, which is broadly overexpressed on tumor cells has already been discussed as an established tumor escape mechanism.

The advent of new immunotherapies has also clearly revealed another problem regarding the kinetics and study endpoints of clinical immunotherapeutic trials (Hoos *et al.* 2010). Evaluation of clinical benefit is usually based on world health organization (WHO) (World Health Organization. 1979) or response evaluation criteria in solid tumors (RECIST) (Therasse *et al.* 2000; Eisenhauer *et al.* 2009). These are criteria that were originally developed for treatment with cytotoxic agents. However, patterns of clinical response may greatly differ in immunotherapeutic trials. Compared to chemotherapeutics, the manifestation of clinical response is often delayed in immunotherapeutic trials and develops after an initial phase of stable disease or even tumor progression. This effect is frequently observed in immunotherapeutic trials and is expressed by a delayed separation of Kaplan-Meyer survival curves (Hoos *et al.* 2010). Furthermore, a well-known discrepancy of many immunotherapies that fail to achieve clinical benefit in progression-free survival but do show an objective effect on overall survival can be explained by this effect. In order to accommodate these observations, new immune related response criteria (irRC) have been proposed and are currently being tested for their significance and applicability in immunotherapeutical trials (Wolchok *et al.* 2009).

Another question that remains in this context is the appropriate general setting of an immunotherapeutic approach. Nearly all trials mentioned in Table 1 focus on late stage patients with metastatic RCC that have often not responded to previous therapies and have acquired a resistance to cytokine treatment and targeted therapy. Some authors have suggested that this might be a rather unfavourable setting for testing the efficiency of an immunotherapeutic approach because the immune system has probably lost the battle against the cancer at a much earlier stge and immune suppressive mechanisms may have progressed to an irreversible state. The use of immunotherapies should therefore be tested preferentially in an adjuvant or minimal residual disease setting (Morse *et al.* 2005; Hoos *et al.* 2007).

6. Tumor associated antigens: the good, the bad, and the ugly

The lack of defined tumor specific antigens in RCC can already be deduced from the list of studies undertaken so far. Only few invoke defined antigens, most make use of autologous tumor tissue alone, in combination with adjuvant or after loading on dendritic cells. The use of non-defined antigens has several inherent problems however. First, the quality of the vaccine is critically dependent on the purity of the antigen and tissue material received from surgery is undoubtedly of varying quality. Furthermore, some studies have shown that only apoptotic and not necrotic cell vaccines can induce a regular immune response (Scheffer *et al.* 2003). Second, a mixture of different unknown antigens always has the potential of partially or preferentially inducing tolerogenic T cells or, even worse, inducing autoimmunity against self-antigens. Last but not least, immunomonitoring of patients, which has become a powerful tool in recent immunotherapeutic studies, can only be carried out accurately if the antigen is known in advance. Compared with full length proteins, antigenic peptides have the advantage of simple chemically defined production in GMP quality. This allows for the combination of different antigenic peptides to a multi-epitope vaccine. Thereby the individuality of a patient's immune response is taken into account, indicating that not every patient will develop an equally strong immune response against one and the same antigen. The major disadvantage of defined T-cell epitopes is that they are usually restricted to for one HLA allotype requiring the patient to match to a certain HLA in

order to benefit from the treatment. This further underlines the need for the identification of additional tumor-associated antigen-derived peptides for less common HLA alleles (Klug *et al.* 2009).

How can we now decide whether an identified antigen is also a good vaccination candidate and what are the hallmarks of these antigens? The most important but often carelessly neglected requirement of any good vaccination candidate is the presence of the antigen on the tumor cell. In terms of an immunotherapy this means that the antigen needs to be accessible in order to be recognized by immune cells, preferentially T cells. At first glance this sounds trivial but in fact most studies have made use of antigens that have actually never been found to be presented by MHC molecules and instead emanated from *in vitro* tested or *in silico* predicted T cell epitopes of previously known tumor associated antigens that were shown to be overexpressed within tumor tissue at the mRNA or protein level. In doing so basic principles of immunology are ignored since T cells are not given insight into tumor cells but have to rely on a showcase of peptide antigens presented by MHC molecules. Indeed it could be shown that the gene expression level of a certain protein and presentation of corresponding protein-derived peptides only reveal a very faint correlation (Weinzierl *et al.* 2007).

The second most important requirement is the immunogenicity of the antigen. This is indeed a crucial point, since many of the tumor antigens are derived from self proteins and hence show only weak immunogenicity if at all because of central or peripheral T-cell tolerance. One great exception are unique antigens that arise from gene mutations or fusion proteins which accumulate during the course of tumor development. These neo-antigens are therefore not affected by tolerance mechanisms, either in the thymus during lymphocyte maturation by clonal deletion or in the periphery by anergy induction. Because of the large set of distinct mutations acquired within each tumor not only within different genes, but also at a multitude of locations within the gene, the application of mutated antigens in tumor vaccination will be restricted to a patient individualized approach.

Other frequently examined antigens including cancer testis or differentiation antigens show a rather restricted expression pattern that can in some cases also be considered to be tumor specific. During the course of tumorigenesis cancer cells often acquire epigenetic alterations that lead to the expression of genes (MAGE, NY-ESO-1) which are usually only transcribed during embryonic development and hence remain silenced within adult tissue. Some of these cancer testis antigens (CTA) have, however, also been found to be expressed in thymic epithelium so that central tolerance by deletion of CTA-specific T cells cannot be excluded (Gotter *et al.* 2004). Differentiation antigens are commonly expressed in malignant and normal cells of the same lineage *e.g.* Melan-A, gp100 and TRP1 in melanoma tumor tissue and benign melanocytes. In cases in which the benign tissue expressing the differentiation antigen is dispensable as, for instance, in prostate cancer patients after prostatectomy, differentiation antigens like PSA become highly specific for the tumor. The great majority of potential vaccination antigens is, however, derived from tumor antigens that are rather ubquitously expressed but show a (high) overexpression on cancer tissue (CA9, Her2/neu, MUC1) (Kessler & Melief 2007). Differentiation and overexpressed antigens are usually subject to central and peripheral tolerance mechanisms. Self tolerance for many antigens, however is not always complete. The prior testing of T-cell immunogenicity for theses antigens is therefore absolutely essential.

Another aspect that should be considered before choosing a vaccination antigen is its impact on tumor oncogenicity. Preferentially, antigens should be targeted that are involved in the oncogenic process and hence indispensable for tumor growth and maintenance of the neoplastic state.

Based on similar but not identical criteria, a ranking of potentially suitable tumor associated antigens for clinical use has been published in an initiative from the National Cancer Institute involving different working groups in the US (Cheever *et al.* 2009). They used an analytical hierachy process generated ranking which further included objective criteria like therapeutic functionality, the expression of the antigen within stem cells, number of patients expressing the antigen, the number of known antigen derived T-cell epitopes and also the cellular localisation of the antigen.

7. Identification of new tumor associated antigens

Having described the hallmarks of optimal tumor associated and specific antigens in the context of an anti-tumor vaccine we would now like to present state-of-the-art technology for the identification of respective antigens with a clear focus on tumor associated HLA ligands. There are several strategies which can be divided into two basic sets: Top-down approaches carried out - by directly analysing the tumor antigens present within or presented by tumor cells, and bottom-up. The latter represent a reverse approach usually starting from the gene level *via* the protein level and subsequently to the HLA ligand level.

7.1 Bottom-up

One of the first approaches developed for the large scale identification of tumor associated antigens is the serological identification of antigens by recombinant expression cloning SEREX (Sahin *et al.* 1995). The method is based on cDNA expression libraries created from tumor cell lines or tissues which are subsequently packaged into lambda-phage vectors that can be used to infect *E. coli* bacteria. During the lytic phase the production of recombinant proteins is induced and clones can be screened for the presence of tumor reactive antibodies using cancer patient sera. Selection, cloning and sequencing of antibody reacting clones allows for the straightforward molecular description of the antigen, as the recombinant proteins are located in the same *E.coli* clone plaque as the respective cDNA (Tureci *et al.* 2005). The major advantage of cDNA expression libraries is that only genes that are actually expressed within the cell/tisue of origin are incorporated and that they can be generated from various sources that include patient derived autologous tumor tissue. SEREX is still a valued, commonly used tool for antigen identification (Wang *et al.* 2009; Kiyamova *et al.* 2010). Several modifications have been incoporated to overcome restrictions for instance regarding the prokaryotic expression system, which does not allow for posttranslational modifications (Kim *et al.* 2007). With the advent of the „omics" era a comparatively high throughput analysis of differences in the gene expression level could be done with relative ease. Now large databases summarizing these data are publicly available (Edgar *et al.* 2002). New approaches aim at gaining a deeper understanding of the tumor and its genetic basis by sequencing the complete tumor genome. The whole genome approach has profited from the rapid progress in next-generation sequencing techniques over the last few years which is fortunately accompanied by decreasing costs (Wong *et al.* 2011). The complete and differential sequencing of tumor tissue and corresponding normal tissue shows alterations

directly on genomic level that the tumor has acquired during its development (see Fig. 2 blue section). The subset of potential tumor antigens can be assessed by the selection of non-silent mutational events. Further reconciliation with databases of known sequence polymorphisms (dbSNP, (Smigielski *et al.* 2000)) or known mutations in cancer (COSMIC, (Bamford *et al.* 2004)) can help to distinguish tumor driver from passenger mutations.

The bottom-up approach yields proteins without any post-translational modifications. A prerequisite for successful peptide vaccination therapy is the presentation on the tumor cell surface by MHC molecules. *In silico* digestion and HLA binding prediction (Feldhahn *et al.* 2009) of the respective parts can shed light on which of these are putative tumor antigens suitable for further evaluation of immunogenicity in T-cell arrays.

A major determinant for peptide:HLA binding is the steric configuration defined by the MHC molecule and the amino acid sequence of the peptide (Bjorkman *et al.* 1987). The HLA molecule forms a peptide binding groove with prominent binding pockets for individual amino acid side chains. The polymorphism of the HLA alleles results in different polypeptides and therefore different binding pocket properties. This yields characteristic peptide sequence motifs for ligands to bind the HLA molecule. The most conserved positions in these motifs form the anchor residues, whose side chains fit best in the binding pockets for strong interaction.

With sufficient ligandome analysis that takes sequence statistics for the given peptide:HLA molecule pairs into consideration one can generate Position Specific Scoring Matrices (PSSMs). Each position holds higher scores for more frequently occurring amino acids. Summing up the scores for each position, these matrices can be used to estimate the binding capability with an HLA molecule for any given peptide sequence. The SYFPEITHI method uses PSSMs generated from naturally processed HLA ligands from the SYFPEITHI database (Rammensee *et al.* 1999) in an expert system fashion also accounting for given chemical conditions. The assumption of independent contribution of each amino acid to the overall binding affinity is one drawback of PSSM approaches. Non-linear fashion machine learning methods can create prediction models that address this issue with different techniques (support vector machine (SVM) based SVMHC (Dönnes & Kohlbacher 2006), artificial neural network (ANN) training method based NetMHC (Buus *et al.* 2003). Prediction quality for all approaches is heavily dependent on the sampling coverage for an an allele-specific HLA ligandome available (discussion of efficiency is out of scope).

There also exist prediction methods for the steps preceding peptide presentation, the proteasomal cleavage of proteins (PAProC (Nussbaum *et al.* 2001), NetChop, (Kesmir *et al.* 2002)) and the TAP transport (TAPPred, (Bhasin & Raghava 2004)). As these steps are very complex and depend on a huge variety of parameters, the results may not adequately reflect the naturally occurring process. The framework for T-cell epitope detection FRED provides easy accession to most prediction methods for MHC binding as well as creating a general infrastructure for the handling of antigen sequence data. It includes the possibility for integrated analysis of protein polymorphisms influences and simultaneous accession of different prediction methods. Analysis pipelines intended for high throughput capability profit immensely from such frameworks. They integrate different algorithms with differing input and output types and therefore provide a flexible means to implement of accelerating the analysis process.

7.2 Top-down

Until now straightforward proteomics approaches have mainly focused on the identification of serum cancer biomarkers rather than target antigens for cancer therapy (Seliger *et al.* 2003). One exception is serological proteome analysis, SERPA, a modified SEREX approach translated to the protein level. In contrast to SEREX, screening with autologous patient sera is not carried out against cDNA expression libraries but against protein lysates separated by two dimensional polyacrylamide gel electrophoresis (2D-PAGE) or directly against protein arrays (Desmetz *et al.* 2009). The comparative full proteome analysis of tumor and benign tissue usually requires the pre-separation of proteins, for example by 2D-PAGE or multidimensional liquid chromatography, due to the overall complexity of the protein lysate. This approach has in the past suffered from low sensitivity and weak reproducibility (Baggerman *et al.* 2005). Identification of tumor associated target antigens has remained rare since standard proteomics approaches often fail to detect low abundant proteins (Joshi *et al.* 2011). Nevertheless proteomics is a rapidly developing field. Taking the steadily increasing mass spectrometric sensitivity (Yates *et al.* 2009), the use of *in vitro* (*e.g.* chemical modification) or *in vivo* (*e.g.* SILAC) differential labeling approaches for quantification (Schulze & Usadel 2010) and also MALDI imaging technology (Fournier *et al.* 2008) into account, proteomics will clearly contribute to a greater extent to TAA identification in the future.

In contrast to standard proteomics, HLA ligandomics is a well established, straightforward approach and perfectly suited for the identification of tumor associated HLA ligands (Schirle *et al.* 2000). HLA molecules can be directly isolated from dissected tumors and autologous normal tissue. The method of choice is immunoaffinity chromatography using antibodies specific for different HLA molecules (see table 2) immobilised on a sepharose matrix. Application of a crude tissue lysate leads to the binding of respective HLA molecules which can be subsequently eluted by acid treatment. At the same time, pH shift also leads to the release of bound peptides from the HLA binding grooves. Due to the narrow mass range of these peptide ligands, they can be easily separated from higher molecular weight substances (>10 kDa) by ultra-filtration. Lyophilisation of the filtrate yields a mixture of different HLA derived peptides ready for concomitant separation and analysis using liquid-chromatography coupled mass spectrometry (LC-MS, see Fig. 2a).

Clone	∝HLA	Reference
W6/32	A,B,C	(Barnstable *et al.* 1978)
B1.23.2	B,C	(Rebai & Malissen 1983)
BB7.2	A2	(Parham & Brodsky 1981)
GAP-A3	A3	(Berger *et al.* 1982)
Spv-L3	DQ	(Spits *et al.* 1983)
Tü-39	DR, DQ, DP	(Maeda & Hirata 1984)
L243	DR	(Lampson & Levy 1980)
IVD-12	DQ	(Kolstad *et al.* 1987)

Table 2. HLA directed antibodies

The greates benefit is that the described procedure yields natural ligands, so that neither the proteasomal cleavage nor intracellular transport and loading onto HLA molecules has to be

determined for the respective peptide. The challenge is the molecular characterisation of the heterogenous mixture of isolated peptides.

Peptide and protein sequencing can be accomplished via tandem mass spectrometry. This is an advanced technique of proteomics analysis and offers a versatile, high-throughput procedure for investigation of protein samples, including sequence identification. The measurement is conducted by recording the mass-to-charge ratio (m/z) of peptide ions with high sensitivity down to the sub-femtomole level. For better identification a second mass analyzer can be added in tandem, where molecules of selected masses are further fragmented (Roepstorff & Fohlman 1984; Johnson *et al.* 1987) and the resulting mass-to-charge ratios measured (see Table 3). Fragmentation of peptides occurs most prominently at the backbone structure, *i.e.* the peptide bonds concatenating the amino acids. The result of a tandem mass spectrometric analysis is a spectrum of fragment m/z values. The fragments containing the N-terminus of the peptide are called „b-ions" whereas those containing the C-terminus are called „y-ions", each enumerated by the number of retained amino acids. *Via* the masses of the fragments the spectra can be annotated with the peptide's sequence.

	b-ions	Mass	y-ions	Mass	
1	(S)	-	YFPEITHI	1019.5197	8
2	SY	251.1026	FPEITHI	856.4563	7
3	SYF	398.1710	PEITHI	709.3879	6
4	SYFP	495.2238	EITHI	612.3352	5
5	SYFPE	624.2664	ITHI	483.2926	4
6	SYFPEI	737.3505	THI	370.2085	3
7	SYFPEIT	838.3981	HI	269.1608	2
8	SYFPEITH	975.4571	I	132.1019	1

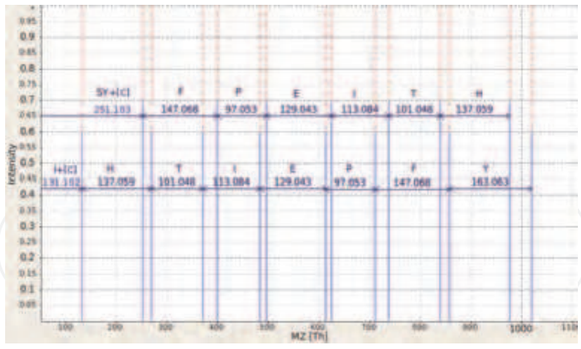

Table 3. Theoretical fragmentation spectrum of SYFPEITHI peptide.

Missing peaks make the spectrum annotation a complex problem. These are due to: technical detection thresholds, complex or incomplete fragmentation, contamination and measurement noise. This can be addressed by computational methods for mass spectrometry proteomics. Spectra can be identified by either algorithmic comparison to a database of known spectra (Eng *et al.* 1994), directly by de novo methods (Bertsch *et al.* 2009) that do not depend on databases or by comparison with a sequence database (Perkins *et al.* 1999).

The latter and in most cases very robust method infers theoretical spectra from a set of compatible sequences and matches the experimentally determined masses with those calculated. The method is robust because it is possible to infer a statistical significance evaluation, *i.e.* a score, from the size of the database as well as the number and quality of matches. Another technique to introduce robustness to the results is the use of a false discovery cut off. The introduction of a decoy database (most commonly the inverse input database) allows for calculation of a false discovery rate, defined as the number of false discoveries (from the decoy) over the number of false and correct discoveries (from both databases) given a score. A false discovery rate of 5% discerns a score within one experiment which guarantees that scores equal or better will be false discoveries only by the chance of p=0.05 (i.e. 5%).

Sophisticated methods for high throughput identification of natural HLA ligands are beginning to emerge. Established proteomics methods are specifically suited for this task. OpenMS (Sturm *et al.* 2008) is an open and flexible framework for proteomics data analysis. As explained, integrated analysis is a major advantage for high throughput oriented analysis. In the case of individualized cancer therapy, different disciplines with diverse methods come together which does not permit a comprehensive framework. One solution for maintaining high throughput capability is a tailored workflow development that integrates analysis tools of different trades. OpenMS/TOPP also comes with tools for workflow development (Kohlbacher *et al.* 2007) for virtually seamless integration of other computational aspects of immunology, such as the *in silico* prediction of HLA presented peptides or database connection and reconciliation containing very different types of data.

7.3 Validation and selection of peptide candidates

The final verification step of identified HLA ligands includes the chemical synthesis of peptides. The tandem mass spectrometric measurement of the synthesized peptide represents a crucial aspect of reliability. As fragmentation patterns define the sequence and are preserved throughout the measurements, the corresponding spectra as well as the retention time of the synthetic and identified peptide will be nearly identical. A putative T-cell epitope needs to be further validated in T-cell priming experiments. For this reason a plethora of protocols has been established to prime naive T cells usually from healthy donors with either natural (*e.g.* dendritic cells) or artificial APCs presenting the synthetic peptide. Proliferation and priming efficiency can subsequently be assayed by a variety of functional tests, *e.g.* enzyme linked immunospot technique (ELISPOT), intracellular cytokine staining (ICS) or tetramer staining.

In order to work reasonably well in a preferably diverse target group, the design of a vaccine also poses the challenge of combining several vaccination candidate peptides to a multi-epitope vaccine. Recently, Toussaint et al. (Toussaint & Kohlbacher 2009) proposed a mathematical framework for the selection of an optimal set of peptides for vaccination. Given a set of candidate epitopes, a target population, information on the respective T-cell reactivities to the peptides (or HLA binding affinities), along with other user-defined information to be incorporated in the selection process, the framework efficiently determines an optimal epitope set.

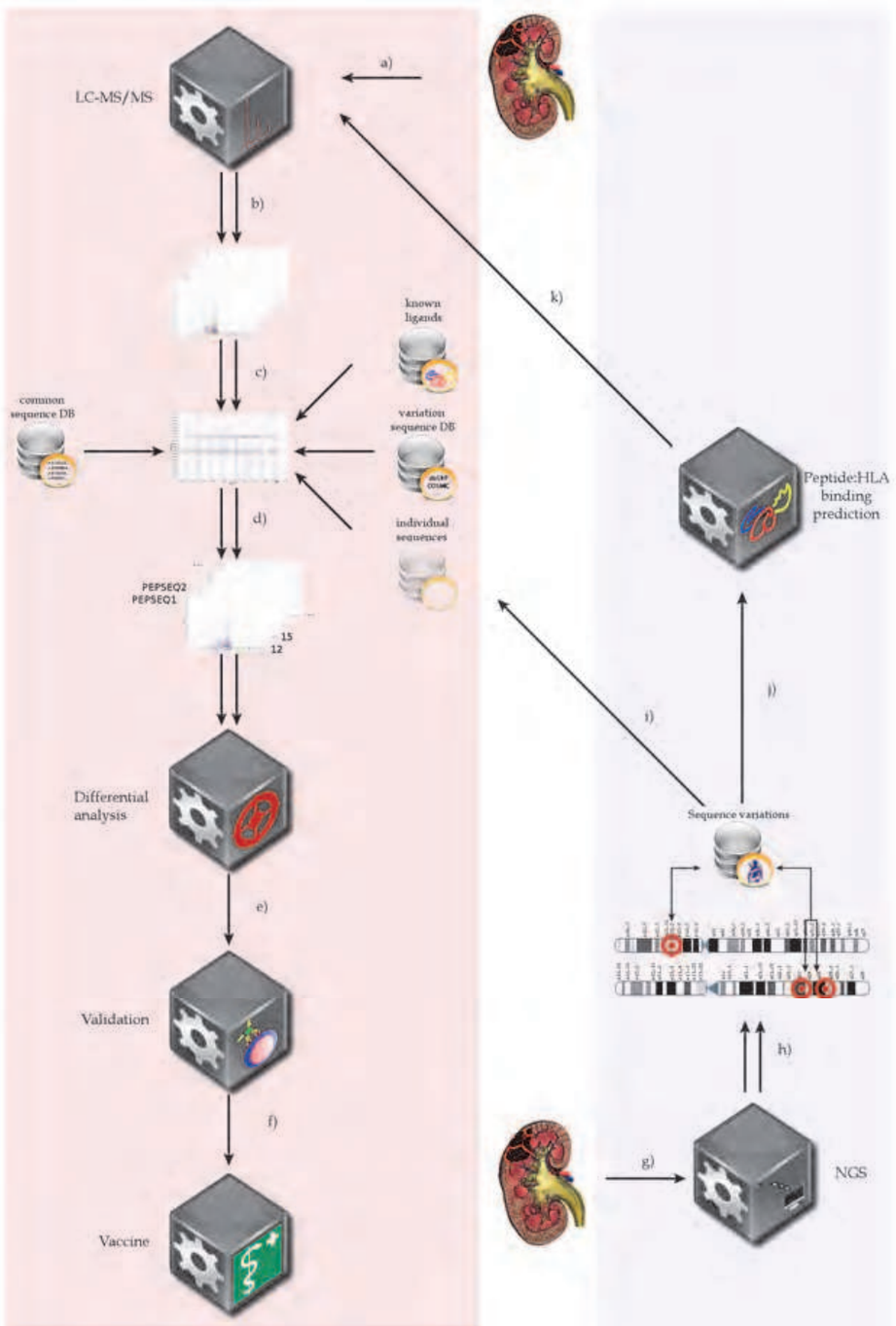

Fig. 2. The workflow for standard HLA ligandome analysis (red) and with mounted bottom-up approach methods (blue) for patient individualisation. a) sample preparation, b) MS analysis, c) peptide identification, d) FDR filter, e) differential analys f) HLA ligand validation, g) vaccine design and application, h) genome sequencing, i) variation detection, j) peptide:HLA binding prediction, k) identification of peptides with sequence variation, l) targeted search. Chromosome figures are modified from a screenshot at http://genome.ucsc.edu (Kent et al. 2002).

8. Outlook

The therapeutic situation in advanced stage renal cell carcinoma leaves much rome for improvement with frequent resistance development to TKIs and still no cure in sight. Despite the lack of convincing clinical succes, targeted immunotherapy remains a promising approach. We are only just beginning to understand the complex relationship between the tumor and the surrounding microenvironment. The sophisticated mechanisms of immune evasion that hamper an active immune response are still a major hindrance on the road to success. First steps to overcoming this are by specifically addressing immunosuppression presented through the advent of immune modulatory antibodies such as Ipilimumab. However improvement of targeted immunotherapy is also clearly dependent on the identification of new tumor associated and also specific antigens as well as HLA ligands (Stevanovic 2002). This stresses the need for concise epitope libraries comprising ligands from different tumor entities and benign tissue for various HLA alleles (Krüger *et al.* 2005).

8.1 Targeting the individual patient

The greatest leap forward in the struggle for a successful immunotherapy, at least from the authors' point of view are expected from a patient individualised approach. Tremendous progress in DNA sequencing during the last few years has enabled us to look at the genetic basis of each individual cancer. Comparison of somatic mutations within different tumors has shown that distinct mutational patterns are not only present between different tumor types but also between tumors of the same entity clearly underlining the need for an individualized approach (Ocana & Pandiella 2010).

Combination of whole genome sequencing with HLA ligandome analysis will in future lead to the identification of mutated tumor specific HLA ligands presented on tumor cells. The advanced utilisation of computer-aided high throughput methods enables fast and accurate identification of vaccine candidates. This demonstrates that individualised anti-tumor therapy is also feasible within a certain time frame (first vaccination estimated 5-7 weeks post surgery see Table 4).

Step	Approx. Duration	Section in Figure 2
Genome sequencing	> 2 Weeks	Blue
Ligandome analysis	1-2 weeks	Red
GMP grade peptide synthesis	>2 Weeks	Red
	∑ ~ 6 Weeks	

Table 4. Current time limitations until vaccine availability

The use of mutated HLA ligands should also eliminate the need for time consuming *in vitro* T-cell immunogenicity testing since these antigens are tumor specific neoantigens and thus foreign to the immune system. Differences in the strength of an immune response towards a

certain mutated peptide antigen can, however, not be excluded. Nevertheless, the probability of response failure can be greatly reduced by a combination of several mutated peptides to a multi-epitope vaccine.

Compared to other treatment strategies, immunotherapy aimed at mutated antigens does not necessarily need to target tumor driver mutations, which are usually key to oncogenic properties. Instead, any mutation specific for a certain tumor which is found to be expressed and presented by HLA molecules can be used. Thereby the amount of possible vaccination candidate peptides is greatly increased. The importance of this fact is obvious given that only 2-5 driver mutations among the 1000-10000 somatic mutations found can be unambiguously identified within each tumor (Stratton 2011).

8.2 Targeting the tumor microenvironment

Failure to eradicate cancer by targeting tumor cells exclusively has extended the focus in anticancer therapy during the last few years to include the tumor microenvironment (Kenny *et al.* 2007). Evidence is accumulating to confirm intense crosstalk between tumor cells and their surroundings. Tumor cells not just passively profit from nutritional factors but actively shape their own environment towards their specific needs in a process of coevolution (Polyak *et al.* 2009). Clear cell renal cell carcinoma are a prime example of this since they are usually hypervascularized. This is nearly always the result of a genetic defect in the hypoxia inducible factor 1 signalling cascade leading to secretion of large amounts of proangiogenic VEGF (Sufan *et al.* 2004). Other well established mechanisms of tumor-stroma interaction are the remodelling of the extracellular matrix by tumor-associated macrophages(Mantovani *et al.* 2006), the expression of stimulatory growth factors by tumor asociated fibroblasts (Bhowmick *et al.* 2004) and of course the attraction of immune suppressive cells (Baglole *et al.* 2006). The idea of targeting the cancer's „safe haven" might therefore not only deprive the tumor from its nutritional basis but hopefully also, at least in part reverse immunosuppression. Targeting the tumor stroma in RCC is already being done: tyrosine kinase inhibitors, which currently represent first line treatment for metastatic RCC, are thought to act primarily on endothelial cells, thereby inhibiting angiogenesis.

Combination therapy of TKIs together with cytokine treatment (Miller & Larkin 2009) as well as TKIs with targeted immunotherapy (Rini *et al.* 2011) are already being tested in clinical trials. But what about targeting the stroma directly with a specific immunotherapy? Stroma cells within tumor cells seem to differ in various aspects from their normal counterparts. Endothelial cells within the tumor show a highly fenestrated chaotic organization and often lack supportive smooth muscle cells as well as a regular basement membrane (Aird 2009). Cancer associated fibroblasts have an activated phenotype with high proliferative capacity, less growth requirements and show a high capacity to recruit endothelial progenitor cells (Orimo & Weinberg 2006; Li *et al.* 2007). Differences between normal and tumor stroma can also be observed on the level of gene expression (Ma *et al.* 2009) and are probably based on different epigenetic alterations (Hu *et al.* 2005). Some groups have reported that tumor associated stroma cells are also subject to clonally selected somatic mutations in tumor suppressor genes (TP53) (Kurose *et al.* 2002) and oncogenes (EGFR) (Weber *et al.* 2005) and further show a high degree of genomic instability (loss of chromosomal heterozygosity) (Moinfar *et al.* 2000). However these results are still controversial and might also be due to technical issues (Polyak, Haviv et al. 2009).

Nevertheless, considering the overall changes within tumor associated stroma might render these cells susceptible to an attack by different immune cells. In order to include the targeting of tumor stroma cells into an immunotherapeutic approach, immunological accessible antigens, especially HLA ligands which are exclusively expressed on tumor stroma need to be identified.

8.3 Targeting cancer stem cells

Another upcoming field in cancer therapy is based on the observation that tumor cells within some cancer types at least show a hierarchical organization which is closely resembling that of normal benign tissue. According to the cancer stem cell hypothesis only a rare population of tumor cells termed cancer stem cells (CSC) or tumor initiating cells (TIC) are ultimately responsible for initiating and driving tumor growth. CSCs share some characteristics with normal tissue stem cells including the potential of self renewal, indefinite division, slow replication rate, greatly increased DNA repair capacity and finally the ability to divide asymmetrically, thereby giving rise to new cancer stem cells and rapidly dividing more differentiated tumor cells that make up the bulk of the tumor (Wang & Dick 2005). Evidence for the existence of a tumor initiating cell population was first provided in acute myeloid leukaemia by John Dick and colleagues in the mid 90s (Lapidot *et al.* 1994; Bonnet & Dick 1997). They demonstrated that tumorigenicity of leukaemia cells was much higher in a subpopulation of $CD34^+CD38^-$ tumor cells. Less than 500 cells exhibiting this particular phenotype were sufficient to engraft immunodeficient mice, whereas even a 100-fold higher number of $CD34^+CD38^+$ tumor cells, which constitute the gross of leukaemic cells, could not initiate engraftment. Even more important engraftment of isolated $CD34^+CD38^-$ cells repeated the composition of the original tumor in that $CD34^+CD38^-$ cells also gave rise to the major leukaemic cell population of $CD34^+CD38^+$ cells.

Several years later, the presence of a cancer stem cell population in solid tumors was shown for breast cancer (Al-Hajj *et al.* 2003). Since then, the existence of a CSC-population has been shown for many different types of cancer including brain, prostate, liver, colon and lung (for a review see (Visvader & Lindeman 2008)). In renal cell carcinoma, the existence of a $CD105^+$ cancer stem cell population has also been postulated but final proof regarding its existence is still missing (Bussolati *et al.* 2008). The attraction of the cancer stem cell model is in part based on its ability to explain long known but still poorly understood clinical phenomena, the relapse of cancer patients after a period of tumor regression and even absence of detectable lesions. CSC are known to be highly resistant to chemo- and radiotherapy. They can remain quiescent for a prolonged period of time and then restart tumor growth at a distant site leading to metastasis. This bears a fundamental clinical impact for the treatment of cancer patients. As long as CSCs are not effectively killed by a treatment we won't be able to completely eradicate a certain tumor (Clevers 2011). Considering the resistance mechanisms to standard therapeutic approaches, immunotherapy may indeed be the only therapeutic option to directly target these cells. Therefore cancer stem cell specific antigens urgently need to be discovered and their potential for a targeted immunotherapy evaluated.

There is ample evidence that specific immunotherapy will play a very important role in the future not only in RCC but rather for the treatment of different cancer types. The first targeted immunotherapy Provenge (Sipuleucel-T) manufactured by Dendreon has just recently been approved by the FDA for the treatment of advanced prostate cancer. Provenge

consists of an autologous dendritic cell vaccine loaded with a fusion protein of GM-CSF with the prostate specific antigen prostatic acid phosphatase (PAP). Although it is far too early to speak of a triumphal course of this new category of cancer treatment at least a start has been made.

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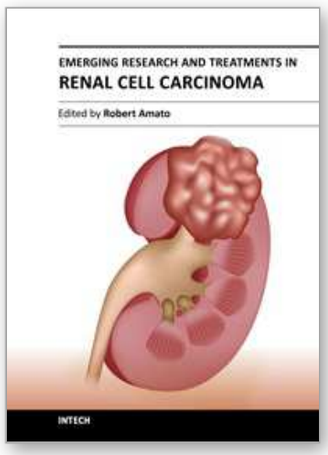

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The field of renal cell cancer has undergone a significant resurgence. This book summarizes up-to-date research and innovative ideas for the future in this rapidly changing field, which encompasses medicine, surgery, radiation oncology, basic science, pathology, radiology, and supportive care. This book is aimed at the clinician or scientist who has an interest in renal cell cancer, whether they are academic or nonacademic. The book covers tumor biology, molecular biology, surgery techniques, radiation therapy, personal testimonies, and present and future treatments of the disease that are on the horizon. The goal was to produce a textbook that would act as an authoritative source for scientists and clinicians and interpret the field for trainees in surgery, medicine, radiation oncology, and pathology.

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