

Epitope-Specific Antibody Response to Mel-CAM Induced by Mimotope Immunization

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Peptide mimotopes of tumor antigen epitopes have been proposed as components of tumor vaccines. In this study, we determined the immunogenicity of melcam mim1 and melcam mim2, peptide mimics of an epitope of the melanoma cell-adhesion molecule (Mel-CAM). BALB/c mice were vaccinated either with mimotopes or mimotopes coupled to tetanus toxoid (TT). The antibody responses of mice to melcam mim1, melcam mim2, and recombinant Mel-CAM were analyzed by an ELISA and immunoblot analyses. TT-coupled mimotopes led to high titers of IgG mainly of the IgG2a subclass to melcam mim1 and melcam mim2. Immunization with each of the mimotope formulations induced antibodies that cross-reacted with recombinant Mel-CAM. Uncoupled mimotopes induced lymphocyte proliferation and cytokine production in spleen cell cultures indicating that both peptide mimotopes also contained T cell epitopes. TT-coupled mimotopes induced T helper (Th)1 (interleukin (IL)-2, interferon- γ) and Th2 (IL-4, IL-5) cytokines, whereas uncoupled mimotopes induced a Th1-biased T cell response. Our results suggest that mimotopes potentially represent a novel vaccine approach to induce a tumor antigen-specific humoral and cellular response.

Key words: antibody response/melanoma/Mel-CAM/mimotope
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Melanoma cell-adhesion molecule (Mel-CAM) is an integral membrane glycoprotein with a molecular mass of 113 kDa and contains several protein kinase recognition motifs in its cytoplasmic domain, suggesting an involvement in cell signalling (Shih, 1999). Mel-CAM is strongly upregulated in melanomas as compared with benign lesions and expressed by more than 80% of metastatic lesions (Kraus *et al*, 1997). Recent data suggest that Mel-CAM is involved in cell–cell adhesion (Satyamoorthy *et al*, 2001). The expression of Mel-CAM on melanoma cells correlates with an invasive phenotype indicating its use as a cell surface marker of metastasis formation and tumor progression (Xie *et al*, 1997). Mel-CAM has been shown to be involved in tumor–endothelial cell interaction that might be part of the extravasation process of tumor cells (Johnson *et al*, 1997; Shih *et al*, 1997). These findings have defined Mel-CAM as a candidate for mediating tumor growth and metastases and therefore blocking Mel-CAM might be a potential target for immunotherapy directed against several Mel-CAM expressing tumors. Fully human antibodies to Mel-CAM inhibited tumor growth and metastasis of human melanoma and spontaneous pulmonary metastasis of osteosarcoma in nude mice, indicating the usefulness of this protein for new

antibody-based immunotherapeutic approaches (Mills *et al*, 2002; McGary *et al*, 2003). The development of an epitope-based synthetic peptide vaccine to induce antibodies against Mel-CAM seems to be a valid new approach. This requires the identification of B cell epitopes capable of inducing immune responses.

Much work has been focused on the difficult problem of identifying the structural characteristics of B cell epitopes and a number of empirical approaches have been used in attempts to predict such epitopes from the primary amino acid sequence of proteins. The predictive value of these methods for potential vaccine use is limited since they identify linear B cell epitopes (Van Regenmortel, 2001). It seems that B cell epitopes capable of inducing functional antibody responses are, however, likely to be conformational in nature and therefore, unlike linear epitopes cannot be predicted from an analysis of the primary amino acid sequence (Mahler *et al*, 2003). Random phage display libraries or solid phase combinatorial peptide libraries have been used to identify peptides that mimic more complex epitopes (Smith, 1991; Felici *et al*, 1995; Zwick *et al*, 1998; Apostolopoulos *et al*, 1999; Yip *et al*, 2002). Peptide sequences that bind to a wide range of ligands such as monoclonal antibodies (mAb), receptors, and carbohydrate moieties have been successfully identified (Apostolopoulos *et al*, 2002; Monzavi-Karbassi *et al*, 2002; Cunto-Amesty *et al*, 2003).

In a previous study, we applied phage display technology to define structural equivalents (mimotopes) of the epitope of the mAb MAd18-5D7 on the cell adhesion molecule Mel-CAM

Abbreviations: IFN, interferon; IL, interleukin; mAb, monoclonal antibody; Mel-CAM, melanoma cell-adhesion molecule; Th1, T helper 1; TT, tetanus toxoid

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(Hafner *et al*, 2002). We used a linear 28mer phage display peptide library and identified two mimotopes, melcam mim1 and melcam mim2. By linear alignment using the BLAST search algorithm it was possible to align the sequence of melcam mim1 to the sequence of Mel-CAM between amino acid positions 307 and 310 (LEPA). In this study, we compared the ability of these mimotopes by themselves or coupled to tetanus toxoid (TT) to induce Mel-CAM cross-reactive antibodies. In addition, the potency of the B cell epitopes in the induction of a peptide-specific T cell reaction was studied.

Results

Induction of anti-peptide-specific immune response To investigate whether the mimotopes assume an immunogenic conformation corresponding to the natural epitope on Mel-CAM, BALB/c mice ($n = 5$ per group) were immunized with either the peptide mimics or the peptide mimics coupled to TT. After a primary immunization and three boosts, sera from individual mice were collected and tested for total IgG anti-mimotope antibodies using ELISA technique. All sera collected from mice immunized with uncoupled mimotopes contained antibodies that bound to the peptides in dilution to at least 1:100. Sera from mice immunized with TT-coupled mimotopes induced anti-mimotope-specific antibodies with an endpoint-titer of at least 1:100,000. Pre-immune sera, ch3 peptide, TT- or adjuvant-immunized mice did not display any melcam mim1- or melcam mim2-specific antibody response (data not shown).

The subclass of IgG that is induced after immunization serves as an indirect measure of the contribution of T helper (Th)1-type cytokines *versus* Th2-type cytokines. In our experiments, we measured production of IgG1 (Fig 1A, C) and IgG2a (Fig 1B, D). In the sera of mice immunized with TT-coupled mimotopes, IgG2a was the predominant isotype, detectable in serum dilutions up to 1:40,640. Lower IgG1 antibody levels were measured in serum dilutions up to 1:10,160. Immunization with unconjugated mimotopes induced only moderate peptide-specific IgG1 levels up to 1:40 (data not shown). Immunization experiments were repeated and resulted in similar antibody responses.

Mel-CAM-specific ELISA antibody assay and inhibition assay Mel-CAM-specific antibody levels were detected by ELISA. TT-coupled mimotopes induced significant Mel-CAM-specific antibody responses ($OD = 1.576 \pm 0.23$ for TT-melcam mim1-immunized mice, $OD = 1.89 \pm 0.127$ for TT-melcam mim1-immunized mice) at a dilution of 1:500; immunization with unconjugated mimotopes induced only moderate Mel-CAM-specific IgG ($OD = 0.759 \pm 0.9$ for melcam mim1-immunized mice, $OD = 0.895 \pm 0.06$ for melcam mim2-immunized mice) at a dilution of 1:40. Control sera from mice immunized with the ch3 peptide or TT did not show any Mel-CAM-specific antibody response (data not shown). We then investigated the specificity of the anti-mimotope antisera by inhibiting their binding to recombinant Mel-CAM. As shown in Fig 2, pre-incubation of peptide immune sera with melcam mim1 or melcam mim2 led to a concentration-dependent binding inhibition of 47% for melcam mim1 and 66% for melcam mim2; pre-incubation of

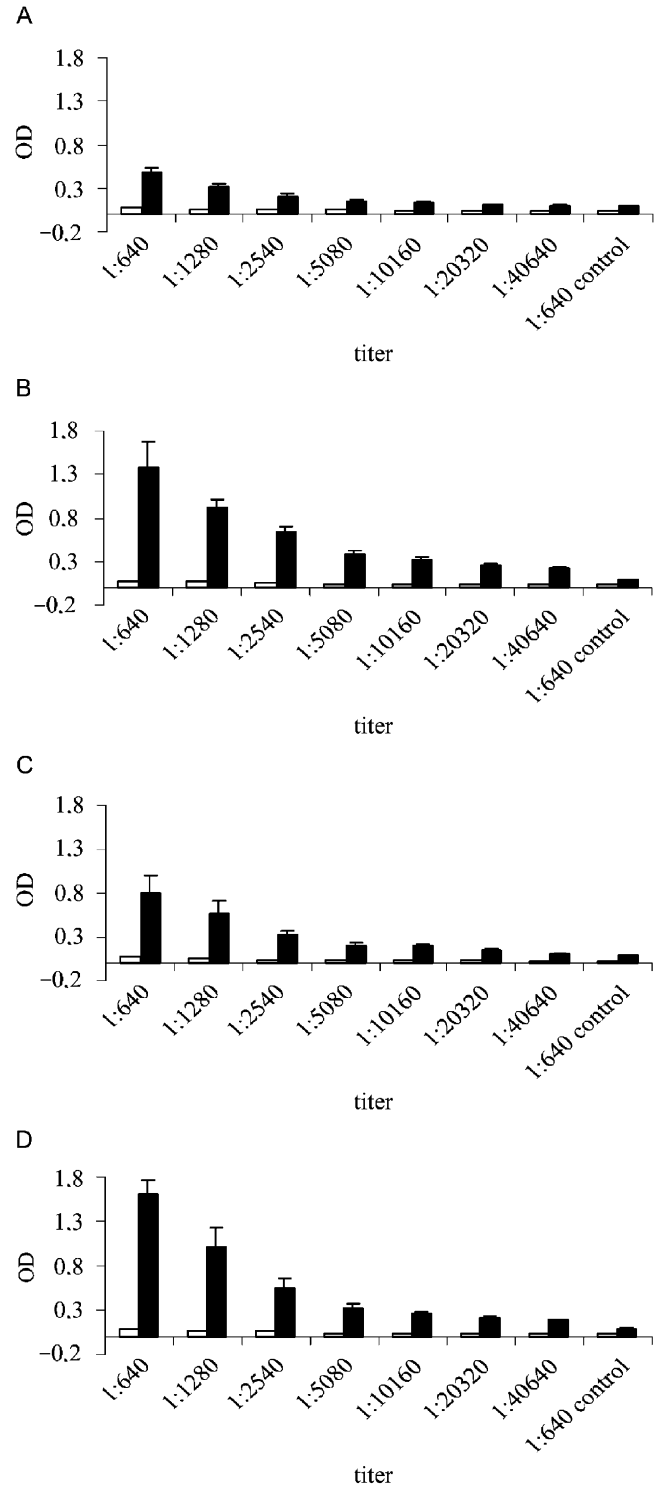
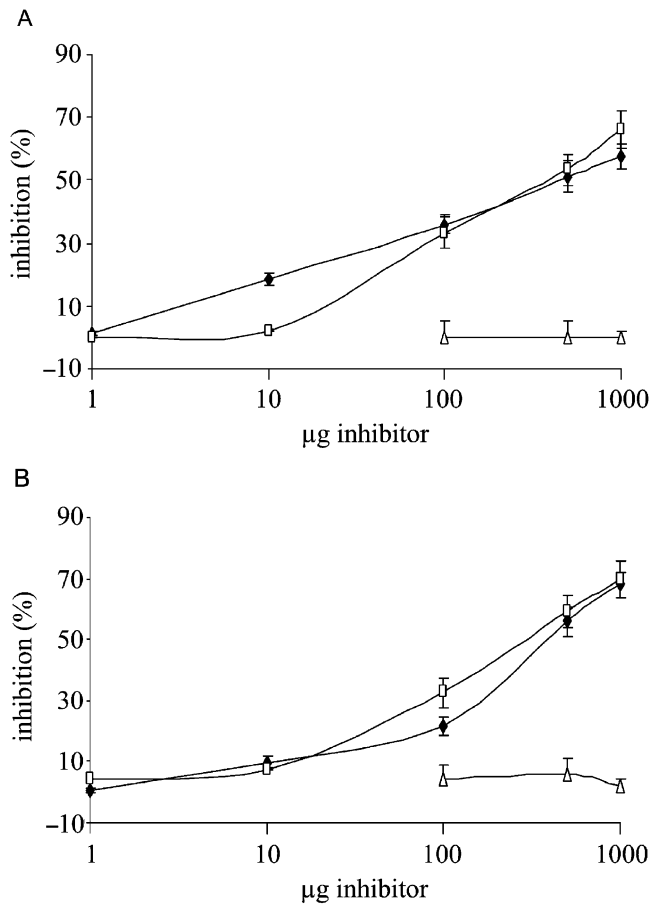


Figure 1

Development of mimotope-specific antibodies. Microtiter plates were coated with melcam mim1 and melcam mim2 and an ELISA assay was performed to measure specific antibody levels. Sera of animals ($n = 5$) immunized with tetanus toxoid (TT)-melcam mim1 and TT as control were analyzed for (A) IgG1 and (B) IgG2a subclasses in dilutions from 1:640 to 1:40,640 in triplicates. Sera of animals ($n = 5$) immunized with TT-melcam mim2 were also analyzed for (C) IgG1 and (D) IgG2a subclasses in dilutions from 1:640 to 1:40,640 in triplicates. □, pre-immune sera; ■, immune sera.

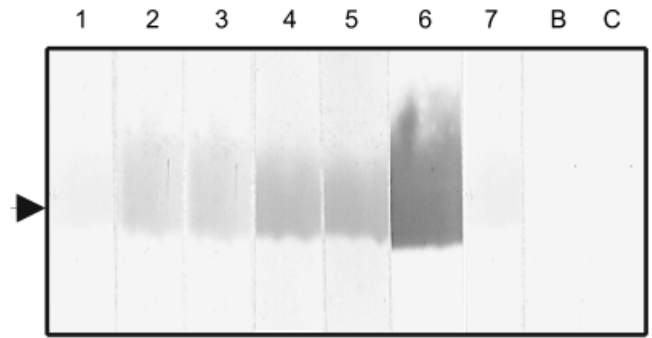
**Figure 2**

Melanoma cell-adhesion molecule (Mel-CAM)-specific ELISA inhibition assay. ELISA was performed to evaluate antibodies specific to recombinant Mel-CAM after immunization with (A) melcam mim1 and melcam mim2 and (B) tetanus toxoid-coupled melcam mim1 and melcam mim2. Sera were analyzed in a dilution 1:40 or 1:500, respectively. Sera were pre-incubated with increasing concentrations of melcam mim1, melcam mim2, or an unrelated peptide ch3 as the inhibitor, and incubated with recombinant Mel-CAM coated to a microtiter plate. Binding results are shown as inhibition (percentage) of binding of mouse sera to Mel-CAM as a function of the free mimotope concentration. ■, melcam mim1 serumpool/inhibition with melcam mim1; □, melcam mim2 serumpool/inhibition with melcam mim2; △, melcam mim1 serumpool/inhibition with ch3.

TT-conjugate immune sera with melcam mim1 or melcam mim2 led to a concentration-dependent binding inhibition of 71% for melcam mim1 and 69% for melcam mim2. No inhibition was observed when immune sera were incubated with the unrelated peptide mimic ch3.

Mel-CAM-specific immunoblot Recombinant Mel-CAM was electrophoresed and blotted onto a nitrocellulose membrane. Strips were incubated with serum pools or mAb MAD18-5D7. Sera from mice immunized with melcam mim1, melcam mim2, or TT conjugates reacted with the recombinant Mel-CAM, whereas no reactivity was observed when strips were incubated with pre-immune serum pool, ch3 peptide, or TT-immunized mice (Fig 3).

Mimotope-specific cellular response We immunized groups of mice with peptides and peptide conjugates. Seven days after boosting mice were sacrificed and spleen

**Figure 3**

Melanoma cell-adhesion molecule (Mel-CAM)-specific immunoblot. Eight micrograms of Mel-CAM were separated by 8% SDS-PAGE, transferred to a nitrocellulose membrane and incubated with a pre-immune serum pool (lane 1), a serum pool from melcam mim1-immunized mice (lane 2), melcam mim2-immunized mice (lane 3), tetanus toxoid (TT)-coupled melcam mim1-immunized mice (lane 4) and TT-coupled melcam mim2-immunized mice (lane 5), monoclonal antibody MAD18-5D7 (lane 6), and TT-immunized mice (lane 7). (B) Buffer control, (C) isotype control; arrow indicates position of recombinant Mel-CAM.

cells were collected. The data presented represent two independent experiments and were performed in triplicates. As shown in Fig 4, spleen cells derived from melcam mim1- or melcam mim2-immunized animals proliferated vigorously upon stimulation with melcam mim1 (stimulation index (SI) = 5.068; c.p.m.: 20139 ± 743) or melcam mim2 (SI = 2.4; c.p.m.: 8375 ± 389). In comparison with spleen cells from naïve mice stimulated with melcam mim1 or melcam mim2, this proliferative response was significant ($p < 0.05$). Spleen cells derived from mice immunized with TT-coupled mimotopes were not activated by any of the mimotopes. More interestingly, melcam mim1 activated spleen cells derived from mice immunized with melcam mim2 (SI = 5.8; c.p.m.: 20339 ± 697), whereas no proliferation of spleen cells was observed with the unrelated peptide mimic ch3 or on naïve spleen cells. The melcam mim1 and melcam mim2 cellular responses were antigen presenting cell (APC) dependent, as detected by the addition of an anti-MHC class II antibody that inhibit proliferation of the spleen cells (data not shown).

Cytokine production in spleen cell cultures To characterize the *in vitro* cytokine production of interleukin (IL)-2, IL-4, IL-5, and interferon (IFN)- γ levels were measured in spleen cell cultures of mimotope-immunized or mimotope-conjugate-immunized animals ($n = 5$ per group). Results are summarized in Table I. Spleen cell cultures of mice immunized with melcam mim1 or melcam mim2 released large amounts of IFN- γ and IL-2 in response to stimulation with melcam mim1 or melcam mim2 ($p < 0.01$ compared with TT-conjugate-immunized mice). Comparable levels of cytokine production were observed in spleen cell cultures of melcam mim2-immunized mice stimulated with melcam mim1, and vice versa. Interestingly, TT-coupled mimotopes induced a Th0-like cytokine profile. No cytokine release in response to melcam mim1 or melcam mim2 was observed from spleen cells of mice immunized with the unrelated peptide ch3, TT or from spleen cells of naïve mice. The data presented in Table I represent the means $\pm$ SD from two independent experiments.

Discussion

In this study, we demonstrated that immunization of mice with two mimotopes that were derived from the melanoma-associated cell adhesion molecule Mel-CAM were able to induce anti-peptide antibodies that recognized the native Mel-CAM antigen. In our previous work, we have shown that both mimotopes were specific antigenic mimics of the mAb MAD18-5D7 epitope of Mel-CAM (Hafner *et al*, 2002). Nevertheless, antigenic mimicry does not guarantee immunogenic mimicry. Mimotope-based vaccine strategies will only be effective if the induced mimotope-specific antibody responses allow recognition of the targeted tumor antigen. We demonstrated by ELISA as well as by immunoblot of recombinant Mel-CAM that the induced anti-mimotope antibodies are Mel-CAM specific (Figs 2 and 3). To provide further evidence that the induced IgG response was directed toward the mAb MAD18-5D7 epitope on Mel-CAM, we performed inhibition experiments. The synthetic mimotopes blocked binding to Mel-CAM in a concentration-dependent manner indicating that induced antibodies were specific (Fig 2).

Synthetic mimotopes are attractive over other forms of vaccination, because they are easily produced and free of contaminating or toxic substances. In order to elicit efficient humoral immune responses against mimotopes, the use of carrier proteins seems to be necessary. Nevertheless, synthetic mimotopes alone were able to induce antibodies with sufficient affinities when they were applied at high density (Olszewska *et al*, 2000). In particular, a carrier protein with T helper activity will influence the immune response (Panina-Bordignon *et al*, 1989). As a prototype of a carrier protein we used TT and compared the effect of TT-coupled peptide mimics or peptide mimics alone on the anti-Mel-CAM response. We demonstrated that mice immunized with melcam mim1 or melcam mim2 could elicit an IgG response against Mel-CAM. Ig titers obtained by mimotope immunizations were, however, substantially lower than those obtained by immunization with TT as a carrier.

The role of cellular response of mimotopes and their influence in the formation of anti-mimotope antibodies against tumor antigens has not been clarified so far in detail. Therefore, we analyzed the humoral and cellular immune responses to mimotopes previously generated by biopannings of a 28mer-phage-displayed peptide library with mAb MAD18-5D7. Upon stimulation with mimotope peptides, we observed a significantly higher proliferation of spleen cell cultures of mice that had been immunized with uncoupled mimotopes (Fig 4). Both mimotopes induced the production of Th1 cytokines in proliferating spleen cells indicating that in addition to B cell epitopes they also contained T cell epitopes (Table I). Interestingly, TT-coupled mimotopes induced both Th1 and Th2 cytokines (Table I). Nevertheless, IFN- γ detected in supernatants collected from spleen cell cultures that had been activated *in vitro* by mimotopes supported Th1-type activation consistent with the observed IgG2a production. Therefore, mimotope immunization against Mel-CAM primes for both humoral and cellular responses. This has been shown previously for carbohydrate mimotopes (Monzavi-Karbassi *et al*, 2001, 2004). One might speculate that our mimotopes include

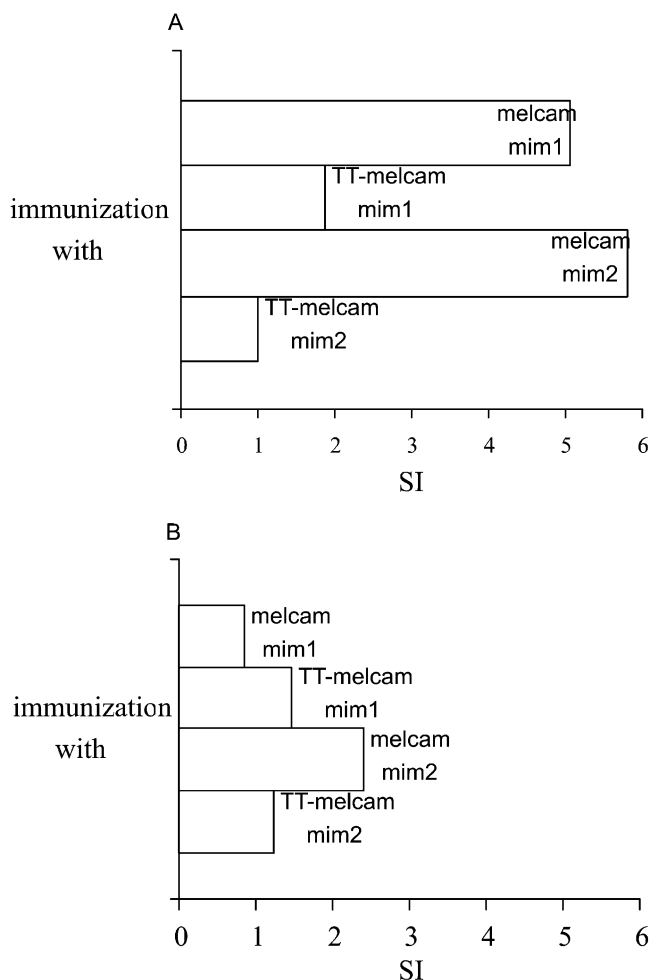


Figure 4
Cell proliferation assay on splenocytes of mice immunized with the indicated mimotope or tetanus-coupled mimotope. Splenocytes were co-cultured with (A) melcam mim1 or (B) melcam mim2. SI, stimulation index = ratio of mean proliferation with peptide and medium control values.

Tumor cell proliferation assay The biological effects of the induced Abs were tested *in vitro*. MelJuso and MD3a cells were incubated with mice sera or with mAb MAD18-5D7. Incubation with TT-melcam mim1 sera enhanced ^3H -thymidine incorporation of 518A2 cells by $19.2\% \pm 2.1\%$, with TT-melcam mim2 sera by $11.7\% \pm 0.98\%$, and with mAb MAD18-5D7 by $11.6\% \pm 2.1\%$ compared with untreated 518A2 cells ($p < 0.01$). The Mel-CAM-negative cell line MD3a was not influenced by anti-TT-melcam mim1 or anti-TT-melcam mim2 antibodies. Anti-TT antibodies did not affect the proliferation of any cell lines used (Fig 5). Experiments were repeated three times under identical conditions.

Complement-dependent cytotoxicity Sera from TT-mimotope-immunized mice were examined for their ability to mediate complement-dependent lysis of Mel-CAM expressing target cells. Anti-TT-melcam mim1 and anti-TT-melcam mim2 sera mediated low but specific lysis of MelJuso cells up to $8.6\% \pm 0.9\%$ ($p < 0.01$) compared with spontaneous cell lysis ($2.1\% \pm 0.7\%$). The data presented in Table II represent the mean values of three independent experiments.

Table I. Release of cytokines from splenocytes of mice immunized with melcam mim1 or melcam mim2 and their corresponding TT conjugates^a

Cytokine release	Immunization with	Stimulation <i>in vitro</i> with		
		melcam mim1	melcam mim2	ch3
IFN- γ	melcam mim1	513.27 $\pm$ 30.22	159.64 $\pm$ 10.98	5.74 $\pm$ 0.46
	melcam mim2	151.33 $\pm$ 13.78	227.92 $\pm$ 20.79	2.30 $\pm$ 0.10
	TT melcam mim1	53.48 $\pm$ 5.42	27.55 $\pm$ 3.52	0.00
	TT melcam mim2	51.86 $\pm$ 4.12	98.55 $\pm$ 10.97	0.00
IL-2	melcam mim1	471.07 $\pm$ 57.34	66.73 $\pm$ 4.54	0.00
	melcam mim2	0.00	112.91 $\pm$ 15.4	0.00
	TT melcam mim1	0.00	0.00	0.21 $\pm$ 0.19
	TT melcam mim2	0.00	0.07 $\pm$ 0.01	0.20 $\pm$ 0.43
IL-4	melcam mim1	10.16 $\pm$ 1.52	0.00	0.21 $\pm$ 0.01
	melcam mim2	0.00	10.02 $\pm$ 2.07	0.30 $\pm$ 0.01
	TT melcam mim1	31.49 $\pm$ 2.54	13.28 $\pm$ 1.38	4.04 $\pm$ 0.21
	TT melcam mim2	2.99 $\pm$ 0.39	17.66 $\pm$ 1.67	3.42 $\pm$ 0.42
IL-5	melcam mim1	13.85 $\pm$ 1.84	0.37 $\pm$ 0.01	0.00
	melcam mim2	0.54 $\pm$ 0.04	42.03 $\pm$ 0.24	0.00
	TT melcam mim1	0.90 $\pm$ 0.02	2.26 $\pm$ 0.62	0.00
	TT melcam mim2	0.00	0.90 $\pm$ 0.05	0.00

^aResults are presented as mean $\pm$ SD after subtraction of baseline. Cytokine release is shown in picograms per milliliter. IFN, interferon; IL, interleukin; TT, tetanus toxoid

cytotoxic T cell epitopes. This might be an explanation for high cellular proliferation and high IFN- γ release of uncoupled mimotopes.

It is now accepted that a Th1-dominant immunity, which is regulated by IL-12 and IFN- γ , plays a pivotal role in the eradication of tumors *in vivo* (Portielje *et al*, 2003). A Th1 response with the expression of the IgG2a isotype is a desirable response as IgG2a Abs are opsonizing and complement-fixing (Hart *et al*, 2004). The Th1-biased immune response of both mimotopes was further indicated by an increase in the IgG2a/IgG1 ratio in the TT-coupled mimi-

tope-immunized mice. However, immunization with TT-coupled mimotopes induced IL-4 as well as IFN- γ producing cells (Table I). These results indicate that both the overall magnitude of the humoral and cellular immune responses were significantly affected by the carrier protein. The fact that immunization with uncoupled mimotopes induced high IFN- γ levels but a low antibody response indicates that the potential of mimotopes to induce a certain quality of immune response can be modulated by a selected carrier system. Mimotope vaccination without carrier proteins may favorably induce a cellular/cytotoxic anti-tumor response. This concept will need further clarification.

Anti-Mel-CAM antibodies can affect tumor growth by both direct and indirect mechanisms (Mills *et al*, 2002;

Table II. Complement-dependent cell lysis^a

Incubation with immune sera against	% lysis of target cell	
	MelJuso	MD3a
TT melcam mim1	8.6 $\pm$ 0.9	0.0
TT melcam mim2	5.2 $\pm$ 0.7	0.5 $\pm$ 0.03
TT	1.2 $\pm$ 0.1	0.9 $\pm$ 0.2

^aTarget cells were incubated with mouse sera and rabbit serum as a source of complement.

Inactivated rabbit serum was used to distinguish specific from spontaneous cell lysis.

Percentage of lysis were calculated using the formula: % lysis = [(experimental - target spontaneous)/(target maximum - target spontaneous)] $\times$ 100.

TT, tetanus toxoid.

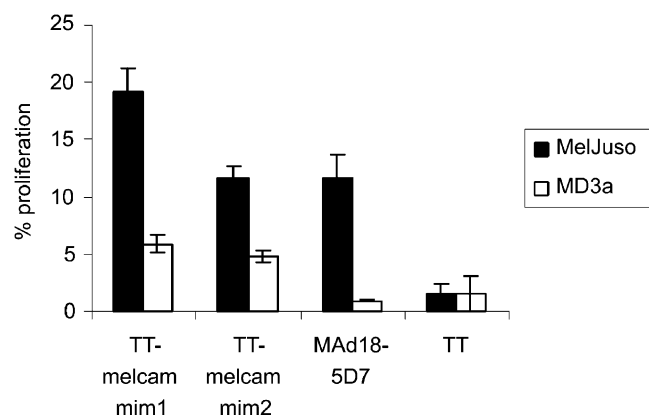


Figure 5
Melanoma cell proliferation assay. ³H-thymidine proliferation assay demonstrating the effects of the murine IgG on MelJuso and MD3a cell line growth.

McGary *et al*, 2003). The direct effect involves homotypic interactions between melanoma cells and heterotypic interactions between melanoma cells and endothelial cells (Shih, 1999). Anti-TT-melcam mim1 antibodies and anti-TT-melcam mim2 antibodies led to a $19.2\% \pm 2.1\%$ or $11.7\% \pm 0.98\%$ enhanced proliferation of MelJuso melanoma cells, respectively. This is comparable with the proliferative effect of mAb MAd18-5D7 on Mel-CAM-positive tumor cells *in vitro* (Fig 5). The indirect mechanisms also involve complement-dependent cytotoxicity. We were able to demonstrate that anti-TT-melcam mim1 as well as anti-TT-melcam mim2 antibodies in association with rabbit complement led to low but specific cell lysis of MelJuso cells (Table II).

The results of this study provide evidence that synthetic mimotopes can resemble T and B cell epitopes of Mel-CAM both as an immunogen and mimic of the natural antigen and suggest that phage display peptide libraries have a broad application for the design of anti-cancer vaccines. A new generation of anti-cancer vaccines could possibly consist of a mixture of mimotopes selected by using tumor-specific mAb.

Materials and Methods

Peptide mimics, recombinant Mel-CAM, cell lines, and antibodies The peptides, GRALPGTAEWTSYPGWTSRLKNLEPAVG (melcam mim1) and RVKHMVLGASSRVPGFSSLPGFQGPFT (melcam mim2), and an unrelated peptide (ch3) were synthesized by piCHEM (Graz, Austria). The purity of the peptides was greater than 95% as assessed by HPLC. The selection process of melcam mim1 and melcam mim2 was described elsewhere (Hafner *et al*, 2002). The recombinant extracellular Mel-CAM domain was a gift of M. Herlyn (The Wistar Institute, Philadelphia, Pennsylvania) and described elsewhere (Shih *et al*, 1997). The human melanoma cell line MelJuSo (Lehmann *et al*, 1987) that expresses high levels of Mel-CAM and the melanoma cell line MD3a (Versteeg *et al*, 1989) with no detectable expression of Mel-CAM were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum and antibiotics (Gibco, Invitrogen, Carlsbad, California). MAd18-5D7 is a mouse IgM mAb and was selected on the basis of its activity of enhancing Mel-CAM-mediated adhesion in aggregation assays (J. P. Johnson, Institute of Immunology, University of Munich, Munich, Germany, unpublished results).

Coupling of mimotopes Mimotopes were coupled to the carrier protein TT (Berna, Bern, Switzerland) using the heterobifunctional cross-linking reagent m-maleimidobenzoyl-N-hydroxysuccinimide (MBS) (Pierce Endogen, Rockford, Illinois). The amino groups of the carrier protein were first activated by the addition of a 25-fold molar excess of MBS for 30 min at room temperature. Excess MBS was removed by a desalting column (PD-10 column, Amersham Bioscience, Little Chalfont, UK). In a second step, peptides were added in a molar ratio of 40:1 peptide-to-carrier protein. Cross-linking occurred to the cysteine residues on the peptides within 3 h at room temperature. Unbound peptides were removed by dialysis against phosphate-buffered saline (PBS).

Immunization with synthetic peptide mimics or with TT-coupled peptide mimics Groups of five 6-wk-old female BALB/c mice (Charles River, Sulzfeld, Germany) were immunized intraperitoneally with synthetic mimotopes or mimotopes coupled to TT. Each injection delivered 20 μ g peptide in 100 μ L 0.9% sodium chloride containing 50 μ L Al(OH)₃ as adjuvants. The control group received TT and adjuvants alone. The immunizations were performed four times in 21-d intervals. Seven days after the last

immunization the animals were sacrificed. Mice were bled prior to immunization, before each immunization and 1 wk after the last immunization. Experiments were performed twice under identical experimental conditions. The Animal Experimentation Committee of the University of Vienna and the Ministry of Education, Science and Culture, Vienna, Austria approved the experiments. The study was conducted according to Declaration of Helsinki Principles.

Peptide-specific ELISA antibody assays Microtiter wells (Maxisorb, Nunc, Roskilde, Denmark) were coated overnight at 4°C with melcam mim1 or melcam mim2 (3 μ g per mL coating buffer 50 mM NaHCO₃, pH 9.4), blocked with 5% milk powder in PBS for 2 h and washed with PBS/0.05% Tween-20. Sera were serially diluted into ELISA wells containing blocking buffer. Isotype specific or total IgG goat anti-mouse secondary antibodies conjugated to alkaline phosphatase (Sigma, St Louis, Missouri) were used for detection of bound antibodies. ELISA were developed by addition of *p*-nitrophenylphosphate (Sigma). The absorbance was measured at 405 nm. Endpoint titers were defined as the dilution producing an OD equal to twice the ELISA background.

Mel-CAM-specific ELISA antibody assay and inhibition assay ELISA for reactivity to recombinant Mel-CAM was performed by coating wells with 2 μ g per mL coating buffer of recombinant Mel-CAM. Sera were serially diluted in blocking buffer and added to the blocked microtiter plates. For inhibition experiments sera were diluted 1:40 (for uncoupled mimotopes) or 1:500 (for TT-coupled mimotopes), and incubated overnight at 4°C with increasing amounts of melcam mim1, melcam mim2, or ch3 (0 μ g per mL up to 1 mg per mL). Subsequently, total IgG goat anti-mouse secondary antibody conjugated to alkaline phosphatase (Sigma) was used for detection of bound antibodies. ELISA was developed by addition of *p*-nitrophenylphosphate (Sigma). The absorbance was measured at 405 nm.

Immunoblot Five micrograms of recombinant Mel-CAM were electrophoresed on an 8% polyacrylamide gel and transferred onto a nitrocellulose membrane (Schleicher and Schuell, Keene, New Hampshire). Sera were diluted 1:100 (for TT-coupled mimotopes) or 1:40 (for uncoupled mimotopes) in 5% MTBS/0.5% Tween-20 and added to the blocked nitrocellulose membrane. mAb MAd18-5D7 was used as a positive control and is described elsewhere (Hafner *et al*, 2002). Bound antibodies were visualized using an AP-conjugated anti-mouse IgG (γ -specific) (Sigma) or an AP-conjugated anti-mouse IgM (μ -specific) (Sigma), respectively, followed by equilibration in AP-buffer (0.1 M Tris base, pH 9.5, 0.1 M NaCl, 1 mM MgCl₂) and development with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate-*p*-toluidine.

Lymphocyte cell proliferation and cytokine production in spleen cell cultures Spleen cells from mimotope-sensitized and control animals were used for detection of T cell proliferation. Briefly, 2×10^6 cells were incubated without or with mimotopes (2 μ g per well), TT (0.2 μ g per well), or concanavalin A (0.2 μ g per well) (Sigma). After 3 d of incubation, 1 μ Ci of ³H-thymidine was added to each well and incubation continued for 16 h. The plates were harvested and the amount of incorporated ³H-thymidine was counted on a 1205 Betaplate Liquid Scintillation Counter (Wallac Oy, Turku, Finland). Stimulation indices were calculated using the formula: SI = ratio of mean proliferation with peptide and medium control values. For determination of IL-2, IL-4, IL-5, and IFN- γ production, spleen cells were cultured with mimotopes, TT, or concanavalin A in 48-well plates (Costar, Cambridge, Massachusetts) at a concentration of 5×10^6 per well. Supernatants were collected from the cultures 48 h after *in vitro* activation and the concentrations of IL-2, IL-4, and IL-5 were measured by purchased appropriate ELISA kits according to the guideline of the manufacturer (Pierce Endogen). IFN- γ levels were measured in 96-well plates (Nunc) and coated with rat anti-mouse IFN- γ (Pierce Endogen) at a concentration of 0.5 μ g per mL carbonate buffer, pH 9.6 for 6 h at room temperature. Thereafter, biotin-labeled rat

anti-mouse IFN- γ antibodies (Pierce Endogen) were applied followed by peroxidase-conjugated streptavidin. For color development tetramethylbenzidine substrate was used and the absorbance measured at 450 nm. The sensitivity of this assay was 15 pg per mL. Results reflect the measured cytokine levels in picograms per milliliter after subtraction of baseline levels of non-stimulated cell cultures.

Tumor cell proliferation assay Tumor cells were plated in 96-well microtiter plates (Costar, Corning, New York) at a density of 5×10^4 cells per well for MelJuso and MD3a cell lines. Cells were allowed to adhere overnight at 37°C. Mouse sera of TT-melcam mim1 or TT-melcam mim2 vaccinated animals or mAb MAd18-5D7 were added to the cells diluted up to 1:100 and incubated for 72 h at 37°C. Cells were pulsed with 0.5 μ Ci ^{3} H-thymidine per well for 6 h and harvested. The incorporated ^{3} H-thymidine was measured in a β -counter (Wallac Oy). Percentage of inhibition was calculated by comparing the counts per minute values of treated cells with those of untreated cells.

Complement-dependent cytotoxicity assay Promega CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega Corp., Madison, Wisconsin) was used for measurement of cell lysis. A naïve rabbit serum in a final dilution 1:5 was used as a source of complement. To calculate unspecific cell lysis, inactivated (56°C/30 min) serum was used. MelJuso and MD3a cells were used as target cells. 1×10^4 cells per well were plated and inactivated mice sera, diluted 1:100, were added. Plates were incubated for 1 h at 37°C. Thereafter complement was added and incubated for 2 h. Assays were performed according to the manufacturer's instructions. Percentages of lysis were calculated using the formula: % lysis = [(experimental – target spontaneous)/(target maximum – target spontaneous)] $\times$ 100.

Statistical analysis The data of tumor cell proliferation and complement-dependent cytotoxicity (CDC) experiments were evaluated statistically by the Pearson χ^2 significance test and the Mann-Whitney *U* test was used to statistically evaluate lymphocyte cell proliferation and cytokine production in spleen cell cultures.

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