universität wien

DISSERTATION

Titel der Dissertation

Generation of a polymimotope vaccine against malignant melanoma

angestrebter akademischer Grad

Doktor/in der Naturwissenschaften (Dr. rer.nat.)

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Dissertationsgebiet (lt. Studienblatt):	Genetik – Mikrobiologie (Stzw)
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Wien, im Jänner 2011

Danksagung

Der experimentelle Teil dieser Arbeit wurde in der Arbeitsgruppe von Priv. Doz. Dr. Stefan Wagner an der Abteilung von ao. Univ.-Prof. Dr. Heimo Breiteneder am Institut für Pathophysiologie und Allergieforschung des Zentrum für Pathophysiologie, Infektiologie und Immunologie der Medizinischen Universität Wien durchgeführt.

Ao. Univ.-Prof. Dr. Heimo Breiteneder und PD Dr. Stefan Wagner danke ich für die Ermöglichung meiner Arbeit an diesem Institut und für die wissenschaftliche Betreuung meiner Dissertation.

Bei der Österreichischen Akademie der Wissenschaften (ÖAW) bedanke ich mich für die finanzielle Unterstützung meiner Dissertation durch ein DOC-fFORTE Stipendium.

Ich danke auch ao. Univ.-Prof. DI Dr. Reingard Grabherr und PD DDr. Angelika Riemer für die Begutachtung dieser Arbeit.

Ein ganz großes Dankeschön an meine LaborkollegInnen des „Lab 16“!
Danke Nina für deine Unterstützung in der Zellkultur und bei den allgemeinen Belangen des Laboralltages, Gerlinde für deine grenzenlose Geduld (v.a. mit mir) bei den Mausexperimenten, und natürlich Christian und Merima für euren wissenschaftlichen Beistand.

Ein riesiges DANKE an Ursula und Julia! Danke, dass ich mit all meinen kleinen und großen Problemchen zu euch kommen kann.

DANKE an meine Familie und meine Freunde! Danke, dass ihr alle für mich da seid, mich unterstützt und mir Kraft gebt!

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Zusammenfassung

"Peptidmimics" von konformationellen Epitopen eines Tumorantigens werden auch als Mimotope bezeichnet und stellen vielversprechende Kandidaten für die Entwicklung von Tumorvakzinen dar. Derartige Mimotopvakzine induzieren eine Tumorantigen-spezifische Immunantwort. Kürzlich konnte für das maligne Melanom gezeigt werden, dass durch Vakzinierung von Kaninchen mit einem an Tetanustoxoid gekoppelten Mimotop des HMW-MAA ("high molecular weight-melanoma associated antigen") Antikörper gebildet wurden, die das Tumorwachstum in einem Melanommausmodell hemmen konnten. HMW-MAA wurde als Zielantigen gewählt, da es von vielen Melanomzellen exprimiert wird und in normalen Geweben fast nicht vorkommt.

Das Ziel dieser Dissertation war die Entwicklung einer Polymimotopvakzine, die eine starke humorale Immunantwort gegen mehrere Epitope des HMW-MAA induzieren sollte. Verschiedene Studien haben gezeigt, dass Vakzine, die gegen mehr als ein Epitop eines Tumorantigens gerichtet sind, eine höhere Erfolgsrate aufweisen. Der Effekt der induzierten Antikörper sollte sowohl *in vitro* (Tumorproliferation, antikörperabhängige, zellvermitteltete Zytotoxizität, komplementabhängige Zytotoxizität) als auch *in vivo* (Melanommausmodell: C57BL/6 Mäuse mit einem subkutanen Tumor der HMW-MAA transfizierten Mausmelanomzelllinie B16F10) untersucht werden.

Mittels einer linearen pIII-12mer Phagenpeptidbank wurden Peptidliganden für eine Reihe von anti-HMW-MAA monoklonalen Antikörpern (mAbs VT80.12, VF1-TP43, VF1-TP34, 149.53 und 225.28S $F(ab')_2$) selektiert. Diese Antikörper erkennen unterschiedliche Epitope des HMW-MAA, weshalb sie sich gegenseitig in ihrer Bindung nicht inhibieren. Es wurden verschiedene Panningstrategien angewendet (Panning mit Oberflächenimmobilisierung und in Lösung mit Protein G- bzw. Streptavidin-Bindung). Für jeden mAb wurde mindestens ein Peptidligand identifiziert. Es waren aber nur die Peptide, die für die mAbs VT80.12 und VF1-TP43 identifiziert wurden, auch in der Lage, die Bindung der entsprechenden mAbs an das HMW-MAA zu inhibieren. Daher wurden diese beiden Peptide jeweils an KLH („keyhole limpet hemocyanin") als immunogenen Träger gekoppelt. Die in Kaninchen induzierten Antikörper zeigten einen starken Hintergrund verursacht durch die anti-KLH Antikörper. Durch die Immunisierung von BALB/c Mäusen konnte dieses Problem gelöst werden. Beide Peptide induzierten Peptid-spezifische Antikörper, aber nur das

VT80.12-Peptid induzierte auch anti-HMW-MAA Antikörper. Diese Antikörper waren jedoch nicht in der Lage, das Wachstum der HMW-MAA exprimierenden humanen Melanomzelllinie 518A2 *in vitro* zu inhibieren.

In dieser Arbeit konnten sehr viele neue Informationen für die Selektion von Mimotopen gewonnen werden, jedoch konnten noch keine idealen Mimotopkandidaten für die Polymimotopvakzine identifiziert und im Melanommausmodell getestet werden.

Summary

Peptide mimics, also called mimotopes, of conformational epitopes of tumor antigens are promising candidates for the design of anti-tumor vaccines. Such mimotope vaccines stimulate a tumor antigen specific immune response. For melanoma, it was recently demonstrated that vaccination of rabbits with a mimotope of the high molecular weight-melanoma associated antigen (HMW-MAA) coupled to tetanus toxoid induced antibodies that suppressed tumor growth in a melanoma xenotransplant severe combined immunodeficiency mouse model. The HMW-MAA was selected as target antigen because it is highly expressed on melanoma cells and has a restricted distribution in normal tissues.

The aim of this thesis was to develop a polymimotope vaccine that would induce a strong humoral immune response against several epitopes of the HMW-MAA. Several studies have demonstrated that vaccines directed against multiple epitopes of a tumor antigen have a higher success rate. The effect of the induced antibodies were then to be tested *in vitro* (tumor proliferation, antibody-dependent cell-mediated cytotoxicity, complement-dependent cytotoxicity) and *in vivo* (melanoma mouse model: C57BL/6 mice harboring a subcutaneous tumor of the mouse melanoma cell line B16F10 transfected with the HMW-MAA).

Peptide ligands for a panel of anti-HMW-MAA monoclonal antibodies (mAbs) – VT80.12, VF1-TP43, VF1-TP34, 149.53, and 225.28S $F(ab')_2$ – which recognize distinct epitopes on the HMW-MAA and do not cross-inhibit each other were selected using a linear pIII-12mer phage display peptide library by surface panning as well as solution-phase panning with protein G or streptavidin capture. Peptide ligands were identified for each mAb, but only one peptide identified for each of the mAbs VT80.12 and VF1-TP43 was able to inhibit the binding of the respective mAb to the HMW-MAA. These two peptides were then coupled to keyhole limpet hemocyanin (KLH) as immunogenic carrier and used for immunization of rabbits. Due to the high background of the KLH-induced antibodies, further immunizations were performed in BALB/c mice. Both peptides induced a peptide specific immune response but only the VT80.12-peptide induced anti-HMW-MAA antibodies. However, these antibodies did not suppress the proliferation of the HMW-MAA expressing human melanoma cell line 518A2 *in vitro*.

In summary, we have produced a lot of new methodologic know-how on the selection of mimotopes but could not yet identify ideal mimotope candidates which could be tested as parts of a polymimotope vaccine in the melanoma mouse model.

Chapter I

Background

Malignant melanoma

Malignant melanoma represents the most common form of fatal skin cancer and one of the most common fatal malignancies among young adults. Worldwide incidence rates have been constantly increasing for at least 30 years more than that of any other cancer, resulting in an estimated increase at a rate of 5% per year [1-4]. In 2009, the American Cancer Society estimated 68,720 new cases and 8,650 deaths for melanoma in the USA [5]. As shown in figure 1, invasive melanoma currently is the fifth most frequently diagnosed cancer in men and the sixth most frequently diagnosed cancer in women in the USA [5,6].

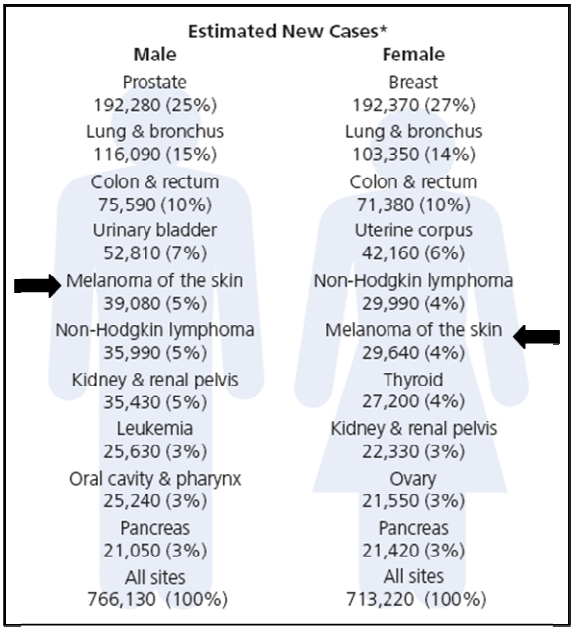

Figure 1. Estimated new melanoma cases are indicated for men and women with black arrows (adapted from Cancer Facts & Figures 2009 [5])

The highest incidence of melanoma worldwide is observed in Australia – with a clear latitude gradient within the continent – and in New Zealand (Table 1). Reported incidence rates are much lower for the USA and Europe. All European countries report a higher incidence in females than males. In contrast, higher incidence for males is reported in Australia, New Zealand, and the USA [4]. In addition, the American Cancer Society reports rapid increases among young white women (3.8% annual increase since 1995 in those aged 15-34 years) and older white men (8.8% annual increase since 2003 in those ≥65 years) [5].

Table 1. Comparative melanoma incidence for selected states and countries worldwide for the time period 1998-2002 (adapted from MacKie *et al.* [4])

Country	**Incidence (per 10^5 subjects)**	
	Male	**Female**
Australia		
Queensland	55.8	41.1
New South Wales	38.5	26.5
Victoria	27.3	23.4
New Zealand	34.8	31.4
US SEER 14 registries	19.4	14.4
Switzerland, Vaud	16.6	19.6
Norway	14.2	14.6
Sweden	11.9	12.1
Denmark	11.9	14.1
Latvia	3.2	4.2
Lithuania	3.7	5.2
Estonia	5.3	6.6
Belarus	2.7	3.5
Serbia	3.8	4.8

Although diagnosis of melanoma has significantly improved [7], leading to an increase in the percentage of patients being diagnosed with "thin" melanomas (≤1 mm) and a decrease in the percentage of patients diagnosed with "thick" melanomas (>4 mm) [8], the mortality rate for melanoma has been continually increasing over the past decade [1,4].

Melanomas generally originate from benign nevi, which are clonally expanded melanocytes that proliferate abnormally but do not progress [9]. The overcoming of senescence leads to a dysplastic nevus, which can progress to a stage of spreading with low invasive potential (radial growth phase). The next stage (vertical growth phase) often results in metastases to local lymph nodes and eventually to distant sites, including other skin sites, pleura, lungs, liver and brain [9]. A schematic view of this process is shown in figure 2.

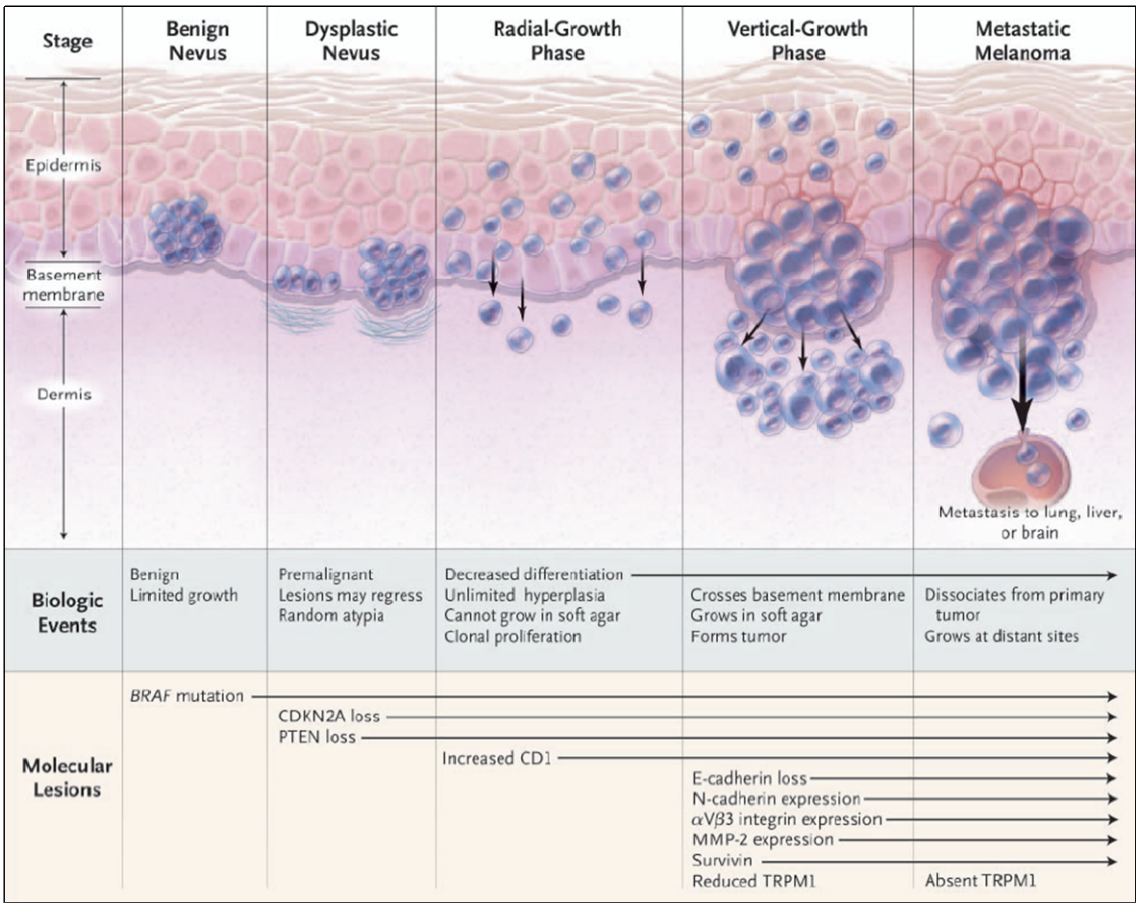

Figure 2. Melanoma development and metastasis (from Miller AJ and Mihm MC [10])

Melanoma associated antigens (MAAs)

Tumor associated antigens or tumor antigens are antigens that are associated with a certain tumor. Thus, melanoma associated antigens (MAAs) or melanoma antigens are highly expressed on melanoma cells. Several MAAs have been identified and characterized. They are grouped into different categories as shown in table 2 and can be expressed at different melanoma stages, i.e. primary or metastatic melanomas [11]. The identification and structural characterization of MAAs opened the possibility to specifically target certain MAAs with immunotherapeutic approaches, as described in following sections.

Table 2. Human melanoma antigens (adapted from Hodi FS [11])

Melanocyte lineage/differentiation antigens (abundant proteins in melanin production) e.g. Tyrosinase, gp75 (tyrosinase related protein-1, TRP-1), gp100, MelanA/MART-1, TRP-2
Oncofetal/cancer-testis antigens (normally expressed in testis and placenta) e.g. MAGE family, BAGE family, GAGE family, NY-ESO-1
Tumor-specific antigens (subtle mutations of normal cellular proteins) e.g. CDK4, β-catenin
Other mutated peptides (activated as a result of cellular transformation) e.g. Mutated introns, *N*-acetylglucosaminyltransferase V gene product, MUM-1, p15
Antigens indentified by monoclonal antibodies e.g. Gangliosides (GM2, GD2, GM3, and GD3), HMW-MAA, p97 melanotransferrin
SEREX antigens e.g. D-1, SSX-2

High molecular weight-melanoma associated antigen (HMW-MAA)

One of the MAAs is the high molecular weight-melanoma associated antigen (HMW-MAA), also known as the chondroitin sulfate proteoglycan 4 (CSPG4) or melanoma chondroitin sulfate proteoglycan (MCSP). This MAA was originally identified with murine monoclonal antibodies (mAbs) on the surface of human melanoma cells at the beginning of the 1980's [12,13].

The HMW-MAA gene is located on human chromosome 15 [14] and encodes a 2322 amino acid (aa) long protein (UniProt accession number: Q6UVK1). A schematic view of the HMW-MAA sequence is shown in figure 3. The first 29 aa residues display a signal peptide and the remaining aa the core protein which contains three major domain structures: a large extracellular domain (1), a short hydrophobic transmembrane region (2), and a short cytoplasmic tail (3). There are 15 potential N-linked glycosylation sites throughout the extracellular domain [1].

The expression of HMW-MAA seems to be regulated by a classical 5' CpG island promoter that precedes the ten exons comprising the human HMW-MAA gene [15]. The level of methylation of the promoter predicts HMW-MAA expression *in vivo*. Unmethylated promoter DNA leads to HMW-MAA expression in human melanoma cells, whereas methylation of promoter DNA results in the absence of HMW-MAA expression in normal human lymphocytes [15].

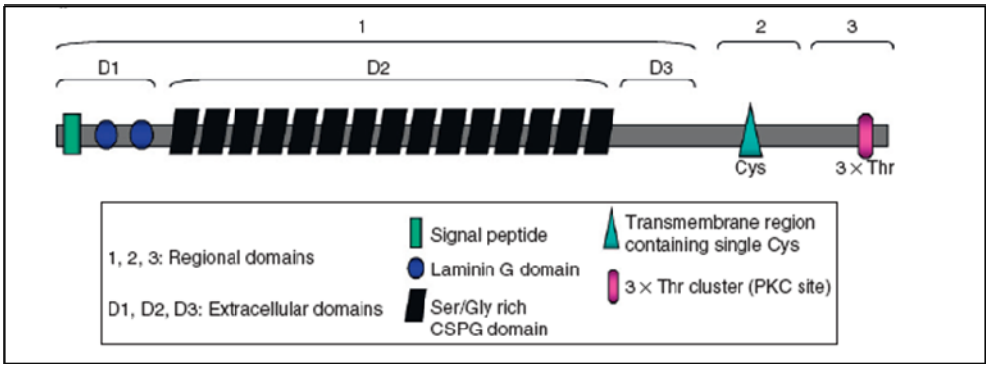

Figure 3. Analysis of the full-length aa sequence of the HMW-MAA protein (from Campoli *et al.* [1])

Potential HMW-MAA homologues have been identified in several animals, i.e. zebra fish, frog, mouse, rat, chicken, dog, cattle, horse, rhesus monkey, and chimpanzee [1]. With the exception of frog chondroitin sulfate proteoglycan, each of the animal homologues shares over 80% aa sequence identity with HMW-MAA and with each other. NG2, the rat homologue, and AN2, the mouse homologue, share more than 90% homology with HMW-MAA.

HMW-MAA is a highly glycosylated integral membrane chondroitin sulfate proteoglycan consisting of an N-linked 280 kDa glycoprotein component and a 450 kDa chondroitin sulfate proteoglycan component expressed on the cell membrane [1,16]. The 280 kDa and 450 kDa components of HMW-MAA contain the same core protein and can be expressed independently. Furthermore, the components are posttranslationally modified in the *trans*-Golgi network through glycosylation and sulfation of carbohydrate moieties. HMW-MAA can be expressed with or without covalently attached chondroitin sulfate glycosaminoglycan [1,17,18]. The number of molecules was determined to be approximately $5x10^6$ per melanoma cell [19].

Several lines of evidence suggest that HMW-MAA plays important roles in cell proliferation, cell migration, invasion, and angiogenesis [1]. HMW-MAA expression correlates with the increase of the proliferative capacity of melanoma cells [20]. In addition, HMW-MAA interacts with extracelluar matrix (ECM) components and promotes cell adhesion and migration, thereby influencing their metastatic potential. Melanoma cells which express HMW-MAA following transfection with HMW-MAA cDNA display higher migratory ability than the parental cells which do not express HMW-MAA [1]. Furthermore, anti-HMW-MAA mAb inhibit melanoma cell attachment to capillary endothelium and interactions with various ECM components including collagen and collagen-fibronectin complexes [21] as well as cytoskeletal reorganization in migrating melanoma cells [1]. Garrigues *et al.* demonstrated that HMW-MAA expression is restricted to cell surface microspike domains of migrating cells *in vitro* [17]. As shown in Figure 4, both the co-localization of HMW-MAA and $\alpha_4\beta_1$ integrin and the interactions between HMW-MAA and ECM components, such as laminin, tenascin, and types II, V, and VI collagen, lead to extensive microspike formation, cytoskeletal rearrangements, melanoma cell spreading and migration, as well as to the activation of focal adhesion kinase (FAK) and ERK 1/2 signaling cascades [1].

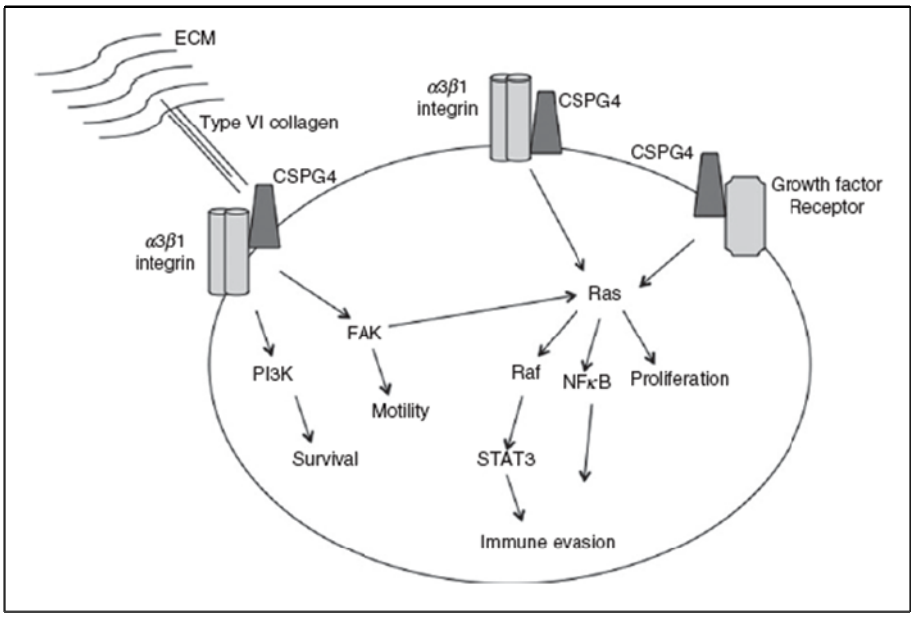

Figure 4. Schematic view of HMW-MAA induced signal transduction (from Campoli *et al.* [1])

HMW-MAA is expressed at high levels on both activated pericytes and pericytes in angiogenic vasculature within the tumor environment as well as by angiogenic blood vessels in normally developing tissues and in neovasculature found in stroma and granulation tissue of healing wounds [1,22]. Therefore, it is suggested that HMW-MAA plays a role in the regulation of angiogensis.

It was originally thought that HMW-MAA has a restricted distribution in normal tissues, having been initially detected only on melanocytes, endothelial cells, and pericytes [1,12,22]. More recently, it became evident that HMW-MAA has a broader distribution, being expressed by a number of normal and malignant cells.
HMW-MAA is also expressed on restricted areas of the interfollicular epidermis as well as the basal layer of normal oral mucosa with the exception of foreskin and perineum, the basal layer of the outer root sheath and the follicular papilla of the hair follicle, chondrocytes, smooth muscle cells, angiomyolipomas, differentiated myofibers of the sarcolemma and neuromuscular junction of human postnatal skeletal muscle, microglial and mesangial cells of the renal glomerulus, and fetal epidermal melanocytes as well as in the vascular paracellular clefts of placental villi [1].

HMW-MAA has been found to be expressed on more than 90% of surgically removed benign nevi and melanoma lesions, with a limited degree of intra- and interlesional heterogeneity [1]. HMW-MAA expression has also been found in astrocytomas, gliomas, neuroblastomas, squamous cell carcinoma of the head and neck, basal breast cancer, mesothelioma, pancreatic carcinoma, some types of renal cell carcinoma, chordoma, chondrosarcoma, soft tissue sarcomas, and hematologic malignancies (expression on blast cells in both childhood and adult acute lymphoblastic leukemia and childhood acute myeloid leukemia) as well as cancer stem cells [1].

Therapy of melanoma

While most early stage melanomas (thin primary tumors; stage 0-II) are highly curable with optimal surgical excision, patients with advanced stage melanoma (lymph node involvement and metastases; stage III-IV) have a poor prognosis and often succumb, due to failure of metastasis control [1,3,23-25].
Metastatic melanoma is highly resistant to conventional therapy, including standard chemotherapy and radiotherapy. The response rate for conventional agents, like cisplatin, dacarbazine or temozolomide, as single agent or in combination, is only 15-25% [26-31]. Therefore, the need to develop and apply novel and improved therapeutic strategies for the treatment of melanoma has been emphasized.

As melanoma is among the most immunogenic of all solid caners, a lot of different immunotherapeutic approaches to treat melanoma have been investigated in preclinical and clinical studies and have been the focus of several review articles in the recent past [3,9,25,26,32-38]. An overview describing the general mechanisms of the investigated strategies is shown in table 3.

Table 3. Approaches for the treatment of melanoma (adapted from Nestle FO [34])

Active (= Vaccination)
Whole cell vaccines (e.g. autologous or allogeneic melanoma cell lines)
Antigen-based vaccines (e.g. peptides, protein, DNA, RNA)
Adjuvant-based vaccines (e.g. dendritic cells)
Passive
Adoptive cell transfer (e.g. tumor-infiltrating lymphocytes (TILs))
Antibodies (e.g. anti-CTLA-4)
Inhibitors (targeting molecules in signal transduction pathways)
Adjuvants (e.g. Toll-like receptor agonists)
Cytokines (e.g. IL-2, IFN-α)

So far, only the immune-modulating agents interferon-α (IFN-α) and interleukin-2 (IL-2) are approved by the US Food and Drug Administration (FDA). IFN-α is administered for the treatment of high-risk melanoma patients. IL-2 is used for the treatment of patients with metastatic melanoma [25,32,33]. For both cytokines, the overall response rates remain low and the benefits must be balanced against several serious side-effects,

such as flu-like symptoms, anorexia, fatigue, depression, thyroid dysfunction, skin rash, altered blood cell counts, and liver toxicity [9,33].

Noteworthy, the development of a vaccine that would show significant clinical benefit in melanoma has not been successful by now, as clinical responses remain at a low rate. However, the extent of research activity in the field and a number of novel approaches indicate that such an approach remains attractive [36].

Encouraging results were obtained by using immunostimulatory mAbs directed to immune-receptor molecules to increase immune responses. MAbs of this type (e.g. anti-CTLA-4, anti-CD137 and anti-CD40) are currently studied in clinical trials [25]. Nevertheless, complications such as autoimmunity and systemic inflammation are problematic side effects associated with these therapeutics.

In addition, adoptive cell transfer of *ex vivo* expanded autologous tumor reactive lymphocytes in combination with lymphodepletion or myeloablation by the use of chemotherapy or radiochemotherapy resulted in 50-70% objective clinical response [39,40]. However, this are time-consuming and cost-intensive treatments, which are therefore at the moment not routinely applicable in the general clinical practice.

Monoclonal antibodies in clinical use

As mentioned above, to date immunotherapeutic approaches to treat melanoma have not been successful. However, chimeric or humanized mAbs directed against tumor antigens of diverse malignancies represent a recent and very significant addition to the therapeutic anticancer armamentarium. Although considerations of side effects, therapy with mAbs is reasonably safe in comparison to other therapeutic modalities used for cancer patients. Today, several mAbs have been approved by the FDA as antibody-based therapeutic anticancer drugs and are routinely used in the clinic. These antibodies are summarized in table 4.

Table 4. FDA-approved tumor antigen specific mAbs for human cancers (adapted from Campoli *et al.* [41])

mAb	Target	Isotype	FDA-approved disease
Rituximab	CD20	Chimeric IgG1	$CD20^+$ low-grade lymphoma diffuse large B-cell lymphoma follicular lymphoma
^{90}Y Ibritumomab + tiuxetan	CD20	Radiolabeled murine IgG1	$CD20^+$ low-grade lymphoma
^{131}I Tositumomab	CD20	Radiolabeld murine IgG1	$CD20^+$ low-grade lymphoma
Alemtuzumab	CD53	Humanized IgG1	Chronic lymphocytic leukemia
Gemtuzumab + ozogamicin	CD33	Recombinant humanized IgG4 -conjugated to calicheamicin	Acute myelogenous leukemia
Trastuzumab	Her2/neu	Humanized IgG1	$Her2/neu^+$ breast cancer
Cetuximab	EGFR	Chimeric IgG1	$EGFR^+$ colon cancer
Panitumumab	EGFR	Fully human IgG2	$EGFR^+$ colon cancer
Bevacizumab	VEGF	Humanized IgG1	Colon cancer recurrent or advanced non-small cell lung cancer metastatic breast cancer

The huge costs of the production of mAbs are the greatest disadvantage of this kind of therapy. Doses used in humans are typically in the mg per kg body weight range, and most regimens involve repetitive applications over longer time periods. Therefore, expensive GMP produced batches are required for quality control, toxicology and clinical trials. An example for the high costs is given in a recent publication where clinicians from the Norfolk and Norwich University Hospital estimated that more than € 2.9 millions were necessary per year to make Herceptin® (Trastuzumab) available to 75 patients who might be eligible for the treatment [42].

Monoclonal antibodies directed to HMW-MAA

Although poorly immunogenic in humans, the HMW-MAA is highly immunogenic in BALB/c mice, as indicated by the high frequency of HMW-MAA-specific antibody-secreting hybridomas generated from BALB/c mice immunized with HMW-MAA-bearing human melanoma cells [12]. Consequently, a large number of mouse anti-HMW-MAA mAbs has been developed (see table 5). Analysis of a panel of mouse anti-HMW-MAA mAb by cross-inhibition experiments classified them into at least six groups that identify distinct and spatially non-overlapping antigenic determinants [12]. In general, these anti-HMW-MAA mAbs are poor mediators of complement- and cell-dependent cytotoxicity of melanoma cells, although they are able to inhibit spreading, migration and invasion of melanoma cells *in vitro* [1].

Table 5. Grouping of a panel of HMW-MAA-specific mAbs according to their epitope specificity (adapted from Campoli *et al.* [12])

Group 1	**Group 2**	**Group 3**	**Group 4**	**Group 5**	**Group 6**
149.53	225.28S	763.74	VF1-TP34	VF1-TP41.2	VF20-VT87.41
VZ68.2	VF4-ZP109	VT80.12	VF4-TP108	VF1-TP43	VF20-VT5-1
	653.25	TP32	VF20-VT1.7		VF18.176
			543		VT67.5
			116		TP61.5
					TP175-11
					724
					9.2.27

It was demonstrated that two anti-HMW-MAA mAbs (225.28S and MK2-23) had anti-tumor effects *in vivo*. The mAb 225.28S was generated by immunizing female BALB/c mice with human melanoma M21 cells [43]. An interesting effect was observed in a follow-up study of more than 300 patients who had received immunoscintigraphy with ^{99m}Tc-labeled 225.28S F(ab')$_2$ fragments as part of a routine melanoma staging protocol. Survival time was significantly longer in patients who had received multiple injections as compared to those who had received only a single dose [44]. This finding suggests that F(ab')$_2$ fragments arrest tumor progression for a limited time and the effect cannot be due to any Fc mediated functions, as no Fc portions were present in the injected material.

Furthermore, Hafner *et al.* were able to demonstrate that the mAb 225.28S was able to suppress tumor growth in a human melanoma xenotransplant model in severe combined immunodeficiency (SCID) mice [45].

In addition, stage IV melanoma patients had a significant increase of survival prolongation upon immunization with the anti-idiotypic mAb MK2-23, which mimics the epitope of the anti-HMW-MAA mAb 763.74 [46,47].

Cancer vaccines – from peptide to mimotope vaccines

Passive administration with tumor antigen specific mAbs, like the treatment with the Herceptin® antibody, has produced promising results in the clinics. However, a number of concerns remains such as repeated treatments and associated costs, limited duration of therapeutic effectiveness, and possible undesired immunogenicity. Therefore, a therapeutic approach capable of inducing active specific immunity would offer sustained protection at a lower cost, preventive therapy and long term immunity.

One of the great problems in developing cancer vaccines is that tumor associated self-antigens do not induce an immune response, since the corresponding reactive B and T cell clones have been eliminated during the establishment of self-tolerance. Especially for melanoma, this kind of malignancy seems to be very efficient regarding tumor escape/evasion of the host immune responses via tumor-induced immunosuppression. The mechanisms of immunoediting include inhibition of maturation of antigen presenting cells or downregulation of MHC class I expression of melanoma cells. In addition, T cell tolerance or anergy is induced upon secretion of immunosuppressive cytokines like IL-10 and transforming growth factor-β (TGF-β) [33], as shown in Figure 5.

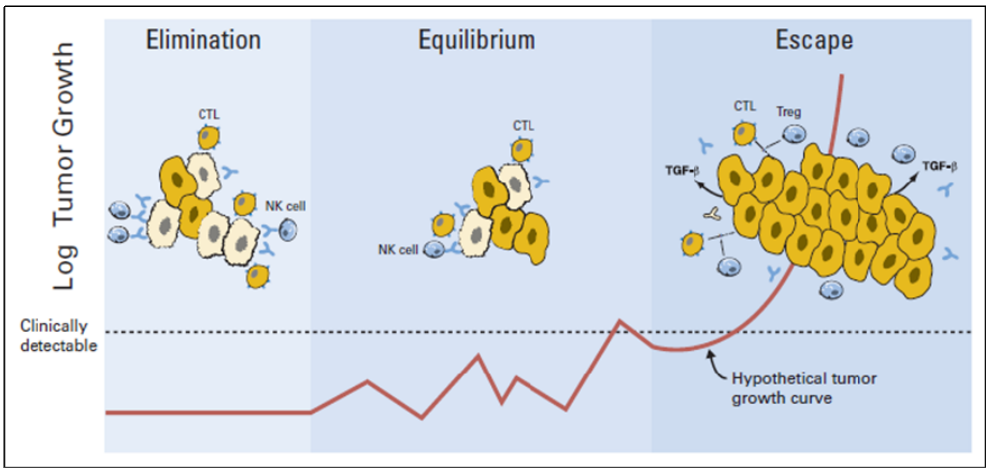

Figure 5. Overview of immunoediting (from Kirkwood *et al.* [33])

As most of the active melanoma vaccines aim to induce a cellular immune response by the induction of cytotoxic T lymphocytes [38], they somehow fail due the above mentioned immunoediting mechanisms used by melanoma.

Therefore, and since some tumor antigen specific mAbs are successfully used in the clinics to treat different cancers, several strategies have been developed to overcome this problem via focusing on B cells. As an example, research groups have predicted putative B cell epitopes of the tumor antigen Her-2/neu and could demonstrate that some of these B cell epitopes coupled to an immunogenic carrier protein induced a humoral immune response directed to the tumor antigen with anti-tumor activity [48,49].

Cancer vaccines designed to elicit an antibody response that targets antigenic sites on a tumor antigen must closely mimic the three-dimensional structure of the corresponding region on the antigen. Studies of the three-dimensional structures of antigen-antibody complexes showed that antigenic epitopes are conformational and vaccine design should consider such parameters to elicit antibodies of high affinity.
Molecular mimicry provides a way to generate vaccine components that elicit and/or enhance an immune response against tumor antigens that are mostly non-mutated self-antigens and therefore poorly or non immunogenic in patients.

The benefits of synthetic peptides and the knowledge of the effects of epitope mimicry led to the development of mimotopes which are peptides that mimic the structure of the epitope of an antigen that is defined by an antibody. The term “mimotope” was introduced by Geysen and his colleagues in the 1980’s [50]. Easy identification of mimotopes was made feasible by the application of the so called “phage display” technology, which was developed by George P. Smith [51].

Mimotopes are selected using random peptide phage display libraries that consist of filamentous phage particles (e.g. bacteriophage M13, fd, and f1) displaying random peptides of defined length on their surface. Most commonly, peptides are either fused to the phage minor coat protein pIII (Gene 3 protein; Fig. 6) or, at a higher copy number, to the major coat protein pVIII (Gene 8 protein; Fig. 6). These are peptides from 4 to 40 aa residues in length, assembled either in linear or in constrained (circular) form [52].

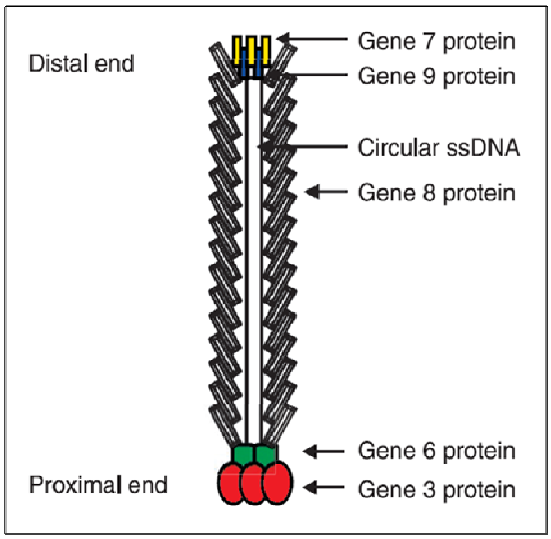

Figure 6. Schematic illustration of the bacteriophage M13 (from Stopar *et al.* [53])

For biopanning, a mAb is immobilized and incubated with a phage display peptide library. Phages displaying specific peptides for the mAb bind to it and can then be eluted by lowering the pH. Amplification of the eluted phages occurs via infection of host cells, mostly *E. coli*. Amplified phages are then used for further rounds of infection to increase the number of specific phages. Single clones are then used to prepare DNA and to determine the sequences coding for the peptides.
A peptide representing a mimotope has to fulfill two requirements: (1) it has to inhibit the binding of the mAb to the antigen and (2) it has to induce a humoral immune response that is directed to the natural antigen.

At the beginning of the 1990's, it was demonstrated that mimotopes were able to mimic discontinuous, i.e. conformational epitopes which are mainly recognized by antibodies [54,55]. Mimotopes can be found for proteins as well as for carbohydrates [56]. Furthermore, mimotopes can be identified with no prior information concerning antibody specificity [57,58].
Mimotopes of several tumor antigens have been generated and were used as vaccines in animal models. One of the first mimotopes of a tumor antigen was described for the prostate-specific membrane antigen [59]. A mAb recognizing an unknown antigen present on the surface of many tumor cells was used to generate a mimotope that was able to prolong the life span of animals inoculated with fibrosarcoma cells [60]. Two mAbs directed against the GD2 ganglioside, expressed on neuroectodermally derived tumors, including neuroblastoma and melanoma, were used in different studies to

generate mimotopes of the antigen. The vaccine-induced antibodies exhibited protection against human GD2-positive melanoma growth in the SCID mouse xenograft model [61] or induced a reduction of spontaneous liver metastases [62].

MAbs used in the clinic also served as sources to generate mimotopes of tumor antigens. Mimotopes of Her-2/neu were generated using trastuzumab [63,64], of epidermal growth factor receptor (EGFR) using cetuximab [65,66], and of CD20 using rituximab [67-70]. Some of these mimotopes [65-67] induced a humoral immune response able to inhibit tumor cell growth *in vitro* and are good candidates for active immunotherapy.

For melanoma, Hafner *et al.* described mimotopes of the melanoma cell-adhesion molecule (Mel-CAM), which induced Mel-CAM specific antibodies in BALB/c mice. These antibodies showed an enhanced proliferation of the Mel-CAM positive human melanoma cell line MelJuSo *in vitro*. However, these antibodies mediated low but specific cell lysis of MelJuSo cells due to complement dependent cytotoxicity [71,72].

In regard to HMW-MAA, mimotopes were successfully identified using the mAb 225.28S [73,74] and the mAb 763.74 [75], respectively. These mimotopes induced HMW-MAA specific antibodies that showed anti-tumor effects *in vitro* [73-75] as well as *in vivo* in a human melanoma xenotransplant SCID mouse model [76].

Multi-epitope vaccines

An interesting effect was observed in a study where SCID mice were treated with a combination of mAbs directed to Her-2/neu. The mixture was more effective than the individual mAbs indicating a synergistic effect [77]. Chen *et al.* observed similar results using a DNA vaccine containing four mimotopes of the MG7 antigen for gastric cancer in BALB/c mice [78]. Wagner *et al.* investigated the effect of a mixture of three peptides in a c-neu transgenic mouse model and observed higher inhibitions of tumor growth with the combination [79]. The authors concluded that targeting several epitopes of a tumor antigen with antibodies was more effective compared to a vaccine targeting only one epitope.

This synergistic effect of targeting multiple epitopes of an antigen was not only investigated for human malignancies, but also for a viral disease of chicken. Wang *et al.* developed a multi-mimotope vaccine for infectious bursal disease virus, which induced high levels of antibodies upon immunization of chicken that showed protective effects against the viral infection [80].

Aim of this thesis

The aim of this study was the development of a mimotope-based polypeptide or polymimotope vaccine that would induce a strong humoral immune response against multiple epitopes of the HMW-MAA. Therefore, peptide ligands for a panel of anti-HMW-MAA mAbs were planned to be selected using the phage display technology. These mAbs recognize distinct epitopes on the HMW-MAA and do not cross-inhibit each other. Phages displaying peptides that inhibit the binding of their corresponding mAb to the HMW-MAA should be selected as mimotopes. These mimotopes were planned to be synthesized and coupled to KLH as an immunogenic carrier protein. Immunization experiments were designed to be performed in BALB/c mice with either single mimotope conjugates or mixtures of mimotope conjugates. After purification of the induced mouse IgGs the *in vitro* effects of these mouse anti-HMW-MAA specific antibodies on HMW-MAA expressing melanoma cells should be assessed. Tumor proliferation, antibody-dependent cell-mediated cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC) should be studied. Finally, the *in vivo* efficacy of the antibodies induced by the mixture of mimotope conjugates that revealed the best *in vitro* results were planned to be tested and compared in a syngenic melanoma mouse model using C57BL/6 mice that harbor a subcutaneous tumor of the mouse melanoma cell line B16F10 which express the HMW-MAA upon transfection with HMW-MAA cDNA.

With this study, the efficacy and superiority of poly- over single-mimotope vaccines *in vitro* and *in vivo* might be proven. The clinical use of mimotope vaccination might still take years, but we hope that this study provides insights towards a new way to prolong overall survival of melanoma patients and ultimately even to cure those patients.

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Chapter II

Successful selection of mimotopes from phage-displayed libraries strongly depends on the selection strategy

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Manuscript submitted to *Molecular Biotechnology*

Abstract

The phage display technology has been successfully applied to study epitopes of a variety of antigens. Different libraries displaying random peptides fused to bacteriophage coat proteins have been developed. Peptide ligands selected from these libraries using monoclonal antibodies (mAbs) can mimic conformational epitopes which are recognized by the antibodies. In the recent past, such peptide mimics, also called mimotopes, have been used to develop antigen specific cancer vaccines. We have focused our research on the high molecular weight-melanoma associated antigen (HMW-MAA) which is highly expressed on melanoma cells. We screened five anti-HMW-MAA monoclonal antibodies with different epitope specificities using a linear pIII-12mer phage display peptide library. Peptide ligands were selected by three different panning strategies: immobilization of the mAb (surface panning), protein G capture of the mAb, or streptavidin capture of the biotinylated mAb (solution-phase panning). Peptide ligands for each mAb could only be identified with the surface panning strategy. We therefore conclude that surface panning is superior to solution-phase panning concerning a straight forward way to identify peptide ligands.

Introduction

The phage display technology uses both filamentous (e.g. M13, fd, f1) and, more recently, lytic bacteriophages (e.g. T4, T7, λ) to display foreign peptides or proteins on the phage surfaces ***(1)***. In the case of filamentous phages, the minor coat proteins pIII, pVI, pVII, and pIX as well as the major coat protein pVIII have been successfully used for peptide presentation ***(1)***. The coat proteins pIII and pVIII are commonly used to display random peptides fused to their N-terminus. Peptides are 4 to 40 amino acid (aa) residues in length, displayed either in linear or in constrained (circular) form ***(2)***. As large foreign peptides can disrupt the structural stability of the phages by hampering assembly and infection as a result of interference with pIII or pVIII function, they can only be displayed as hybrid phage particles ***(3,4)***. Therefore, phage display systems have been classified according to the arrangement of the coat protein genes into three different types: (1) type 3, (2) type 33, and (3) type 3+3 (the same applies to pVIII as well as pVI). Type 3 systems display foreign peptides on every copy of pIII. In type 33 vectors, the phage genome bears two genes encoding for the wild-type and recombinant molecule. Type 3+3 systems have two genes for pIII on separate genomes, the wild-type version on a so called helper phage, the recombinant version on a phagemid ***(4)***. The latter two system types supply mosaic phages.

Epitope study and other relevant research fields have been successfully investigated by using phage display ***(5-7)***. One attractive application of this technology is the identification of mimotopes which are small peptides that structurally mimic a given antibody-binding site but are composed of different amino acids ***(8)***. Mimotopes are able to mimic conformational epitopes both of protein and carbohydrate antigens ***(9)***, and should induce antibodies against the target antigen upon immunization. Therefore, they have been used in vaccines to induce immune responses against bacterial polysaccharides or tumor antigens.

Regarding tumor antigen specific vaccines, several monoclonal antibodies (mAbs) directed against diverse tumor antigens have been used to identify mimotopes which were then investigated as vaccines. For malignant melanoma, the high molecular weight-melanoma associated antigen (HMW-MAA) has presented an interesting target antigen in the recent past. Mimotopes were identified using different phage display peptide libraries by screening several anti-HMW-MAA mAbs. The mAb 763.74 was screened with both a linear pVIII-15mer and a cyclic pVIII-12mer (XCX_8CX) library ***(10)***, the mAb 149.53 with a linear pVIII-15mer library ***(11)***, and the mAb 225.28S with

a linear pVIII-9mer ***(12,13)***, a linear pVIII-15mer ***(11)*** as well as with a cyclic pIII-12mer ($CX_{10}C$) library ***(14)***. All studies followed a surface panning strategy, i.e. immobilization of the mAb by coating to a plastic surface, resulting in the identification of peptide ligands, except for the cyclic pVIII-12mer.

Our intent was to identify peptide ligands for five different anti-HMW-MAA mAbs using surface as well as solution-phase panning strategies to evaluate under which conditions tight-binding peptides can be selected. Therefore, we have applied a commercially available phage display peptide library from New England BioLabs (Ipswich, MA, USA), which expresses 12-mer peptides at the N-terminus of the minor coat protein pIII of bacteriophage M13 (Ph.D.-12™). This library has been successfully used not only for the generation of mimotopes of tumor antigens ***(8,15)*** but also for epitope mapping of antibodies ***(16-18)*** as well as diverse other antigens ***(19-22)***.

Materials and Methods

Monoclonal anti-HMW-MAA antibodies

The mAbs VT80.12, VF1-TP34, 149.53, 225.28S F(ab')$_2$,VF1-TP43 and TP61.5 were developed and characterized as described elsewhere ***(23-26)***.

Biotinylation of mAbs

NHS-LC-Biotin (Pierce, Rockford, IL, USA) was diluted in dimethylformamide at a concentration of 40 mg/ml. Five microliters of this solution was added to 1 mg/ml mAb in PBS and incubated for 45 min at room temperature (RT). Excess NHS-LC-Biotin was removed by dialysis against PBS. Successful biotinylation was proven by streptavidin detection in a dot blot assay. Briefly, biotinylated mAb was dotted onto a nitrocellulose membrane (Whatman, Dassel, Germany) and incubated with alkaline phosphatase (AP)-conjugated streptavidin (GE Healthcare, Little Chalfont, UK). Color development was done with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium.

Cell lines

The human melanoma cell line 518A2 which expresses high levels of HMW-MAA and M14, the human melanoma cell line with no detectable expression of HMW-MAA, were maintained in RPMI 1640 medium (Lonza, Verviers, Belgium). The medium was supplemented with 10% (v/v) FCS and 1% (v/v) antibiotic-antimycotic mix (both from Gibco, Paisley, UK). Both cell lines were cultured in a humidified atmosphere containing 5% CO_2 and 95% ambient air at 37°C.

Preparation of cell lysates

A total of $5x10^7$ melanoma cells was suspended in 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% (v/v) Triton X-100; 1x complete EDTA-free protease inhibitor mix (Roche, Mannheim, Germany)), extensively vortexed and incubated on ice for 15 min. After disruption, samples were centrifuged 10 min at 800 x *g* at 4°C. Supernatants were removed from cell debris and stored at -20°C until use. Protein concentration was determined using a bicinchoninic acid (BCA) protein assay (Pierce).

Microsomal preparations

Cells (~5x10^7) were disrupted in 1 ml lysis buffer (50 mM Na-phosphate, pH 7.4; 2 mM EDTA; 250 mM saccharose; 1x complete EDTA-free protease inhibitor mix (Roche)) using a dounce tissue grindler. Unbroken nuclei were separated by centrifugation at 1000 x *g*. Thereafter, supernatants were centrifuged at 32000 x *g* for 1 h at 4°C. Pellets were solubilized in buffer containing 100 mM Na-phosphate, pH 7.4; 2 mM EDTA; 500 mM NaCl and 1% Triton X-100. Samples were stored at 4°C until use. Protein concentration was determined using a BCA protein assay (Pierce).

Biopanning

The Ph.D.-12™ library that expresses linear 12mer peptides fused to the pIII minor coat protein of bacteriophage M13 was purchased from New England Biolabs (Ipswich, MA, USA). Biopanning protocols for surface and solution-phase panning were executed following the manufacturer's instructions with some minor modifications.

Surface panning (direct coating).

MaxiSorp immunotubes (Nunc, Roskilde, Denmark) were coated with 10 µg mAb in 1 ml 50 mM Na-carbonate buffer, pH 9.6, overnight (o/n) at 4°C. Unspecific binding sites were blocked with TBST (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.1% (v/v) Tween-20) containing 3% (w/v) milk powder for 1 h at RT. Afterwards, the immobilized mAb was incubated with 1x10^{11} phages and 10 µg isotype control (BD Biosciences, Franklin Lakes, NJ, USA) in 1 ml TBST for 1 h at RT with agitation. Unbound phages and phage-isotype complexes were removed by extensively washing with TBST. Bound phages were eluted with 1 ml elution buffer (0.2 M Glycine-HCl, pH 2.2). The eluate was neutralized by adding 150 µl 1 M Tris-HCl, pH 9.1.

Solution-phase panning with surface protein G capture.

MaxiSorp immunotubes (Nunc) were coated with 20 µg protein G (AbD Serotec, Düsseldorf, Germany) in 1 ml 100 mM $NaHCO_3$, pH 8.6 o/n at 4°C. Unspecific binding sites were blocked with 100 mM $NaHCO_3$, pH 8.6 containing 5 mg/ml BSA for 1 h at 4°C. Meanwhile, 1x10^{11} phages were preincubated with 10 µg mAb in TBST at RT. Protein G capture of the phage-mAb complex was allowed for 1 h at RT. Bound phages were eluted with 1 ml elution buffer (0.2 M Glycine-HCl, pH 2.2; 1 mg/ml BSA) and neutralized by adding 150 µl 1 M Tris-HCl, pH 9.1. An incubation step of phages with the isotype control was included for the 2nd as well as for the 3rd round of selection.

Solution-phase panning with surface streptavidin capture.

MaxiSorp immunotubes (Nunc) were coated with 100 µg streptavidin in 1 ml 100 mM $NaHCO_3$, pH 8.6 o/n at 4°C. Unspecific binding sites were blocked with 100 mM $NaHCO_3$, pH 8.6 containing 5 mg/ml BSA and 0.1 µg/ml streptavidin for 1 h at 4°C. Meanwhile, $1x10^{11}$ phages were preincubated with 10 µg biotinylated mAb in TBST at RT. Streptavidin capture of the phage-biotinylated mAb complex was allowed for 30 min at RT. Bound phages were eluted as described above for the solution-phase panning with surface protein G capture.

For all three panning strategies, phages were amplified in *E. coli* ER2738. The amplified phages were purified by precipitation with 20% polyethylene glycol (PEG) 6000, 2.5 M NaCl and used in the next round. Three rounds of selection were performed with increasing Tween-20 concentrations (1^{st} round: 0.1%; 2^{nd} round: 0.3%; 3^{rd} round: 0.5%). After that, individual plaques were picked up randomly of the unamplified 3^{rd} round eluate to screen for specific single phage clones.

Phage ELISA

MaxiSorp immunoplates (Nunc) were coated with 5 µg/ml mAb in 50 mM Na-carbonate buffer, pH 9.6 o/n at 4°C. Nonspecific binding sites were blocked with PBS containing 3% milk powder (3% MPBS). Phage precipitates were diluted in 3% MPBS, added to mAb-coated plates, and incubated for 1 h at RT. After washing with PBS, bound phages were detected using a horse radish peroxidase (HRP)-conjugated mouse anti-M13 antibody (GE Healthcare) diluted 1:5000 in 3% MPBS. Color development was carried out with o-phenylendiamin (Fast o-Phenylendiamin HCl; Sigma, St. Louis, MO, USA). The reaction was stopped by addition of 0.18 M H_2SO_4 and the absorbance was measured at 450 nm.

ssDNA isolation and sequencing

Single phage clones that bound specifically to the respective mAb were amplified in *E.coli* ER2738 (o/n-culture 1:100 diluted) for 4.5 h at 37°C with vigorous shaking. Bacterial cells were pelleted by centrifugation and single stranded DNA from each phage clone was isolated from 2 ml phage containing supernatant using the QIAprep Spin M13 Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Agarose gel electrophoresis (1.2%) was carried out to check ssDNA integrity. PCR was

performed using the SequiTherm EXCEL II DNA Sequencing Kit (Epicentre Biotechnologies, Madison, WI, USA) with an IRD-800 labeled -96 gIII sequencing primer (5´- CCC TCA TAG TTA GCG TAA CG -3´; MWG Biotech, High Point, NC, USA). DNA sequence analysis was performed with an automatic LI-COR fluorescent sequencer 4000 L (LI-COR, Lincoln, NE, USA) and the AlignIR V2.0 software (LI-COR).

Competitive ELISA

MaxiSorp immunoplates (Nunc) were coated with 5 µg/ml mAb in 50 mM Na-carbonate buffer, pH 9.6 o/n at 4°C. Nonspecific binding sites were blocked with 3% MPBS. Phage particles (10^{11} pfu/well) and cell lysates (0.5, 1, and 1.5 mg/ml) were added simultaneously and incubated for 2 h at RT. After washing with PBS, bound phages were detected as described for phage ELISA.

Synthesis of peptides

The peptides NHLDTVMSLRLRC (80.12p3), NYQDLQRTHFKSGPGPGC (43.12p3), and an unrelated peptide AEGEFTRTQPGRFPGGGGGC (control peptide) were synthesized using F-moc strategy by piCHEM (Graz, Austria). The purity of the peptides was ~95%, as assessed by HPLC.

ELISA inhibition assay

Maxisorp immunoplates (Nunc) were coated o/n at 4°C with 4 µg/ml of mAb T61.5 in coating buffer (50 mM Na-carbonate, pH 9.6). Ten nanograms of biotinylated mAb was incubated with increasing concentrations (0, 10, 50, and 100 µg/ml) of synthetic peptide in TBST (0.5% Tween-20) containing 1% (w/v) BSA o/n at 4°C. The next day, microtiter plates were blocked with TBST/3% milk powder and incubated for 3 h at RT with 100 µg/ml microsomal preparations diluted in TBST/1% BSA. After washing, mAb preincubated with peptides was added and incubation was continued for one additional hour at RT. Bound biotinylated mAb was detected using AP-conjugated streptavidin (GE Healthcare), followed by addition of p-nitrophenylphosphate (Sigma). Absorbance was measured at 405 nm. Percentage of inhibition was calculated as follows: 100 – (OD (inhibited)/OD (uninhibited) x 100).

Sequence and structural alignments

Identified peptide ligands were aligned with the HMW-MAA aa sequence (UniProt accession no. Q6UVK1) using the pairwise local alignment BLAST ***(27)***.

Using the ModBase Database of Comparative Protein Structure Models ***(28)***, four models spanning the extracellular domain of HMW-MAA were chosen for mapping of mimotope residues onto the molecular surface of HMW-MAA using MIMOX, a Web Tool for Phage Display Analysis ***(29)***. Laminin alpha 2 chain LG4-5 domain pair (PDB accession no. 1dykA) was used as template to model aa 27-380 of HMW-MAA, the crystal structure of mammalian fatty acid synthase (PDB accession no. 2vz8B) for aa 260-1733, the C-cadherin ectodomain (PDB accession no. 1l3wA) for aa 1259-1832, and the crystal structure of mammalian fatty acid synthase in complex with NADP (PDB accession no. 2vz9A) for aa 680-2234.

Results

To select the positive clones that bind to the anti-HMW-MAA mAbs, a random 12-mer phage display peptide library composed of $1x10^{11}$ independent phage clones was incubated with the mAbs. For each biopanning, the phage titer of the amplified eluate was determined in plaque forming units (pfu) for inputs and outputs to determine the degree of selection.

Surface panning with the mAb VT80.12

After three rounds of selection, the total number of phages that bound to the mAb VT80.12 was increased from $3x10^5$ pfu/ml in the 1st round to $2x10^9$ pfu/ml in the 3rd round (Fig. 1A). Twenty-four individual phage clones were tested by phage ELISA for their binding ability to the mAb VT80.12 (Fig. 1B). Twenty-two positive clones were subjected to DNA sequencing yielding two predominant DNA sequences that encoded the aa sequences NHLDTVMSLRLR ("80.12p3"; eight phage clones) and HFYQFSLLNDMQ ("pVT80-2"; ten phage clones). Phage particles, expressing the two sequences, were co-incubated with a cell lysate from the HMW-MAA expressing human melanoma cell line 518A2 (HMW-MAA^{pos}) or the HMW-MAA negative human melanoma cell line M14 (HMW-MAA^{neg}) on ELISA plate-immobilized mAb VT80.12. Only phages displaying the 80.12p3-peptide were competitively removed in a concentration-dependent way up to 35% (Fig. 2A). Therefore, the peptide 80.12p3 was chemically synthesized with an additional C-terminal cysteine residue for conjugation purposes. This peptide inhibited the binding of the biotinylated mAb VT80.12 to the HMW-MAA only up to 23% in an ELISA inhibition experiment (Fig. 2B).

Surface panning with the mAb VF1-TP43

Three rounds of selection were performed with the mAb VF1-TP43. The phage titer was increased from $1x10^5$ pfu/ml in the 1st round to $3x10^9$ pfu/ml in the 3rd round (Fig. 3A). Thirty individual phage clones were tested for their specificity to the mAb VF1-TP43 (Fig. 3B). Twenty positive clones were subjected to DNA sequencing yielding one DNA sequence encoding the aa sequence NYQDLQRTHFKS ("43.12p3"). In a competitive ELISA experiment, binding of the phage-displayed peptide was reduced up to 33% by a HMW-MAA containing cell lysate (Fig. 4A). The peptide 43.12p3 was synthesized with an GPGPG-linker and an additional C-terminal cysteine residue. It was

able to inhibit the binding of the biotinylated mAb VF1-TP43 to the HMW-MAA in a dose-dependent way up to 92% (Fig. 4B).

Surface panning with the mAb VF1-TP34

Three rounds of selection were performed with the mAb VF1-TP34 showing a distinct increase of the phage titer from $1x10^7$ pfu/ml to $1x10^9$ pfu/ml (Fig. 5A). Thereafter, 141 individual clones were picked up randomly and tested in phage ELISA for their specificity. Only nine phage clones were able to bind specifically to the mAb VF1-TP34. DNA sequencing yielded the two deduced aa sequences IICHPYPKRCVN ("pTP34-1"; eight individual clones) and SSTIQKHLETRR ("pTP34-2"; one clone). Both peptides (as phage-displayed peptides) were not competitively removed by a HMW-MAA containing cell lysate (data not shown).

Surface panning with the mAb 149.53

Four rounds of selection were performed with the mAb 149.53, because the increase of the phage titer was not sufficient after three rounds (Fig. 5B). Thereafter, the phage titer was increased from $2x10^6$ pfu/ml to $1x10^9$ pfu/ml. Overall, 118 individual clones of the fourth round were tested for their binding ability to the mAb 149.53. Twenty-six positive clones were subjected to DNA sequencing resulting in the one deduced aa sequence YVCPPMIHLCYS ("p149.53"). The binding of this phage-displayed peptide to the mAb 149.53 was not inhibited by a cell lysate from HMW-MAA^{pos} cells.

Surface panning with the mAb 225.28S F(ab')$_2$

After three rounds of selection, the phage titer was increased from $2x10^6$ pfu/ml in the 1st round to $2x10^8$ pfu/ml in the 3rd round (Fig. 5C). Twenty-four indiviudal phage clones were tested by phage ELISA for their binding ability to the F(ab')$_2$-fragment of the mAb 225.28S. Twenty-three positive clones were subjected to DNA sequencing yielding one DNA sequence that encoded the aa sequence SWIHWNQADKLF ("p225Fab"). The binding of this phage-displayed peptide to the mAb 225.28S F(ab')$_2$ was not inhibited by a cell lysate from HMW-MAA^{pos} cells.

Identification of peptide ligands by solution-phase panning

Due to the fact that direct coating of an antibody may alter its native 3-dimensional structure – although antibodies should bind preferentially with their Fc-part to the surface of microtiter plates – phage displayed peptides that could mimic conformational epitopes, which are recognized by the variable regions of an antibody, might not be identified. We therefore decided to perform the panning procedure under more native conditions for the mAbs, i.e. as solution-phase panning and used two different strategies.

First, as protein G binds antibodies of the IgG1 isotype specifically via their Fc-part, this possibility seemed to be a very elegant one of identifying specific phages for our mAbs VT80.12, VF1-TP34, and 149.53. Therefore, protein G was immobilized and incubated with the mAb-phage complex. There was no increase of phage titer from the 1st to the 3rd round (~10^7 pfu/ml for each round) and the amplified, precipitated phages from each round were negative for the respective mAb in phage ELISA experiments.

In a second solution-phase panning procedure, streptavidin was immobilized to catch the biotinylated mAbs VT80.12 and VF1-TP34, respectively. Despite increasing phage titer (from ~10^6 pfu/ml in the 1st round to ~10^9 pfu/ml in the 3rd round), the amplified, precipitated rounds were not specific for the mAbs.

Sequence and structure alignments

The peptides 43.12p3 and p225Fab could be aligned to the extracellular domain of HMW-MAA at position 387-397 and 445-449, respectively, by BLAST (Fig. 6). No significant similarities were found for the other peptides.

The peptide 80.12p3 could be located at position 570-527 of HMW-MAA using MIMOX (Table 1). Locations of the peptides 43.12p3, p225Fab, and 80.12p3 on the molecular surface of the HMW-MAA are illustrated in Fig. 7.

Discussion

We have performed biopannings of five anti-HMW-MAA mAbs that recognize different epitopes of HMW-MAA described by lacking cross-reactivity to each other, using the Ph.D.-12™ library from New England BioLabs. Biopannings were performed in three ways: (1) surface panning/direct coating of the mAb, (2) solution-phase panning with surface protein G capture of the mAb, and (3) solution-phase panning with surface streptavidin capture of the biotinylated mAb, yielding different results regarding selection of peptide ligands for each panning strategy.

At least one peptide sequence for each mAb was obtained with the surface panning protocol. Phages binding specifically to the mAb VT80.12 were easily obtained, resulting in two peptide ligands. Whereas the peptide 80.12p3 inhibited the binding of the mAb VT80.12 to the HMW-MAA (Fig. 2), the other peptide (pVT80-2) did not, indicating that the latter peptide did not mimic the antigen binding site of the mAb properly. As inhibitions obtained with the synthetic peptide 80.12.p3 were lower than the ones obtained with the phage-associated peptide, it is possible that the phage particle provides some inhibitory effects for this peptide. This can be explained by the fact that the interaction between the antibody and the phage-displayed peptide depends on the peptide's microenvironment that is provided by the phage particle during the affinity selection ***(30)***.

One peptide ligand was identified for the mAb VF1-TP43. All sequenced phage clones both from the 2^{nd} (data not shown) and 3^{rd} round (Fig. 3) displayed only this aa sequence indicating that this was the dominant peptide ligand for the mAb VF1-TP43. The peptide 43.12p3 inhibited the binding of HMW-MAA to the mAb VF1-TP43 both as phage-displayed peptide (Fig. 4A) and as synthetic peptide (Fig. 4B), excluding the possibility that the conformation of the phage-displayed peptide was constrained by the structural context of the bacteriophage surface. Furthermore, the additional GPGPG-linker of the synthetic peptide might stabilize the peptide's conformation leading to a higher inhibition obtained with the synthetic peptide than the phage-displayed one. These results clearly indicate that the synthetic peptide adopts a conformation in solution that is comparable with that on the phage surface ***(14)***. This proves that this mimotope is a genuine antigenic mimic of the HMW-MAA-epitope recognized by the mAb VF1-TP43. Hence, the peptide 43.12p3 will be considered as a mimotope candidate for immunization experiments to induce HMW-MAA specific antibodies.

Biopanning of the mAb VF1-TP34 was difficult yielding only ~6% positive individual phage clones displaying two peptide sequences. Peptide pTP34-1 contains two cysteine residues at position 3 and 10, which are likely to form a disulfide bridge, thus transforming the linear peptide into a circular one displayed on the phage particle. In our case, regarding coupling the synthetic peptide to an immunogenic carrier protein via an additionally introduced C-terminal cysteine residue for vaccination purposes, the chemical synthesis and the coupling process would only be possible with great effort. Due to this "unpreferable" sequence, the peptide pTP34-1 would be excluded for further examinations. As this peptide was more frequently found than the other peptide (pTP34-2), the first seemed to be the dominant ligand obtained with the library used. However, both peptides did not inhibit the binding of HMW-MAA to the mAb VF1-TP34, indicating that these peptide ligands do not mimic the epitope.

For the mAb 149.53 four rounds of selection were needed to obtain sufficient phage titers (Fig. 5B). After that, one peptide sequence containing two cysteine residues at position 3 and 10 could be obtained for all of the sequenced phage inserts. Therefore, the same conclusions can be made as for the peptide pTP34-1.

With the $F(ab')_2$-fragment of the mAb 225.28S one peptide sequence was obtained. Comparing this sequence to already described mimotopes identified with the mAb 225.28S ***(11-14)*** revealed no sequence similarities. Peptide ligands obtained by Ferrone *et al.* ***(11)***, Hafner *et al.* ***(14)***, and Wagner *et al.* ***(13)*** yielded the same consensus sequence. We suggest that the $F(ab')_2$-fragment, which binds properly to HMW-MAA, has a slightly different three-dimensional structure due to the missing Fc portion compared to the mAb 225.28S, thus preferring different types of peptide constraints.

All identified peptides have one to four aromatic aa residues as well as many hydrophobic aa side chains, suggesting that they might partially mimic carbohydrate moieties, which would be in line with the fact that HMW-MAA is a highly glycosylated integral membrane chondroitin sulfate proteoglycan. The presence of both aromatic and hydrophobic residues is often a characteristic of peptides that mimic carbohydrates, resembling sugar moieties in their size and cyclic shape ***(31)***. However, local alignment or mimotope mapping of the peptides with the HMW-MAA sequence yielded matches for the peptides 43.12p3, p225Fab, and 80.12p3 (Fig. 6+7; Table 1), that could be allocated to surface-exposed residues of the extracellular domain of HMW-MAA.

Both solution-phase panning strategies either by protein G or streptavidin capture did not yield any specific peptide ligand. This might be attributed to restrictions regarding concentrations of either mAb or the catching molecules, incubation time, washing conditions as well as elution conditions, which affect the two important parameters of affinity selection, i.e. stringency and yield.

We therefore conclude that screening a panel of several peptide libraries rather than just one and optimization of screening conditions should lead to the identification of high-affinity peptides. It is possible that a single library will not provide tight-binding peptides for a lot of antibodies as the preference of an antibody for particular peptide ligands is usually not known in advance.

Acknowledgments

We thank Hubert Pehamberger for financial support.

Tables

Table 1 Mimotope mapping. The peptide 80.12p3 was mapped to the HMW-MAA surface using the conservative match mode of the web tool MIMOX ***(29)***. The crystal structure of mammalian fatty acid synthase (PDB accession no. 2vz8B) was used to model aa 260-1733 of HMW-MAA.

aa position	570	568	566	567	569	565	563	561	541	536	534	527
HMW-MAA	Q	H	L	E	T	I	M	S	L	R	L	R
80.12p3	N	H	L	D	T	V	M	S	L	R	L	R

Figures

Fig. 1 Specificity of phages selected for the mAb VT80.12. Amplified panning rounds (A) and 24 selected single phage clones (B) were tested for their specificity to the mAb VT80.12 (■) or a mouse IgG1 isotype control (□) in phage ELISA experiments.

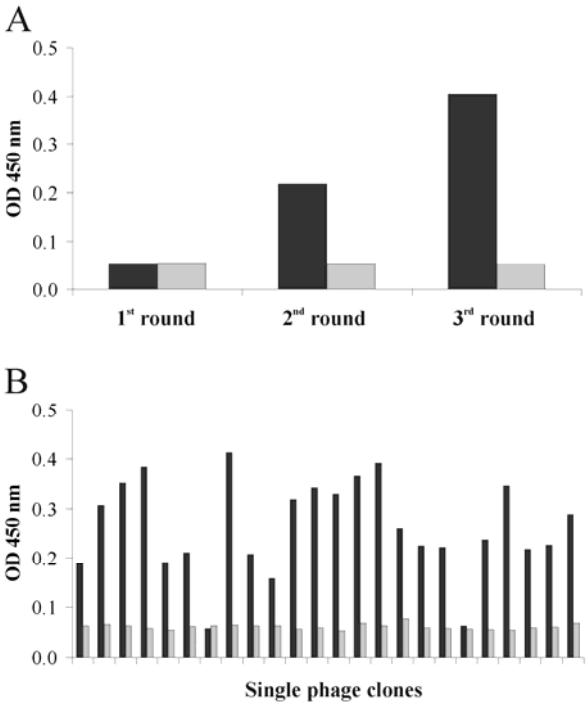

Fig. 2 Mimicry of the phage-displayed peptide "80.12p3". (A) In a competitive ELISA experiment, the binding of the phage-displayed peptide 80.12p3 to the mAb VT80.12 was competed by a cell lysate from HMW-MAA^{pos} cells (■) but not from HMW-MAA^{neg} cells (□). (B) Microtiter plates were coated with the mAb TP61.5 and incubated with a microsomal preparation of 518A2 melanoma cells to catch the HMW-MAA. Biotinylated mAb VT80.12 was preincubated with increasing concentrations of synthetic peptide 80.12p3 (●) and a control peptide (▲), and then incubated with the HMW-MAA. Percentage of inhibition was calculated as follows: 100 – (OD (inhibited)/OD (uninhibited) x 100).

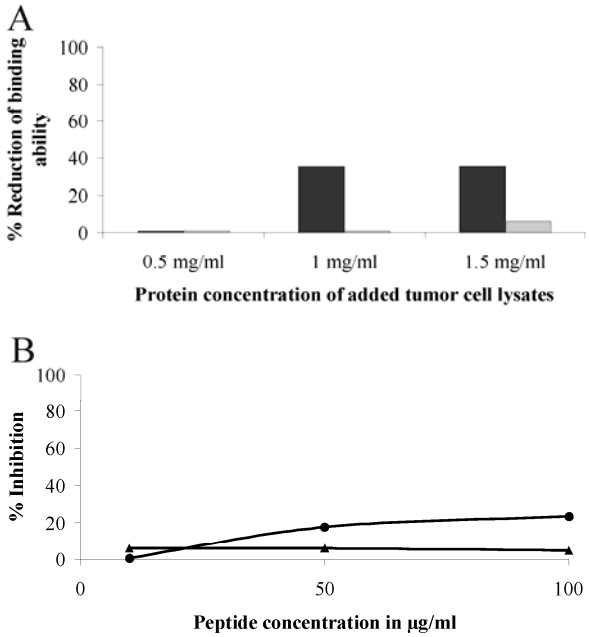

Fig. 3 Specificity of phages selected for the mAb VF1-TP43. Amplified panning rounds (A) and 30 selected single phage clones (B) were tested for their specificity to the mAb VF1-TP43 (■) or a mouse IgG1 isotype control (□) in phage ELISA experiments.

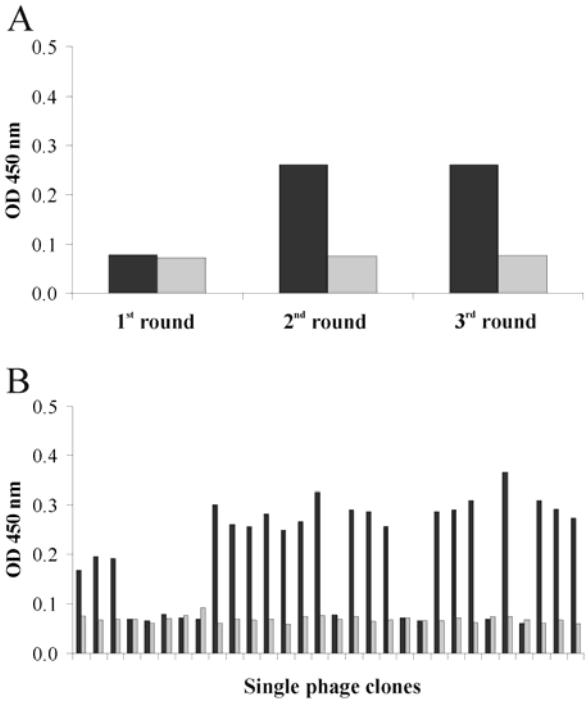

Fig. 4 Mimicry of the phage-displayed peptide "43.12p3". (A) A cell lysate from HMW-MAA^{pos} cells (■) but not from HMW-MAA^{neg} cells (□) was able to compete the binding of the phage-displayed peptide 43.12p3 in a concentration dependent way. (B) Graph showing percentage of inhibition as calculated from reduction in binding ability, when biotinylated mAb VF1-TP43 was preincubated with increasing concentrations of synthetic peptide 43.12p3 (●) and a control peptide (▲) ,and then incubated with the HMW-MAA.

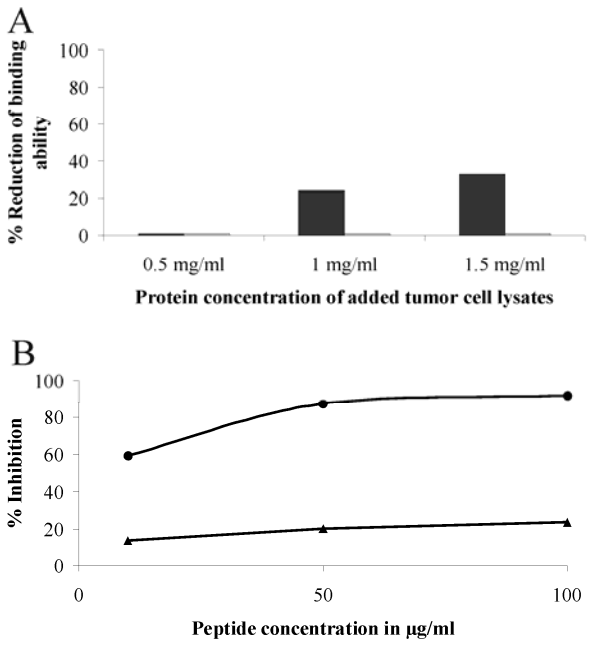

Fig. 5 Specificity of phages selected for the mAbs VF1-TP34 (A), 149.43 (B), and 225.28S F(ab')$_2$ (C). Amplified panning rounds were tested for their specificity to the respective mAb (■) and isotype-matched control antibody (□).

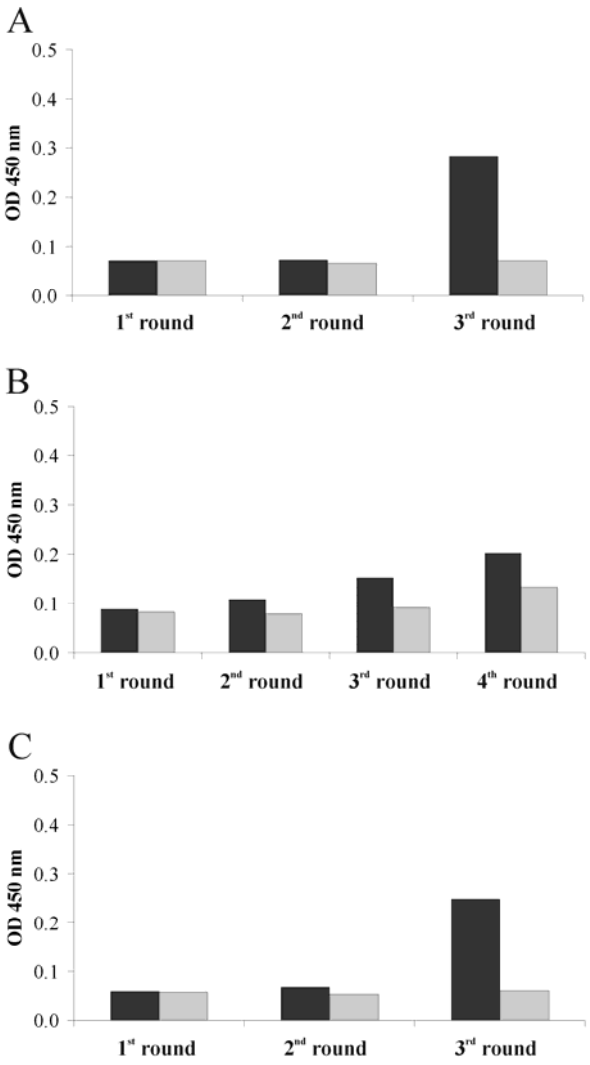

Fig. 6 Local alignment of identified peptide ligands with HMW-MAA. The sequences of the peptides 43.12p3 (magenta) and p225Fab (cyan) could be aligned with HMW-MAA (UniProt accession no. Q6UVK1) using BLAST ***(27)***. Identical residues are indicated in bold, similar ones in italics. The length of the whole peptide sequences is indicated with colored solid lines. The signal peptide and the transmembrane region of HMW-MAA are indicated in bold and underlined letters at the N-terminus and C-terminus, respectively.

```
   1 MQSGPRPPLPAPGLALALTLTMLARLASAASFFGENHLEVPVATALTDIDLQLQFSTSQP
  61 EALLLLAAGPADHLLLQLYSGRLQVRLVLGQEELRLQTPAETLLSDSIPHTVVLTVVEGW
 121 ATLSVDGFLNASSAVPGAPLEVPYGLFVGGTGTLGLPYLRGTSRPLRGCLHAATLNGRSL
 181 LRPLTPDVHEGCAEEFSASDDVALGFSGPHSLAAFPAWGTQDEGTLEFTLTTQSRQAPLA
 241 FQAGGRRGDFIYVDIFEGHLRAVVEKGQGTVLLHNSVPVADGQPHEVSVHINAHRLEISV
 301 DQYPTHTSNRGVLSYLEPRGSLLLGGLDAEASRHLQEHRLGLTPEATNASLLGCMEDLSV
 361 NGQRRGLREALLTRNMAAGCRLEEEEYEDDAYGHYEAFSTLAPEAWPAMELPEPCVPEPG
 421 LPPVFANFTQLLTISPLVVAEGGTAWLEWRHVQPTLDLMEAELRKSQVLFSVTRGARHGE
 481 LELDIPGAQARKMFTLLDVVNRKARFIHDGSEDTSDQLVLEVSVTARVPMPSCLRRGQTY
 541 LLPIQVNPVNDPPHIIFPHGSLMVILEHTQKPLGPEVFQAYDPDSACEGLTFQVLGTSSG
 601 LPVERRDQPGEPATEFSCRELEAGSLVYVHRGGPAQDLTFRVSDGLQASPPATLKVVAIR
 661 PAIQIHRSTGLRLAQGSAMPILPANLSVETNAVGQDVSVLFRVTGALQFGELQKQGAGGV
 721 EGAEWWATQAFHQRDVEQGRVRYLSTDPQHHAYDTVENLALEVQVGQEILSNLSFPVTIQ
 781 RATVWMLRLEPLHTQNTQQETLTTAHLEATLEEAGPSPPTFHYEVVQAPRKGNLQLQGTR
 841 LSDGQGFTQDDIQAGRVTYGATARASEAVEDTFRFRVTAPPYFSPLYTFPIHIGGDPDAP
 901 VLTNVLLVVPEGGEGVLSADHLFVKSLNSASYLYEVMERPRHGRLAWRGTQDKTTMVTSF
 961 TNEDLLRGRLVYQHDDSETTEDDIPFVATRQGESSGDMAWEEVRGVFRVAIQPVNDHAPV
1021 QTISRIFHVARGGRRLLTTDDVAFSDADSGFADAQLVLTRKDLLFGSIVAVDEPTRPIYR
1081 FTQEDLRKRRVLFVHSGADRGWIQLQVSDGQHQATALLEVQASEPYLRVANGSSLVVPQG
1141 GQGTIDTAVLHLDTNLDIRSGDEVHYHVTAGPRWGQLVRAGQPATAFSQQDLLDGAVLYS
1201 HNGSLSPRDTMAFSVEAGPVHTDATLQVTIALEGPLAPLKLVRHKKIYVFQGEAAEIRRD
1261 QLEAAQEAVPPADIVFSVKSPPSAGYLVMVSRGALADEPPSLDPVQSFSQEAVDTGRVLY
1321 LHSRPEAWSDAFSLDVASGLGAPLEGVLVELEVLPAAIPLEAQNFSVPEGGSLTLAPPLL
1381 RVSGPYFPTLLGLSLQVLEPPQHGALQKEDGPQARTLSAFSWRMVEEQLIRYVHDGSETL
1441 TDSFVLMANASEMDRQSHPVAFTVTVLPVNDQPPILTTNTGLQMWEGATAPIPAEALRST
1501 DGDSGSEDLVYTIEQPSNGRVVLRGAPGTEVRSFTQAQLDGGLVLFSHRGTLDGGFRFRL
1561 SDGEHTSPGHFFRVTAQKQVLLSLKGSQTLTVCPGSVQPLSSQTLRASSSAGTDPQLLLY
1621 RVVRGPQLGRLFHAQQDSTGEALVNFTQAEVYAGNILYEHEMPPEPFWEAHDTLELQLSS
1681 PPARDVAATLAVAVSFEAACPQRPSHLWKNKGLWVPEGQRARITVAALDASNLLASVPSP
1741 QRSEHDVLFQVTQFPSRGQLLVSEEPLHAGQPHFLQSQLAAGQLVYAHGGGGTQQDGFHF
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1861 PDSAPGEIEYEVQRAPHNGFLSLVGGGLGPVTRFTQADVDSGRLAFVANGSSVAGIFQLS
1921 MSDGASPPLPMSLAVDILPSAIEVQLRAPLEVPQALGRSSLSQQQLRVVSDREEPEAAYR
1981 LIQGPQYGHLLVGGRPTSAFSQFQIDQGEVVFAFTNFSSSHDHFRVLALARGVNASAVVN
2041 VTVRALLHVWAGGPWPQGATLRLDPTVLDAGELANRTGSVPRFRLLEGPRHGRVVRVPRA
2101 RTEPGGSQLVEQFTQQDLEDGRLGLEVGRPEGRAPGPAGDSLTLELWAQGVPPAVASLDF
2161 ATEPYNAARPYSVALLSVPEAARTEAGKPESSTPTGEPGPMASSPEPAVAKGGFLSFLEA
2221 NMFSVIIPMCLVLLLLALILPLLFYLRKRNKTGKHDVQVLTAKPRNGLAGDTETFRKVEP
2281 GQAIPLTAVPGQGPPPGGQPDPELLQFCRTPNPALKNGQYWV
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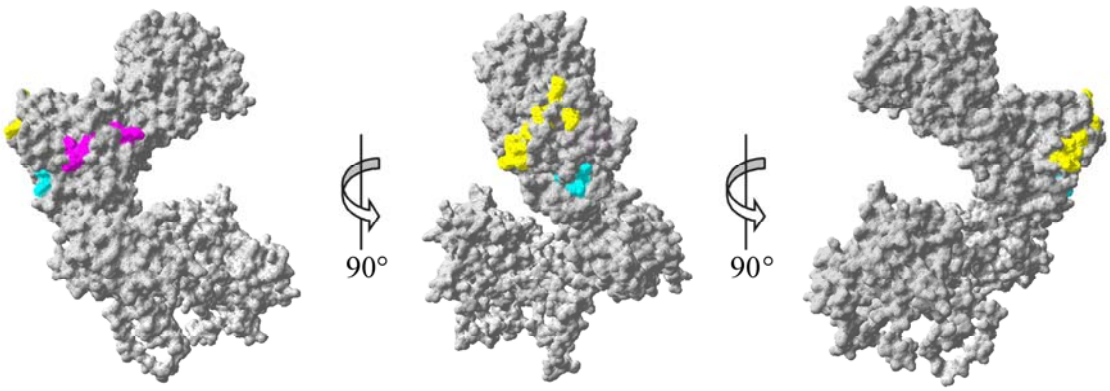
Fig. 7 Mimotope mapping. The peptides 43.12p3 (magenta), p225Fab (cyan), and 80.12p3 (yellow) are displayed on the putative surface of HMW-MAA using Swiss PDB Viewer (Swiss Institute of Bioinformatics, Lausanne, Switzerland). The crystal structure of mammalian fatty acid synthase (PDB accession no. 2vz8B) was used to model aa 260-1733 of HMW-MAA.



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Chapter III

Specificity of mimotope-induced anti-high molecular weight-melanoma associated antigen (HMW-MAA) antibodies does not ensure biological activity

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Manuscript submitted to *Vaccine*

Abstract

Vaccines based on peptide mimics (mimotopes) of conformational tumor antigen epitopes have been investigated for a variety of human tumors including breast cancer, tumors expressing the carcinoembryonic antigen, B cell lymphoma, neuroblastoma, and melanoma. In our previous work, we designed a vaccine based on a mimotope of the high molecular weight-melanoma associated antigen (HMW-MAA) that elicited HMW-MAA specific antibodies with anti-tumor activity *in vitro* and *in vivo*. In this study, we aimed to identify additional HMW-MAA mimotopes with distinct epitope specificities as potential components of a polymimotope melanoma vaccine.

Random peptide phage libraries were screened with the anti-HMW-MAA monoclonal antibodies (mAbs) VT80.12 and VF1-TP43 yielding one peptide ligand for each mAb. Both peptides confirmed epitope mimicry in inhibition ELISAs and were coupled to the carrier protein keyhole limpet hemocyanin (KLH) for immunization experiments. Although both HMW-MAA mimotopes elicited peptide specific antibodies in rabbits or BALB/c mice, HMW-MAA specific antibodies were only obtained in mice upon immunization with the mimotope defined by the mAb VT80.12. However, despite being cross-reactive to HMW-MAA these antibodies had no effect on the *in vitro* tumor proliferation of HMW-MAA expressing human melanoma cells.

This work describes constraints related to mimotopes generated by phage display and discusses the potential relevance of mimotope-selecting mAbs and applied immunization models on the outcome of mimotope vaccination. From our results we conclude that these aspects should be considered when using mimotopes as vaccine components.

1. Introduction

For at least two decades, the high molecular weight-melanoma associated antigen (HMW-MAA) has been the focus of several studies to implement effective immunotherapy for melanoma. Originally identified with murine monoclonal antibodies (mAbs) on the surface of human melanoma cells, HMW-MAA has been found to be overexpressed in at least 80% of primary and metastatic melanomas and more recently, in several other tumors as well as cancer stem cells [1,2]. However, HMW-MAA expression is not restricted to transformed cells, as it has been found on various cells of normal tissue including melanocytes, hairfollicles and basal cells of the epidermis as well as endothelial cells, chondrocytes and pericytes [1]. Its expression on both activated and resting pericytes in tumor vessels has suggested that HMW-MAA is critical to tumor angiogenesis [2]. Furthermore, HMW-MAA is known to contribute to the malignant phenotype of melanoma by activating several signaling cascades (e.g. Rho GTPases, $p130^{cas}$, FAK) that are involved in adhesion, migration and spreading of melanoma cells [3].

The idea of HMW-MAA-directed immunotherapy has been primarily supported by preclinical studies that showed prolonged survival rates of melanoma patients in association with anti-HMW-MAA specific antibodies (Abs) induced by active immunization [4-6]. Since then, a number of antibody- as well as T cell-based immunotherapies have been investigated. Recently, Maciag *et al.* generated a *Listeria monocytogenes* (*Lm*)-based vaccine against HMW-MAA [7]. Immunization of C57BL/6 mice bearing B16F10-HMW-MAA melanomas induced HMW-MAA specific $CD8^+$ and $CD4^+$ T cells that were equally required for tumor inhibition, as *in vivo* depletion of each of these cells resulted in uncontrolled tumor growth [7]. Noteworthy, this study provided the first syngenic melanoma mouse model for HMW-MAA as well as novel information on the contribution of T cells in anti-HMW-MAA immunity.

In contrast, we emphasized the induction of humoral anti-HMW-MAA immunity by using peptide mimics (mimotopes) as immunogens. Mimotopes are small peptides that mimic conformational B cell epitopes of antigens and can be selected by screening random peptide phage libraries with a mAb of interest. As mimotopes do not necessarily share the identical amino acid sequence with the original antigen, they are ideal antigen surrogates able to overcome immunotolerance [8-11]. Regarding melanoma,

this is of particular interest as most melanoma associated antigens including the HMW-MAA are known to be self-antigens [1,12].

In previous studies, we identified a linear HMW-MAA mimotope (225D9.2+) which mimics an epitope recognized by the anti-HMW-MAA mAb 225.28S. Immunization with this mimotope coupled to tetanus toxoid induced HMW-MAA specific Abs in rabbits that inhibited tumor cell proliferation *in vitro* [13]. Passive administration of these Abs in a xenogenic melanoma SCID mouse model inhibited tumor growth up to 40% in a therapeutic setting and up to 62% in a prophylactic setting [14].

As epitope loss is commonly found in melanoma [15] and several studies indicate that targeting multiple epitopes of a tumor antigen has synergistic effects and correlates with higher success rates, efficacy of a melanoma vaccine might be improved by the co-application of several peptides [16-18].

In this study, we report the selection of additional HMW-MAA mimotopes with distinct epitope specificities and discuss constraints related to mimotope vaccines regarding their immunogenicity and antigenicity.

2. Materials and methods

2.1. Monoclonal anti-HMW-MAA antibodies

The mAbs VT80.12, VF1-TP43, and TP61.5 were developed and characterized as described elsewhere [19-21].

2.2. Biotinylation of Abs (mAbs, rabbit IgGs)

NHS-LC-Biotin (Pierce, Rockford, IL, USA) was diluted in dimethylformamide at a concentration of 40 mg/ml. Five microliters of this solution was added to 1 mg/ml mAb or rabbit IgGs in PBS and incubated for 45 min at room temperature (RT). Excess NHS-LC-Biotin was removed by dialysis against PBS.

2.3. Cell lines

The human melanoma cell line 518A2 which expresses high levels of HMW-MAA and M14, a human melanoma cell line with no detectable expression of HMW-MAA, were maintained in RPMI 1640 medium (Lonza, Verviers, Belgium). The medium was supplemented with 10% (v/v) FCS and 1% (v/v) antibiotic-antimycotic mix (both from Gibco, Paisley, UK). Both cell lines were cultured in a humidified atmosphere containing 5% CO_2 and 95% ambient air at 37°C.

2.4. Microsomal preparations

Microsomal preparations were performed using ~$5x10^7$ cells according to the protocol described elsewhere [22]. Protein concentration was determined using a bicinchoninic acid (BCA) protein assay (Pierce).

2.5. Phage display, affinity selection and sequence analysis

Peptide ligands for the mAb VT80.12 were selected from a pVIII-15mer phage display peptide library [23]. Therefore, the mAb VT80.12 was immobilized and incubated with ~10^{10} phages. Phages displaying peptides that bound to the mAb VT80.12 were eluted and amplified in *E. coli* TG1.

Peptide ligands for the mAb VF1-TP43 were selected using the Ph.D.-12™ Phage Display Peptide Library, a pIII-12mer library, purchased from New England Biolabs (Ipswich, MA, USA). Biopanning was executed following the manufacturer's instructions.

After three rounds of selection, single phage clones that bound to the respective mAb were identified by phage ELISA and subsequently subjected for DNA sequencing.

2.6. Synthesis of peptides

The peptides GRQYYEGRKPDYRAAC (15/3/6) and NYQDLQRTHFKSGPGPGC (43.12p3) were synthesized using F-moc strategy by piCHEM (Graz, Austria). The purity of the peptides was ~95%, as assessed by HPLC.

2.7. ELISA inhibition assay

MaxiSorp immunoplates (Nunc, Rosklide, Denmark) were coated overnight (o/n) at 4°C with 4 µg/ml of mAb T61.5 in 50 mM Na-carbonate buffer, pH 9.6. Ten nanograms of biotinylated mAb were incubated with increasing concentrations (0, 10, 50, 100, and 500 µg/ml) of synthetic peptides in TBST (0.5% (v/v) Tween-20) containing 1% (w/v) BSA o/n at 4°C. After blocking with TBST/3% (w/v) milk powder, plates were incubated for 3 h at RT with 100 µg/ml microsomal preparations diluted in TBST/1% BSA. After washing, the mAb preincubated with peptides was added and incubation was continued for an additional hour at RT. Bound biotinylated mAb was detected using alkaline phosphatase (AP)-conjugated streptavidin (GE Healthcare, Little Chalfont, UK), followed by addition of p-nitrophenylphosphate (Sigma). Absorbance was measured at 405 nm. Percentage of inhibition was calculated as follows: 100 – (OD (inhibited)/OD (uninhibited) x 100).

2.8. Conjugation of peptides

Peptides were coupled to the carrier protein keyhole limpet hemocyanin (KLH; Sigma, St. Louis, MO, USA) or bovine serum albumin (BSA; Pierce) using the heterobifunctional crosslinker reagent m-maleimidobenzoyl-N-hydroxysuccinimide (MBS; Pierce) as described previously [13]. Conjugation of the peptides to the carrier proteins was verified in a dot blot assay. Therefore, peptide conjugates were dotted onto nitrocellulose (NC) membrane (Whatman, Dassel, Germany). After blocking with PBST (0.5% Tween-20) containing 3% milk powder, NC strips were incubated with biotinylated mAb, followed by AP-conjugated streptavidin (GE Healthcare). Color development was done with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium.

2.9. Immunizations

New Zealand white rabbits were immunized at Charles River Laboratories (Kisslegg, Germany). Immunizations were done thrice in 14- to 28-day intervals (day 1, 29, and 43) each with 200 µg of the peptide-KLH conjugates or KLH alone adsorbed to CFA (first immunization) or IFA (second and third immunization). Serum samples were taken before treatment (preimmune serum) and ten days after the first (day 11) and second immunization (day 39). Rabbits were sacrificed two weeks after the third immunization (day 57).

Female BALB/c (4 groups, n = 3; 6-8 weeks old; Charles River Laboratories, Sulzfeld, Germany) were immunized i.p. three times in 14-day intervals (day 1, 15, and 29) each with 15 µg of the peptide-KLH conjugates or KLH alone adsorbed to aluminium hydroxide (Alum; Serva, Heidelberg, Germany) in a total volume of 150 µl PBS solution. Sham-treated mice received PBS buffered alum only. Sera were taken from the tail vein on day 0 (preimmune serum), 22, and 41. Mice were treated according to European Union Rules of Animal Care, with permission 66.009/152-II/10b/2009 from the Austrian Ministry of Science.

2.10. Purification of rabbit or mouse IgG Abs

Total IgG from sera of rabbits were purified according to the protocol described elsewhere [13].

For purification of mouse IgG Abs, serum samples taken after the third immunization or after sacrifice of mice were pooled for each group and diluted with an equal volume of binding buffer (20 mM Na-phosphate, pH 7.0). The HiTrap Protein G HP column (GE Healthcare) was equilibrated with binding buffer and the sample applied. Bound Abs were eluted with 100 mM glycine-HCl, pH 2.7 and neutralized by addition of 1 M Tris-HCl, pH 9.0.

Purification of rabbit or mouse IgG was monitored using nonreducing 8% SDS-PAGE. Protein concentration was determined by a BCA protein assay (Pierce).

2.11. Peptide specific Ab response

MaxiSorp immunoplates (Nunc) were coated with 10 µg/ml peptide-BSA conjugates or KLH or BSA in 50 mM Na-carbonate buffer, pH 9.6, o/n at 4°C. Nonspecific binding sites were blocked with TBST/3% milk powder.

Purified rabbit Abs were diluted at concentrations of 0.05, 0.1, 0.5, 1, 5, and 10 µg/ml in TBST/0.5% BSA, added to antigen-coated plates, and incubated for 2 h at RT. After washing, bound Abs were detected using AP-conjugated swine anti-rabbit IgG (Dako, Glostrup, Denmark) diluted 1:1000 in TBST/0.5% BSA. Color development was performed as described for the ELISA inhibition assay.

Mouse sera were diluted 1:1000 in TBST/0.5% BSA and incubated for 2 h at RT. AP-conjugated rabbit anti-mouse IgG + IgM (Jackson ImmunoResearch, West Grove, PA, USA) diluted 1:5000 in TBST/0.5% BSA was used as a second step Ab. Visualization of bound mouse Abs was performed as described above.

2.12. HMW-MAA specific Ab response

2.12.1. ELISA protocol

For the detection of the HMW-MAA specific Ab response, HMW-MAA was purified from microsomal preparations as described for the ELISA inhibition assay. After blocking and catching, plates were incubated for 2 h at RT with increasing concentrations (12.5, 50, and 200 µg/ml) of purified rabbit Abs diluted in TBST/1% BSA. Bound IgG was detected as described for the peptide-specific Ab response.

2.12.2. FACS protocol

Flow cytometric analysis of melanoma cells stained with sera from immunized mice or purified Abs from immunized rabbits was performed as previously described [24]. Briefly, $5x10^5$ cells were incubated for 1 h on ice with a 1:10 dilution of pooled mouse sera for each group or 100 µg purified rabbit Abs in 100 µl PBS/1% BSA. One microgram of mAb VT80.12 served as positive control staining of HMW-MAA. Cells were then washed twice with PBS/0.5% BSA and incubated for an additional 30 min on ice with FITC-labeled goat anti-mouse IgG or FITC-labeled goat anti-rabbit IgG (both diluted 1:2000 in PBS/1% BSA; AbD Serotec, Düsseldorf, Germany). After washing, cells were resuspended in PBS/0.5% BSA and 20,000 gated events were analyzed by flow cytometry on a BD FACScanto using BD FACSDiva software (BD Biosciences,

Franklin Lakes, NJ, USA). Histogram overlays were done applying FlowJo software (Tree Star, Ashland, OR, USA).

2.12.3. Immunohistochemistry (IHC) protocol

Immunohistochemical staining of established 518A2 tumor tissues was performed as described by Wagner *et al.* [14]. Briefly, tumor sections were incubated with either blocking buffer (negative control), 30 µg/ml biotinylated mAb VT80.12 (positive control) or 100 µg/ml biotinylated purified Abs of rabbits either immunized with 15/3/6-KLH conjugate or KLH. After washing with TBST, slides were incubated with StreptABComplex/HRP (Dako) for 30 min. Specific Ab binding was visualized by a DAB chromogen solution (Dako) following hematoxylin counterstaining. Stained slides were viewed by an Olympus Vanox AHBT3 microscope and photographed with a Zeiss AxioCam MRc5 camera.

2.13. Inhibition of tumor cell growth in vitro

Tumor cells were seeded in 96-well tissue culture plates (Costar; Corning, NY, USA) at 1500 cells/well. Cells were allowed to adhere o/n at 37°C. Purified Abs from mice immunized with either the 15/3/6-KLH conjugate or KLH were added at increasing concentrations (0, 0.01, 0.1, and 1 mg/ml) and incubation was continued for an additional 72 h at 37°C. Cells were pulsed with 0.5 µCi of [methyl-^{3}H]thymidine/well (GE Healthcare) for another 6 h at 37°C and then harvested. Incorporated [^{3}H]thymidine was measured by a Wallac MicroBeta TriLux 1450 counter (PerkinElmer, Waltham, MA, USA). Percentage of inhibition of proliferation was calculated by comparing the cpm values of treated cells with those of untreated cells, which were set at 100%.

3. Results

3.1. Identification of peptide ligands

Biopanning of the mAb VT80.12 was performed with a linear pVIII-15mer phage display peptide library. After three rounds of selection, the total number of phages that bound to the mAb VT80.12 was increased from $1x10^5$ CFU/ml in the 1st round to $1x10^9$ CFU/ml in the 3rd round. Twenty individual phage clones of each the 2nd and 3rd round were tested for their ligand specificity in phage ELISA. Among these, eighteen (2nd round) and nineteen (3rd round) phage clones bound to the mAb VT80.12. DNA sequencing of these 37 phage clones yielded one peptide sequence GRQYYEGRKPDYRAA ("15/3/6"). Phages (10^6-10^{13} CFU/ml) displaying this sequence showed a concentration-dependent inhibition up to 70% in ELISA experiments (data not shown). Therefore, the peptide 15/3/6 was chemically synthesized with an additional C-terminal cysteine residue for conjugation purposes. In ELISA inhibition experiments, the peptide inhibited the binding of the mAb VT80.12 to the HMW-MAA up to 93% (Fig. 1A).

Biopanning of the mAb VF1-TP43 was performed with a linear pIII-12mer phage display peptide library. Three rounds of selection yielded an increase of phage titer from $1x10^5$ pfu/ml (1st round) to $3x10^9$ pfu/ml (3rd round). Among 30 tested phage clones, 20 were specifically recognized by the mAb VF1-TP43 in phage ELISA. DNA sequencing yielded one peptide sequence NYQDLQRTHFKS ("43.12p3"). In a competitive ELISA experiment, binding of the phage displayed peptide to the mAb VF1-TP43 was reduced up to 33% by a HMW-MAA containing cell lysate (data not shown). The peptide 43.12p3 was synthesized with a GPGPG-linker and an additional C-terminal cysteine residue. In subsequent inhibition experiments, this peptide inhibited the binding of the mAb VF1-TP43 to the HMW-MAA up to 100% (Fig. 1B).

3.2. Peptide specific Ab response in rabbits

The peptide 15/3/6 was coupled to KLH or BSA as carrier protein. Conjugation to the carrier protein was confirmed in a dot blot assay (data not shown). New Zealand white rabbits were immunized with the conjugate 15/3/6-KLH or the carrier protein KLH alone. After three immunizations, Abs were purified using a HiTrap protein A HP column. Twenty milliliters of serum yielded ~20 mg of IgG Abs. The purity was greater than 95% as confirmed by SDS-PAGE (data not shown). Peptide specific Abs were

determined by ELISA after incubation of coated peptide-BSA conjugate, BSA or KLH with purified IgG Abs. Both, 15/3/6- and KLH-specific Abs were already detectable at a concentration of 0.05 µg/ml and increased in a dose- dependent manner (Fig. 2). Abs purified from the KLH-immunized rabbit did not bind to the peptide. No Abs directed against BSA could be determined.

3.3. HMW-MAA specific Ab response in rabbits

HMW-MAA specificity of rabbit Abs was determined in ELISA by testing their binding ability to the HMW-MAA. Binding to the HMW-MAA negative cell line M14 was detected for both, the peptide-conjugate and the KLH-induced Abs (Fig. 2). Even when the HMW-MAA catching mAb was directly incubated with purified rabbit Abs and subsequently detected with AP-conjugated swine anti-rabbit IgG, high background levels were measured (data not shown).

In a second approach, HMW-MAA specificity of rabbit Abs was investigated by FACS analysis. The HMW-MAA positive human melanoma cell line 518A2 (HMW-MAA^{pos}) or the HMW-MAA negative human melanoma cell line M14 (HMW-MAA^{neg}) were incubated with each 100 µg purified rabbit Abs and detected with a FITC-conjugated goat anti-rabbit IgG. As expected, the mAb VT80.12 specifically bound to the HMW-MAA^{pos}, but not to the HMW-MAA^{neg} cells. However, HMW-MAA specificity of the 15/3/6-KLH conjugate induced Abs could not be observed, as Abs induced by KLH showed a similar staining pattern to HMW-MAA^{pos} as well as to HMW-MAA^{neg} cells (Fig. 3A).

In a third approach, we used solid 518A2 tumors established in C.B.17 SCID/SCID mice [14] to detect HMW-MAA reactive rabbit Abs by IHC. Whereas staining of melanoma cells was observed with the biotinylated mAb VT80.12, none was observed with either an isotype-matched control mAb (data not shown) or PBS buffer. Also in this setup, we detected a high background staining of anti-KLH Abs on 518A2 tumor tissue, and therefore no HMW-MAA specific anti-15/3/6 Abs (Fig. 3B).

3.4. Peptide specific Ab response in BALB/c mice

The peptides 15/3/6 or 43.12p3 were coupled each to KLH or BSA. Female BALB/c mice (4 groups, n = 3) were immunized with 15 µg of the peptide-KLH conjugates, KLH or were sham-treated with PBS buffered alum. Serum samples of each group were tested for the presence of peptide specific Abs in ELISA. Peptide specificity was confirmed for both anti-15/3/6-KLH and anti-43.12p3-KLH Abs as they bound to the corresponding peptide-BSA conjugates (Fig. 4). As expected, KLH specific Abs were detected in the KLH-group (Fig. 5) as well as in the groups immunized with the peptide-KLH conjugates (data not shown). Sham-treated mice did not develop Abs against the peptides 15/3/6 or 43.12p3, KLH or BSA (data not shown).

3.5. HMW-MAA specific Ab response in BALB/c mice

Preimmune serum samples or serum samples after the third immunization of individual mice were pooled for each group. Serum pools were diluted 1:10 and incubated with HMW-MAA^{pos} or HMW-MAA^{neg} cells. Bound Abs were detected with a FITC-labeled goat anti-mouse IgG by FACS analysis. None of the mouse Abs of preimmune serum pools showed reactivity to HMW-MAA^{pos} or HMW-MAA^{neg} cells (data not shown). Immunization with the 15/3/6-KLH conjugate induced HMW-MAA specific Abs, whereas the 43.12p3-KLH conjugate did not (Fig. 5). KLH-induced Abs showed negligible background staining on HMW-MAA^{pos} as well as HMW-MAA^{neg} cells.

3.6. Inhibition of tumor cell growth

The effect of anti-15/3/6-KLH Abs on tumor growth *in vitro* was determined by a [^{3}H]thymidine proliferation assay. HMW-MAA^{pos} or HMW-MAA^{neg} cells were incubated with increasing concentrations of purified mouse Abs. Upon treatment with anti-15/3/6 Abs, proliferation of HMW-MAA^{pos} cells was inhibited to a maximum of ~17% (Fig. 6). Almost identical results were obtained, when either HMW-MAA^{pos} cells were treated with anti-KLH Abs or HMW-MAA^{neg} cells were incubated with anti-15/3/6 or anti-KLH Abs. Results given in Fig. 7 represent mean values of 3 independent experiments.

4. Discussion

In this study we aimed to select two mimotopes defined by the anti-HMW-MAA mAbs VT80.12 and VF1-TP43 that should serve together with our previously described 225D9.2+ mimotope [13,14] as components of a polymimotope vaccine against melanoma. We therefore screened two linear phage display peptide libraries (pVIII-15mer with mAb VT80.12, pIII-12mer with mAb VF1-TP43) yielding one peptide ligand for each mAb. Both peptides (15/3/6: ligand to VT80.12; 43.12p3: ligand to VF1-TP43) inhibited binding of the respective mAb to the HMW-MAA up to 100%, thereby proofing epitope mimicry and their definition as mimotopes. Upon immunization of either rabbits or BALB/c mice, each mimotope coupled to the carrier protein KLH demonstrated immunogenicity by inducing mimotope specific Abs. However, only the 15/3/6-KLH conjugate induced HMW-MAA specific Abs in BALB/c mice, but these Abs failed to inhibit tumor cell proliferation *in vitro*.

Epitope mimicry to HMW-MAA was confirmed for both peptides 15/3/6 and 43.12p3 in ELISA inhibition experiments (Fig. 1). Thus, constraints referring to phage display were preliminary excluded.

An important issue of this study was the applied animal model in the context of the carrier protein used for vaccination. Initially, immunization experiments were performed in rabbits as high amounts of antibodies can be obtained from the blood for subsequent *in vitro* tests. Although mimotope specific Abs were induced for the tested mimtope, these showed no HMW-MAA specificity in ELISA experiments (Fig. 2). Instead, we observed a high background staining of KLH specific Abs on the HMW-MAA-positive or -negative cell lysate, as well as on the HMW-MAA catching mAb. Similarly, FACS staining (Fig. 3) as well as immunohistochemistry (Fig. 4) failed to confirm HMW-MAA specific Abs. Immunizations were then repeated in BALB/c mice to assess whether the results from the rabbit immunizations were attributed to the carrier protein KLH or the applied animal model. Using this model, FACS analysis confirmed HMW-MAA specificity of mimotope-induced Abs (Fig. 6), indicating that the undesired binding of rabbit anti-KLH Abs was likely attributed to the rabbit Abs and not to the carrier protein KLH. However, we cannot exclude a possible contribution of the used adjuvants (CFA/IFA in rabbits vs. alum in BALB/c mice) to the unspecific binding of rabbit anti-KLH Abs, as adjuvants are known to differentially deliver antigen

and activate the immune system [25]. We therefore conclude that the choice of carrier protein has an impact on the outcome of vaccination and might depend on the used immunization model.

Although the 43.12p3-KLH conjugate induced mimotope specific mouse Abs, these failed to cross-react with the natural antigen HMW-MAA (Fig. 6), indicating that immunogenicity of mimotopes does not necessarily correlate with their specificity. The discrepancy between epitope specificity of an anti-HMW-MAA mAb and epitope specificity of HMW-MAA mimotope-induced Abs is best described by previous studies of Wagner *et al.* [13] and Hafner *et al.* [26]. Both studies deal with the identification of distinct HMW-MAA mimotopes which were defined by the same mAb (225.28S) and coupled to the same carrier protein (tetanus toxoid, TT) for subsequent immunization experiments in rabbits. Using the identical immunization protocol, a HMW-MAA specific Ab response was only obtained upon immunization with the mimotope-TT conjugate described by Wagner *et al.* [13]. These findings clearly indicate that – despite conforming affinity selections as well as conforming immunization protocols – not every mimotope truly mimics the relevant epitope of the original antigen. On the one hand, such constraints might originate during affinity selection as phage displaying peptides may bind to residues of the mAb that are not part of the paratope. Although such peptides might inhibit the binding of their defined mAb to the original epitope, these will fail to induce a specific immune response to the original antigen. In addition, B cell epitopes are known to be conformational in nature [27]. Hence, it is possible that mAbs recognize certain conformations of epitopes which are not displayed in the same structural context by the natural antigen. The above-described constraints probably outline the major pitfall of the phage display technique that complicates the selection of "true" mimotopes, solely based on their binding characteristics to mAbs. On the other hand, it is to state that immunization with mimotopes does not guarantee the induction of Abs that display the appropriate paratope to the natural antigen, emphazising also a possible contribution of the used animal model to the outcome of mimotope vaccination. This might be of particular relevance for other investigators who apply mimotopes for vaccination strategies.

Immunization of BALB/c mice with the 15/3/6 mimotope conjugate resulted in the induction of mimotope- and HMW-MAA specific Abs (Fig. 5 and 6). However, these Abs showed no anti-tumor activity in *in vitro* proliferation assays (Fig. 7). Importantly, the mAb VT80.12 itself does not inhibit tumor cell proliferation neither directly nor

indirectly by mediating ADCC or CDC [28]. We therefore have to question whether anti-tumor activity *in vivo* of the mimotope defining mAb should be a prerequisite to induce antibodies with tumor inhibiting properties. As a matter of fact, certain mAbs (e.g. Rituximab [29-32], Cetuximab [33,34], Trastuzumab [11,35]) which are currently applied for cancer therapies and successfully served as sources to generate mimotope vaccines with anti-tumor activity *in vitro* fulfill this requirement even if *in vivo* results for the these mimotope vaccines are not available yet. As reported by Hafner *et al.*, passive administration of the anti-HMW-MAA mAb 225.28S significantly reduced tumor volume in a human melanoma xenotransplant SCID mouse model, although this mAb had no effect on human melanoma cells *in vitro* [36]. This supports the hypothesis of using mAbs with *in vivo* rather than *in vitro* anti-tumor activity for the selection of mimotopes.

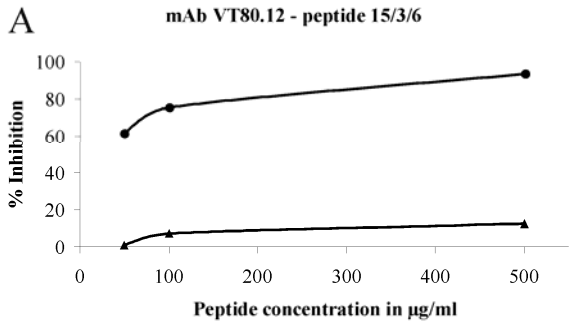
To date, only one mimotope-based vaccine targeting the HMW-MAA has demonstrated anti-tumor activity *in vitro* [13] and *in vivo* [14]. The identification of additional HMW-MAA mimotopes as components of an effective polymimotope vaccine might depend on the *in vivo* anti-tumor activity of the selecting mAbs *per se* but also on the used animal model as well as on the paratope conformation of the mimotope-induced Abs. In addition, we suggest that the efficacy of every mimotope based vaccine should be evaluated in an animal model by active immunization.

Acknowledgments

We thank Christine Hafner for providing the linear pVIII-15mer phage display peptide library.

Figures

Fig. 1. Inhibition of mAb binding to HMW-MAA by synthetic peptides. Microtiter plates were coated with mAb TP61.5 and incubated with microsomal preparation of 518A2 melanoma cells to catch HMW-MAA. Biotinylated mAbs were preincubated with increasing concentrations of generated peptide mimics, followed by incubation with HMW-MAA. (A) Biotinylated mAb VT80.12 was preincubated with synthetic peptide 15/3/6 (●) and a control peptide (▲). (B) Biotinylated mAb VF1-TP43 was preincubated with synthetic peptide 43.12p3 (■) as well as the control peptide (▲). Binding of biotinylated mAbs was measured using an AP-conjugated streptavidin. Percentage of inhibition was calculated as follows: 100 – (OD (inhibited)/OD (uninhibited) x 100).



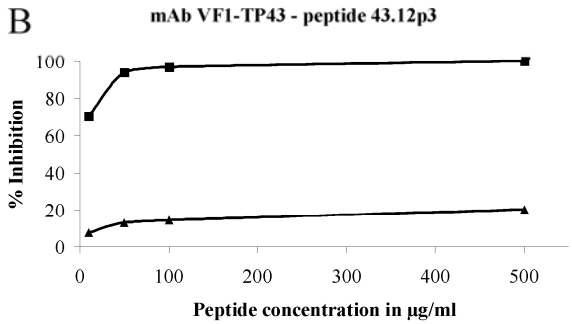


Fig. 2. Antigen and HMW-MAA specific immune response of immunized rabbits. Left panel: Microtiter plates were coated with 15/3/6 coupled to BSA, KLH, or BSA and incubated with increasing concentrations of purified rabbit Abs from immunizations with 15/3/6-KLH or KLH to detect peptide and KLH specific Abs. Right panel: Microtiter plates were coated with mAb TP61.5 and incubated with microsomal preparations of the melanoma cell lines 518A2 or M14. Purified rabbit Abs were analyzed by administration of increasing Ab concentrations.

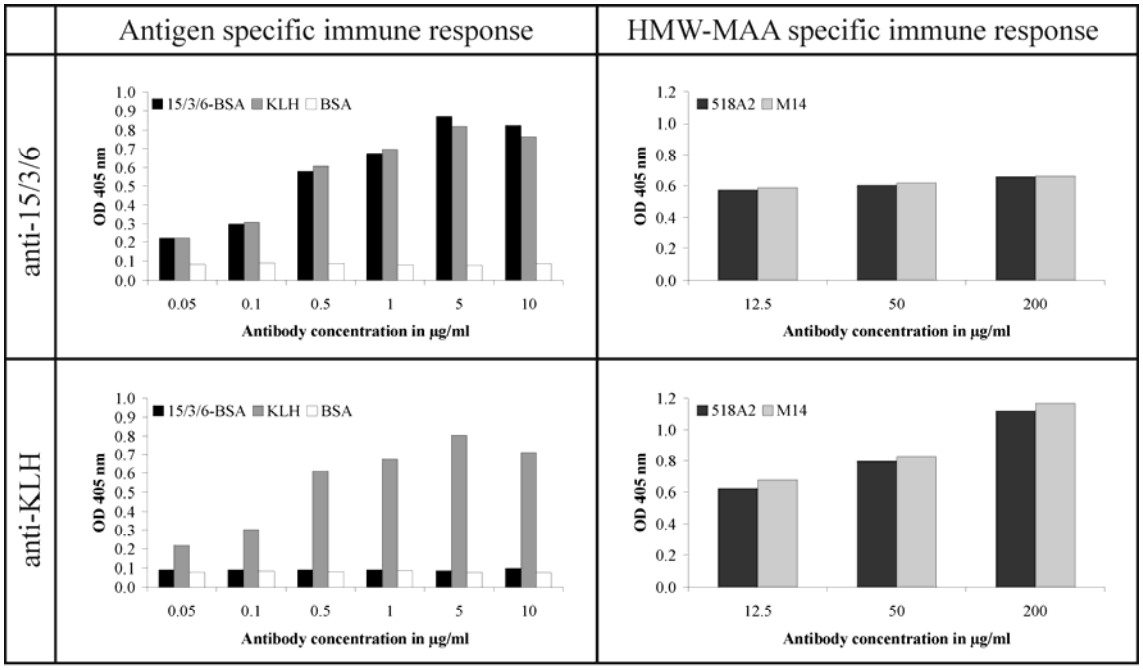


Fig. 3. HMW-MAA specific Ab response determined by FACS and IHC. (A) The human melanoma cell lines 518A2 and M14 were each incubated with 1 µg mAb VT80.12 (as positive control to detect HMW-MAA) or 100 µg purified rabbit Abs from immunizations with 15/3/6-KLH or KLH. Bound antibodies were detected with FITC-labelled goat anti-mouse IgG or FITC-labelled goat anti-rabbit IgG. Histogram overlays show unstained (white) vs. stained cells (grey). (B) Immunohistochemistry on 518A2 tumor tissues of C.B.17 SCID/SCID mice [14]. HMW-MAA staining was done with PBS buffer (negative control), mAb VT80.12 (positive control), and biotinylated IgG fraction of either 15/3/6-KLH antiserum (anti-15/3/6) or KLH antiserum (anti-KLH). Bound Abs were detected with StrepAB/HRP and visualized by DAB-chromogen solution and subsequent hematoxylin counterstaining.

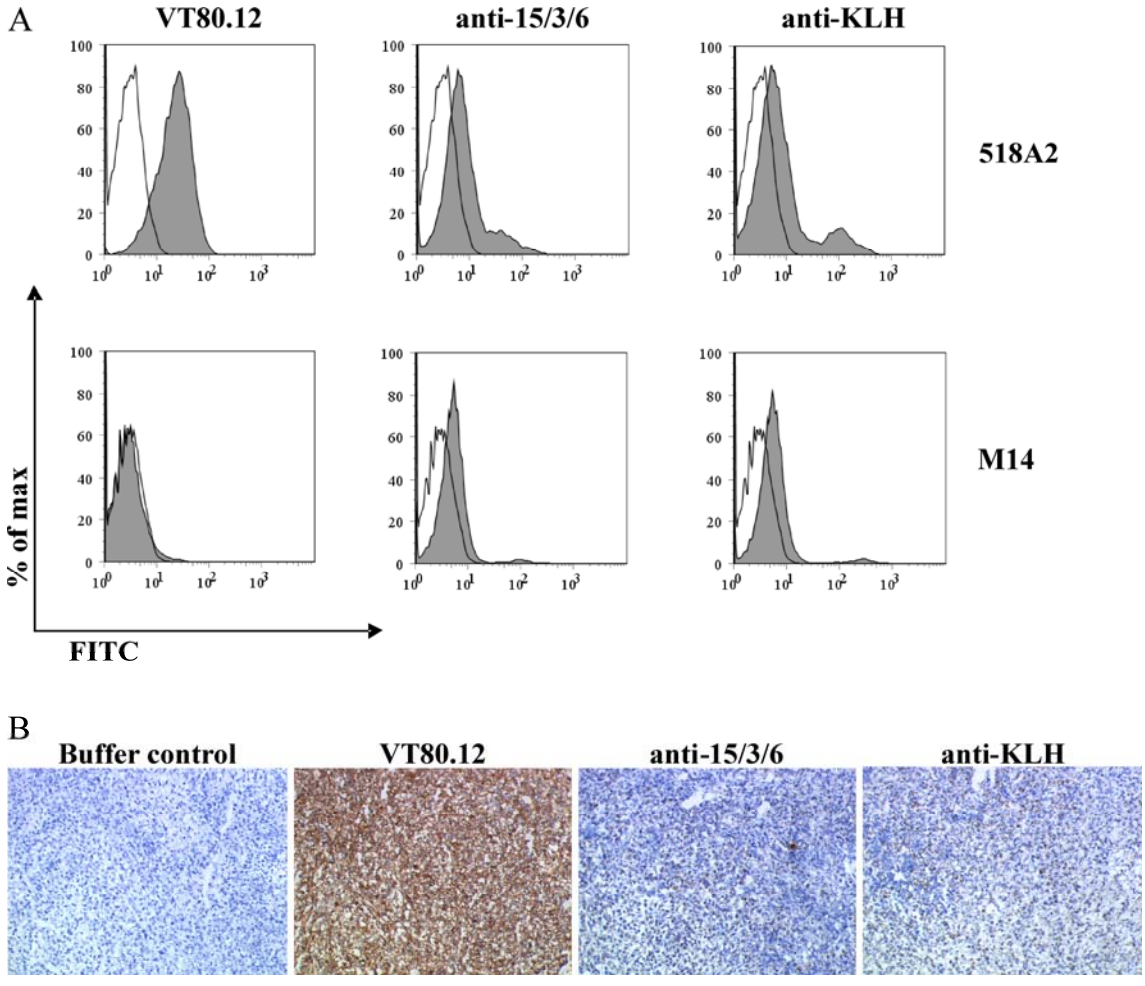


Fig. 4. Antigen specific immune response of immunized BALB/c mice. Serum samples from day 0 (preimmune serum; white bars), day 22 (grey bars), and day 41 (black bars) were tested in ELISA. Results are shown for each mouse individually. Peptide specificity of sera from mice immunized with 15/3/6-KLH or 43.12p3-KLH was assessed using the respective peptide-BSA conjugates. Sera from KLH immunized mice were tested for specificity of KLH.

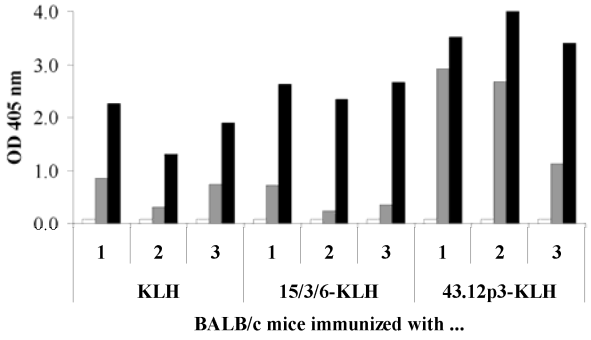


Fig. 5. HMW-MAA specific immune response of immunized BALB/c mice. Human melanoma cell lines 518A2 and M14 were either incubated with 1 µg mAb VT80.12 (as positive control) or a 1:10 diluted serum pool of each group. Bound antibodies were detected with FITC-labelled goat anti-mouse IgG. Histogram overlays show unstained (white) and stained cells (grey).

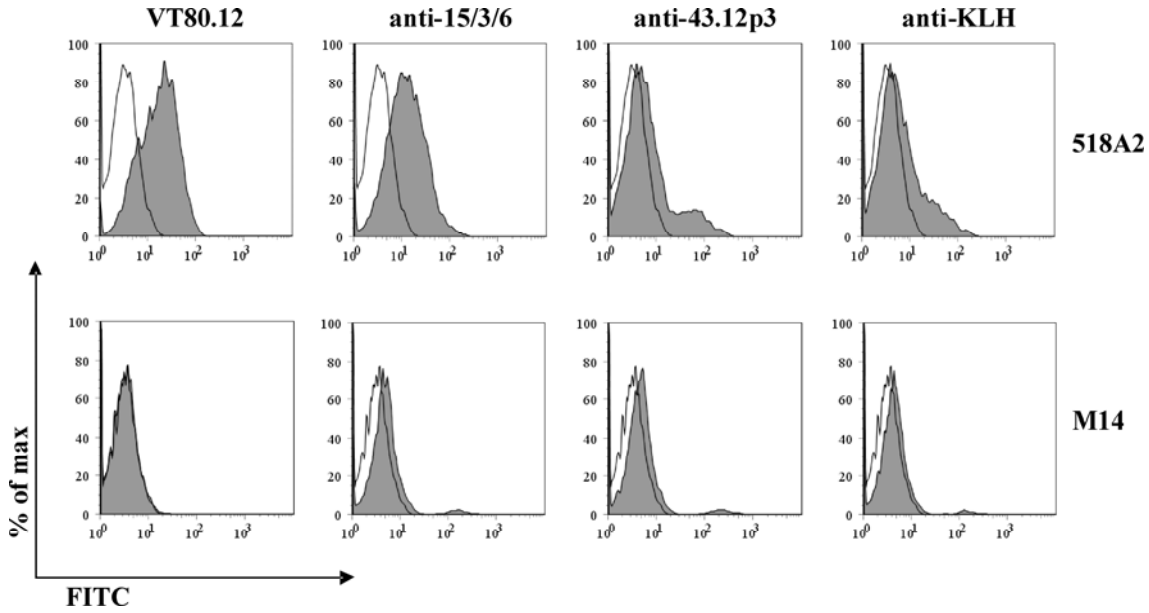
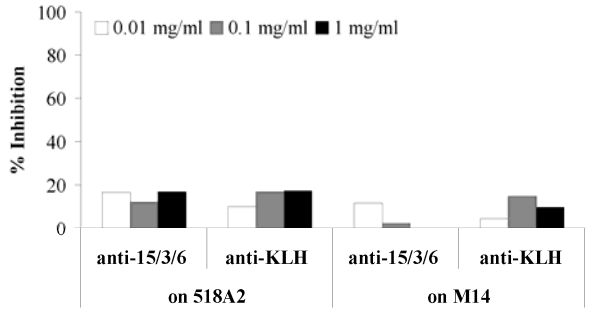


Fig. 6. Inhibition of cell proliferation in vitro. [^{3}H]Thymidine proliferation assay demonstrating the effects of the purified mouse Abs on HMW-MAA expressing melanoma cells. The 518A2 or M14 cells were incubated with increasing concentrations of purified Abs from the mouse immunizations with 15/3/6-KLH or KLH for 72 h and pulsed with [^{3}H]thymidine. Data are shown in percentage of inhibition compared with untreated cells; cpm values of untreated cells were set at 100%. The values are the mean of three independent experiments. Percentage of inhibition was calculated as follows: 100 – (OD (inhibited)/OD (uninhibited) x 100).



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Chapter IV

Concluding Remarks

Concluding Remarks

The aim of this thesis was the generation of a polymimotope vaccine against malignant melanoma. This vaccine should target several epitopes of the HMW-MAA, a tumor-antigen which is highly expressed on human melanoma cells, using the mimotope approach to elicit a strong humoral anti-HMW-MAA immune response. The efficacy of this polymimotope vaccine should then be tested in a syngenic melanoma mouse model. The results obtained with this thesis should provide new know-how regarding the superior efficacy of multi- over mono-epitope targeting vaccines.

Limitations regarding (1) the application of different panning strategies using phage display peptide libraries to identify mimotopes, (2) the presence of *in vitro* or *in vivo* biological activity of an antibody which is used for the identification of mimotopes, (3) the animal model used for immunization to obtain tumor antigen specific antibodies, and (4) the possible discrepancy between immunogenicity and antigenicity of mimotopes have been extensively discussed in chapter II and III.

In addition to the already discussed limitations, there are three issues that need to be addressed.

First, regarding phage display, the efficacy of selection can be enhanced to a certain extent by manipulating stringency which is the degree to which peptides with higher fitness are favored over peptides with lower fitness and is inversely proportional to yield which is the fraction of phage particles with a given fitness that survive selection [1,2]. Usually, peptides with high fitness should be isolated, but stringency can only be increased to a certain extent, as increasing stringency usually implicates decreased yield. Therefore, high yield of the fittest clones is of paramount importance in the first round of selection, whose input consists of all clones of a certain phage display peptide library [2]. Considering the facts that each clone is represented by only 100 phage particles on average and the yield for the fittest clones is sometimes not greater than 1%, it is very likely that such clones are going to be lost during the first round of selection and can therefore never be recovered [2]. Clones that are obtained after the first round of selection can be amplified and are thus represented by millions of phages which can be applied in following rounds of selection. Thereafter, yield can be

decreased in favor of high stringency for the subsequent rounds. However, there is a limit to stringency, because in practice selection techniques are imperfect and there is an unavoidable background yield of all phages regardless of their fitness [2]. If stringency is set too high, the yield of a specifically selected phage will fall far below the background of a nonspecifically isolated phage, and all power of discrimination in favor of high fitness is lost [2].

Second, regarding the fact that a peptide which binds its selector antibody (thus qualifying it as an antigenic mimic) is not able to elicit antibodies directed against the target antigen when used to immunize naive animals (thus failing as an immunogenic mimic), there are additional considerations which should be mentioned [2].
Small peptides are often flexible and might adopt a certain conformation when binding the selector antibody but other conformations when eliciting new antibodies upon immunization [3,4]. Therefore, only few of these antibodies, if any, would cross-react with the authentic epitope. Furthermore, a peptide might be an antigenic mimic without being a true structural mimic. Such a peptide would bind the selector antibody in an entirely different way than does the original authentic epitope, *via* altogether different interactions [2].
However, the induction of mimotope specific antibodies is a much more difficult process and varies considerably from one mimotope to another. This may be reasonable since the immune system can differentiate different antigens and produces very different responses [5].

Third, as short synthetic peptides representing B cell epitopes, continuous/linear or discontinuous/conformational, are generally poor immunogens, they require conjugation to a carrier protein providing T helper cell epitopes and usually the co-administration of an adjuvant in order to induce an antibody response [6-8].
Because it is now difficult to obtain carriers such as tetanus toxoid (TT) or diphtheria toxoid, it seems to be a good and cheap alternative to use KLH, as it is a large (>2000 kDa), highly glycosylated protein frequently used as a carrier protein in animals and humans due to its potent immunogenicity [9,10]. Nevertheless, there are epitopes on the surface of KLH that cross-react with a variety of different carbohydrate antigens [10]. May *et al.* immunized mice with mimotopes, which were identified for the glucuronoxylomannan (GXM) component of the capsular polysaccharide of

C*ryptococcus neoformans*, conjugated to KLH as carrier. They observed that KLH alone induced antibodies that cross-reacted with GXM, but did not confer protection against cryptococcal infection, suggesting that the cross-reactive epitope on KLH was not protective [10].

Additionally, different carriers may influence the immunogenicity of a mimotope conjugate. In this concern, Li *et al.* showed that antibodies induced upon immunization of mice with a CD20 mimotope coupled to KLH were capable of mediating more efficient complement killing of $CD20^+$ cells when compared to the antibodies from mice vaccinated with the respective TT-conjugate [11]. Wondimu *et al.* observed the opposite when using GD2 ganglioside mimotopes coupled to KLH or synthesized as MAP (multiple-antigen peptides). Here, the KLH-conjugate was less efficient than the MAP [12].

In conclusion, the immunogenicity of a mimotope conjugate can be increased by using a correct carrier and caution needs to be taken when creating vaccines for carbohydrate antigens using KLH as carrier protein [10-12].

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Appendix

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September 5, 2006	Received Masters degree (Mag. rer. nat.)
09/2006 – 12/2007	Project management and laboratory work within "EuroPrevall" ("The Prevalence, Cost and Basis of Food Allergy in Europe" – a European Commission funded project within the 6th framework programme, FOOD-CT-2005-514000) and a Biomay AG (Vienna, Austria) sponsored project on recombinant allergens at the Department of Pathophysiology and Allergy Research, Medical University of Vienna, Austria
10/2006 – current	PhD studies in Genetics/Microbiology, University of Vienna, Austria PhD thesis at the Department of Pathophysiology and Allergy Research, Medical University of Vienna, Austria
05/2007	Research visit at the Paul Ehrlich Institute, Langen, Germany
01/2008 – 12/2010	Recipient of a DOC-fFORTE-fellowship of the Austrian Academy of Sciences

List of Publications

PAPERS as first author

Gaier S*, Marsh J*, Oberhuber C*, Rigby NM, Lovegrove A, Alessandri S, Briza P, Radauer C, Zuidmeer L, van Ree R, Hemmer W, Sancho AI, Mills C, Hoffmann-Sommergruber K, Shewry PR. **Purification and Structural Stability of the Peach Allergens Pru p 1 and Pru p 3.** Mol. Nutr. Food Res. 2008; 52(Suppl 2): 220-9

Gaier S, Oberhuber C, Hemmer W, Radauer C, Rigby NM, Marsh JT, Mills CE, Shewry PR, Hoffmann-Sommergruber K. **Pru p 3 as a marker for symptom severity for peach allergic patients in a birch pollen environment.** J Allergy Clin Immunol. 2009; 124(1): 166-7

Gaier S, Balazs N, Radauer C, Ferrone S, Hafner C, Breiteneder H, Wagner S. **Successful selection of mimotopes from phage-displayed libraries strongly depends on the selection strategy.** Manuscript submitted to *Molecular Biotechnology*

Gaier S*, Latzka J*, Hofstetter G, Balazs N, Ferrone S, Scheiner O, Breiteneder H, Pehamberger H, Wagner S. **Specificity of mimotope-induced anti-high molecular weight-melanoma associated antigen (HMW-MAA) antibodies does not ensure biological activity.** Manuscript submitted to *Vaccine*

PAPERS as coauthor

Oberhuber C, Bulley SM, Ballmer-Weber BK, Bublin M, **Gaier S**, DeWitt AM, Briza P, Hofstetter G, Lidholm J, Vieths S, Hoffmann-Sommergruber K. **Characterization of Bet v 1 related allergens from kiwifruit using sera from patients with combined kiwifruit and birch pollen allergy.** Mol. Nutr. Food Res. 2008; 52(Suppl 2): 230-40

Mandalari G, Adel-Patient K, Barkholt V, Baro C, Bennett L, Bublin M, **Gaier S**, Graser G, Ladics GS, Mierzejewska D, Vassilopoulou E, Vissers YM, Zuidmeer L, Rigby NM, Salt LJ, Defernez M, Mulholland F, Mackie AR, Wickham MS, Mills EN. ***In vitro* digestibility of beta-casein and beta-lactoglobulin under simulated human gastric and duodenal conditions: A multi-laboratory evaluation.** Regul Toxicol Pharmacol. 2009; 55(3): 372-81

PRESENTATIONS at national and international congress meetings as presenting author

Gaier S, Oberhuber C, Scheiner O, Hoffmann-Sommergruber K. **Molecular characterisation of Pru p 1, the Bet v 1 homologue from peach**. Poster presentation at the Annual Meeting of the Austrian Society of Allergology and Immunology (ÖGAI), December 2005, Graz, Austria

Gaier S, Oberhuber C, Scheiner O, Hoffmann-Sommergruber K. **Pru p 1, a major food allergen from peach (*Prunus persica*)**. Poster presentation at the vfwf – university lecture at the Medical University of Vienna, May 2006, Vienna, Austria

Gaier S, Oberhuber C, Rigby N, Marsh J, Hemmer W, Mills C, Shewry P, Scheiner O, Hoffmann-Sommergruber K. **IgE reactivity patterns of peach allergic patients in Austria**. Poster presentation at the Pirquet Symposium, December 2006, Vienna, Austria

Gaier S, Oberhuber C, Rigby N, Marsh J, Mills C, Shewry P, Scheiner O, Hoffmann-Sommergruber K. **The effect of thermal processing and *in vitro* gastrointestinal digestion on the stability of the peach allergens, Pru p 1 and Pru p 3**. Poster presentation at the Annual Meeting of the Austrian Society of Allergology and Immunology (ÖGAI), December 2007, Alpbach, Austria

Gaier S, Oberhuber C, Hemmer W, Rigby N, Marsh J, Mills C, Shewry P, Scheiner O, Hoffmann-Sommergruber K. **LTP – a relevant allergen for Austrian *Rosaceae* allergic patients?**. Poster presentation at the Annual Meeting of the Austrian Society of Allergology and Immunology (ÖGAI), December 2007, Alpbach, Austria

Gaier S, Oberhuber C, Rigby N, Marsh J, Hemmer W, Mills C, Shewry P, Scheiner O, Hoffmann-Sommergruber K. **Allergenicity of peach allergens Pru p 1 and Pru p 3 related with protein stability.** Poster presentation at the 27th EAACI Congress, June 2008, Barcelona, Spain

Gaier S, Oberhuber C, Rigby N, Marsh J, Hemmer W, Mills C, Shewry P, Scheiner O, Hoffmann-Sommergruber K. **pH conditions exert effects on the stability of the peach allergens Pru p 1 and Pru p 3.** Poster presentation at the Joint Annual Meeting of Immunology of the Austrian and German Societies (ÖGAI, DGfI), September 2008, Vienna, Austria

Gaier S, Latzka J, Ferrone S, Pehamberger H, Scheiner O, Breiteneder H, Wagner S. **Selection of HMW-MAA mimotopes for the generation of a polymimotope vaccine against malignant melanoma.** Poster presentation at the Joint Annual Meeting of Immunology of the Austrian and German Societies (ÖGAI, DGfI), September 2008, Vienna, Austria

Gaier S, Ferrone S, Hafner C, Pehamberger H, Breiteneder H, Wagner S. **HMW-MAA mimotopes for the generation of a polymimotope vaccine against malignant melanoma.** Poster presentation at the Joint Meeting of the 7th World Congress on Melanoma and 5th Congress of the European Association of Dermato-Oncology (EADO), May 2009, Vienna, Austria

Gaier S, Ferrone S, Hafner C, Pehamberger H, Breiteneder H, Wagner S. **Design of a polymimotope vaccine against malignant melanoma using HMW-MAA mimotopes.** Poster presentation at the 2nd European Congress of Immunology (ECI), September 2009, Berlin, Germany

OTHER PRESENTATIONS at national and international meetings

Hoffmann-Sommergruber K, Sancho A, Holzhauser T, Skov P, Alessandri S, Wal J, Rigby N, Marsh J, Bernard H, **Gaier S**, Lauer I, Ma Y, Oberhuber C, Barkholt V, Griesmeier U, Bublin M, Radauer C, Scheurer S, Reese G, Zuidmeer L, Akkerdaas J, Neubauer A, Fernández-Rivas M, Ballmer-Weber B, Vieths S, van Ree R, Shewry P, Mills C. **The Europrevall allergen library: a collection of high quality purified natural and recombinant allergens for in vitro diagnosis.** Poster at the 26th EAACI Congress, June 2007, Göteborg, Sweden

Oberhuber C, **Gaier S**, Untersmayr-Elsenhuber E, Hafner C, Hoffmann-Sommergruber K. **Adverse reactions to dark chocolate – a case report.** Poster at the World Allergy Congress, December 2007, Bangkok, Thailand

Forstenlechner P, Ma Y, **Gaier S**, Oberhuber C, Scheiner O, Hoffmann-Sommergruber K. **Cloning of wheat profilin**. Poster presentation at the Annual Meeting of the Austrian Society of Allergology and Immunology (ÖGAI), December 2007, Alpbach, Austria

Bruckmüller MU, Oberhuber C, **Gaier S**, Vázquez-Cortéz S, Fernández-Rivas M, Hoffmann-Sommergruber K. **Melon is a relevant elicitor of food allergy in Southern Europe.** Poster at the Joint Annual Meeting of Immunology of the Austrian and German Societies (ÖGAI, DGfI), September 2008, Vienna, Austria