

Reduction of Human Melanoma Tumor Growth in Severe Combined Immunodeficient Mice by Passive Transfer of Antibodies Induced by a High Molecular Weight Melanoma-Associated Antigen Mimotope Vaccine

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Abstract Purpose: The high molecular weight melanoma-associated antigen (HMW-MAA) is an attractive target for immunotherapy of malignant melanoma. We have recently generated a vaccine based on the HMW-MAA mimotope 225D9.2⁺ that was able to induce anti-HMW-MAA antibodies with antitumor activity *in vitro*. Here, we investigated the antitumor activity of these antibodies in a human melanoma xenotransplant severe combined immunodeficient (SCID) mouse model.

Experimental Design: Tumors were established by injecting the human melanoma 518A2 cells into C.B.17 SCID/SCID mice. In tumor prevention experiments, 200 μ g purified total IgG antibodies were injected intravenously the same day or on day 5 in therapeutic experiments. Antibody administration was repeated every fourth day and tumor volumes were measured. Antibody specificity and tumor infiltration by macrophages were investigated by immunohistochemistry.

Results: Within 35 days after cell inoculation, antibody treatment reduced tumor growth up to 40% in the therapeutic and up to 62% in the tumor prevention experiments compared with the control mice. In tumors of all groups, a similar distribution of the HMW-MAA and no differences in infiltration of macrophages were detected by immunohistochemistry.

Conclusions: Here, we showed that antibodies induced by the 225D9.2⁺ mimotope effectively inhibited melanoma tumor growth. Additional mechanisms besides antibody-dependent cell cytotoxicity like disruption of interactions of melanoma cells mediated by extracellular matrix components seem to be involved in tumor growth inhibition. Based on our findings, we suggest that active immunization with this mimotope might be a promising strategy for treatment of melanoma.

Development of an effective treatment against melanoma is an important challenge in dermato-oncology. Despite new therapeutic strategies (e.g., chemotherapy and radiotherapy), the overall survival time of patients with distant melanoma metastases remains <1 year (1). This situation demands efforts to develop new biological therapies.

The high molecular weight melanoma-associated antigen (HMW-MAA) is an established target to attack melanoma because it is highly expressed on the surface of melanoma cells (2). Recent findings have shown that the HMW-MAA is involved in the activation of several signaling pathways modulating melanoma cell adhesion, spreading, migration, and invasion (2). The HMW-MAA has been exploited for immunotherapeutic approaches with monoclonal antibodies (mAb; ref. 3). To improve efficacy of immunotherapy directed against the HMW-MAA, active vaccination against the HMW-MAA was investigated using the anti-idiotypic mAb MK2-23, which mimics the determinant recognized by the anti-HMW-MAA mAb 763.74 (4, 5). The results of these studies revealed a moderate patients' improvement, yet these approaches never entered extensive clinical trials.

Melanoma therapy by vaccination needs to be revisited in the light of several recent findings in animal model systems and patients. A retrospective analysis of >300 patients with melanoma who underwent immunoscintigraphy with ^{99m}Tc-labeled F(ab')₂ fragments of the anti-HMW-MAA mAb 225.28S suggested that repeated administrations of radiolabeled F(ab')₂ fragments of the mAb had a statistically significant beneficial effect on the median survival of melanoma patients (6). The

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Translational Relevance

Because vaccination against tumor antigens, which are in most cases overexpressed self-antigens, is a difficult and often ineffective approach, antibody therapies have emerged. They represent the most advanced form of immunotherapy at the moment. In this study, we describe the antitumor effect of antibodies induced by a HMW-MAA mimotope in a human melanoma xenotransplant SCID mouse model. Extrapolating the presented results, therapeutic efficacy against malignant melanoma and even a prophylactic efficacy could be expected by active immunization with this mimotope coupled to an immunogenic carrier protein. In addition, our mimotope represents a peptide that can easily be synthesized and standardized to meet the regulatory requirements for clinical trials and facilitate the monitoring of the humoral antitumor antigen immunity elicited by the active specific immunotherapy. The results obtained with this HMW-MAA mimotope in mice make it likely that it will display a similar immunogenicity and tumor-protective effect in patients with malignant melanoma.

repeated intravenous application of the mAb 225.28S significantly suppressed the tumor cell growth of human melanomas in a severe combined immunodeficient (SCID) mouse xenotransplant model *in vivo* and resulted in distinct modulation of the expression of 307 genes (7). However, the mechanisms underlying these observations have not been well defined.

In our previous work, we described for the first time the identification of a linear HMW-MAA mimotope, which mimics the HMW-MAA determinant recognized by the mAb 225.28S (8). This was accomplished by biopanning a phage-display peptide library with the anti-HMW-MAA mAb 225.28S. The mimotope showing the best HMW-MAA mimicry induced an anti-HMW-MAA IgG response in rabbits, which was able to inhibit human melanoma tumor growth *in vitro* (8). Similar works have been reported on the identification of other tumor antigen-associated mimotopes using phage-display peptide libraries (9–11). Here, we extend these findings and report effects of the antibodies induced by the mimotope *in vivo* in a human melanoma xenotransplant mouse model.

Materials and Methods

Cell culture and mice. The human melanoma cell line 518A2 (a gift from Dr. Peter Schrier, Medical School Leiden) was cultured as described previously (8). Six-week-old, female, pathogen-free C.B.17 SCID/SCID mice were obtained from Harlan Winkelmann. Animals were housed in microisolator cages in laminar flow racks and received autoclaved food and water *ad libitum* throughout the experimental period. All experiments were approved by the Animal Experimentation Committee of the University of Vienna and the Ministry of Education, Science and Culture.

Immunization and purification of rabbit IgG antibodies. The linear peptides AEGERTRINPWPALGGGGG (225D9.2⁺) and PESFDGDPASNTAPLQPGGGGG (P4) were manufactured synthetically (piChem) and the peptide 225D9.2⁺ was coupled to the carrier protein tetanus toxoid (TT; Berna) using the heterobifunctional cross-linking reagent *m*-maleimidobenzoyl-*N*-hydroxysuccinimide (Pierce) as described previously (8).

Immunizations with the peptide conjugate 225D9.2⁺-TT or TT alone as a control were done in rabbits at Charles River Laboratories. Immunizations were done three times in 14- to 21-day intervals, each with 200 µg peptide conjugates adsorbed to complete and incomplete Freund's adjuvant. Seven days after the last immunization, the animals were sacrificed. Total IgG from sera of rabbits was purified using a HiTrap Protein A HP column (Amersham Biosciences) as described previously (8).

Specificity of the antibodies was tested in a DotBlot assay. One microgram of the peptide 225D9.2⁺ or P4, the peptide 225D9.2⁺ coupled to keyhole limpet hemocyanin (KLH; Sigma), or KLH alone were dotted in duplets onto a nitrocellulose membrane. Blot strips were blocked with TBS-Tween 20/3% bovine serum albumin and thereafter incubated with TBS-Tween 20, 100 µg/mL IgG fraction of the 225D9.2⁺-TT antiserum, or IgG fraction of the TT antiserum for control. Bound antibodies were detected by alkaline phosphatase-conjugated anti-rabbit IgG (Jackson ImmunoResearch). Color development was done with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium.

Passive antibody transfer and analysis of tumor growth. For *in vivo* tumor prevention experiments, 1×10^6 518A2 human melanoma cells resuspended in 100 µL PBS were injected subcutaneously into the lower left flank of SCID mice. On day 1 and then in 4-day intervals, one group of mice ($n = 8$) received 200 µg IgG fraction of the 225D9.2⁺-TT antiserum intravenously in a volume of 200 µL saline solution, the second group ($n = 8$) received 200 µg IgG fraction of the TT antiserum in a volume of 200 µL saline solution, and the third group ($n = 5$) received 200 µL saline solution. Tumor size was measured every fifth day using calipers.

For *in vivo* therapeutic experiments, 1×10^7 518A2 human melanoma cells per mouse were inoculated subcutaneously into the lower left flank on day 1. On day 5, when tumors reached a mean diameter of 3 mm, and in 4-day intervals, one group of mice ($n = 8$) received 200 µg IgG fraction of the 225D9.2⁺-TT antiserum intravenously in a volume of 200 µL saline solution, the second group ($n = 8$) received 200 µg IgG fraction of the TT antiserum in a volume of 200 µL saline solution, and the third group ($n = 5$) received 200 µL saline solution.

The experiments were repeated three times under identical experimental conditions. Results were compared with nonparametric Mann-Whitney *U* test using the SPSS 14.0 program (SPSS). $P \leq 0.05$ was considered as statistically significant.

Immunohistochemistry Tumor tissues were embedded in Tissue-Tek OCT compound (Sakura), frozen in liquid nitrogen, sectioned at 5 µm, fixed in acetone, and treated with 0.3% H₂O₂. The mAb 225.28S or rabbit antibodies were biotinylated using NHS-LC-biotin (Pierce) at a concentration of 40 mg/mL. NHS-LC-biotin (5 µL) was added to 1 mg antibodies and incubated for 45 min at room temperature. Excess NHS-LC-biotin was removed by dialysis against PBS. An avidin/biotin block (Vector Laboratories) was done based on the manufacturer's instructions to reduce background staining. Tumor sections were then incubated with 60 µg/mL biotinylated mAb 225.28S, 100 µg/mL biotinylated IgG fraction of the 225D9.2⁺-TT antiserum, or biotinylated IgG fraction of the TT antiserum and blocking buffer for controls. After extensive washing with TBS-Tween 20 and incubation with StrepAB Complex/HRP (DAKO)-specific antibody binding was visualized with DAB-chromogen solution (DAKO) and immediately viewed by an Olympus Vanox AHB T3 microscope and photographed with a Zeiss AxioCam MRc5 camera.

For macrophage staining, unfixed cryostat sections (5 µm) of tumor tissue were blocked 30 min and incubated overnight at 4°C with the F4/80 antibody (clone A3-1; Serotec) diluted 1:200 in PBS. Further steps were done as described above. The slides were scanned at low power to identify the so-called hotspots: areas with the greatest density of F4/80-positive cells. The "hotspot region" was then scanned to identify the best fields for counting. Microscopic fields with the highest degree of immunoreactivity were chosen for analysis. Counting was done on three separate $\times 200$ fields ($\times 20$ objective and $\times 10$ ocular, 0.74 mm² per $\times 200$ field) within these hotspots by two independent observers blinded to the conditions. The macrophage count was defined as the number of F4/80-positive cells within a $\times 200$ field.

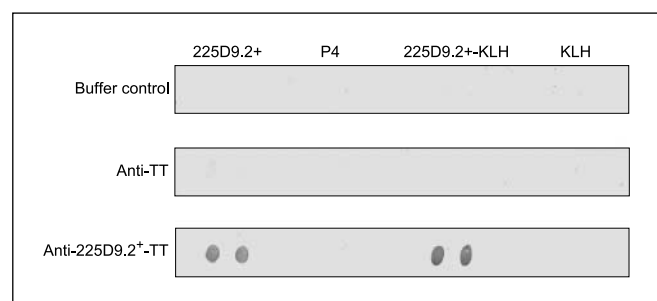


Fig. 1. Synthetic mimotope 225D9.2⁺ of the mAb 225.28S epitope on the HMW-MAA is specifically recognized by the IgG fraction of the 225D9.2⁺-TT antiserum (anti-225D9.2⁺-TT). 225D9.2⁺ but not the control peptide P4 dotted in duplicates is recognized by anti-225D9.2⁺-TT antibodies. No binding was observed using the IgG fraction of the TT antiserum (anti-TT) or detecting anti-rabbit IgG antibodies (buffer control). The mimotope conformation is preserved after conjugation to the carrier molecule KLH (225D9.2⁺-KLH). Anti-225D9.2⁺-TT antibodies recognized the mimotope conjugate but did not interact with the carrier molecule KLH.

The mean was calculated and *P* values were determined using the Student's *t* test. *P* ≤ 0.05 was considered statistically significant.

Results

The vaccine construct. The mimotope was coupled via the linker to the immunogenic carrier protein TT to ensure immunogenicity and availability of sufficient T helper epitopes. Specific immunogenicity of the mimotope was also preserved in the conjugated form 225D9.2⁺-TT. The peptide 225D9.2⁺ and the conjugate 225D9.2⁺-KLH, but not the control peptide P4 representing a peptide derived from the sequence of HER-2/*neu* (12), were specifically recognized by the IgG fraction of the 225D9.2⁺-TT antiserum (Fig. 1). No binding was observed by the control IgG fraction of the TT antiserum (Fig. 1).

Effect of the anti-HMW-MAA mimotope antibodies in vivo. The ability of the IgG fraction of the 225D9.2⁺-TT antiserum to delay tumor development and tumor progression was investigated in a human melanoma xenograft SCID mouse model. The treatment schedules were started according to the experimental design given in Fig. 2 with the IgG fraction of the 225D9.2⁺-TT antiserum, with the IgG fraction of the TT antiserum or saline solution. Mice were treated with eight or seven consecutive injections every 4 days in the tumor prevention and the therapeutic experiments, respectively.

In the tumor prevention experiments, first tumors were observed in untreated and control mice on day 7. Within 10 days, all mice in these two groups developed tumors. A delay in tumor onset was observed in the group treated with 200 μg IgG fraction of the 225D9.2⁺-TT antiserum (Fig. 3A). At the given endpoint on day 35, the tumor volumes were 426 ± 77 and 275 ± 69 mm³ in the control groups, whereas the tumor volume in the mice treated with the IgG fraction of the 225D9.2⁺-TT antiserum was significantly (*P* = 0.01 or 0.03, respectively) smaller at 164 ± 60 mm³.

In the therapeutic experiments, rapid tumor progression was observed in the control groups. In contrast, mice treated with the IgG fraction of the 225D9.2⁺-TT antiserum showed slower progression of established tumors in early stages. As shown in Fig. 3B, control mice reached a total tumor volume of 835 ± 147 mm³ within 30 days following the first antibody injection. At this time point, the tumor volume of mice treated with the IgG fraction of the 225D9.2⁺-TT antiserum was significantly smaller (*P* = 0.012) at 501 ± 124 mm³. No significant difference in tumor progression compared with sham-treated mice was observed in the group of mice receiving the IgG fraction of the TT antiserum, reaching 952 ± 193 mm³ tumor volume within 30 days after the first injection.

Immunohistochemistry. To evaluate the mechanism of anti-tumor response observed in mice treated with the IgG fraction

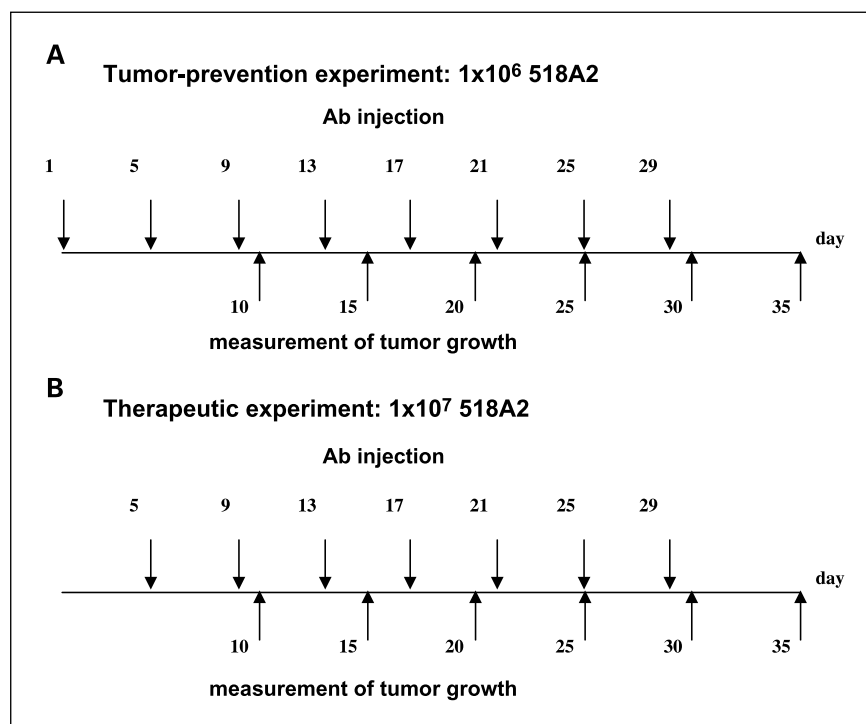


Fig. 2. Immunization schedule. Tumors were established in C.B.17 SCID/SCID mice with 1×10^6 518A2 cells for tumor prevention experiments (A) or 1×10^7 cells for therapeutic experiments (B). Injection of antibodies were started on day 1 in tumor prevention experiments (A) and day 5 in therapeutic experiments (B) and repeated in 4-day intervals. Measurement of tumor growth was started on day 10 and repeated every 5 d.

of the 225D9.2⁺-TT antiserum, we looked for HMW-MAA protein expression levels in tumor sections. Tumor tissues of sham-treated mice (group 1), mice treated with the IgG fraction of the TT antiserum (group 2), or mice treated with the IgG fraction of the 225D9.2⁺-TT antiserum (group 3) were used. In all three tumor sample groups, staining of melanoma cells was observed with biotinylated mAb 225.28S and with biotinylated IgG fraction of the 225D9.2⁺-TT antiserum with comparable intensity (Fig. 4A and B). An isotype-matched antibody and the IgG fraction of the TT antiserum as controls did not show any staining of melanoma cells in all tumor tissues.

Macrophage infiltration in tumors. The F4/80 antigen is a surface marker suitable for detecting mature mouse macrophages. Comparing numbers of macrophages in melanoma xenotransplants of saline-treated animals and animals treated with the IgG fraction of the TT antiserum or the IgG fraction of the 225D9.2⁺-TT antiserum, we observed no differences of macrophage infiltration in immunostaining of tumor tissue (Fig. 4C). The numbers of F4/80-positive cells in three $\times 200$ fields of five tumor samples per group were counted by two independent observers blinded to the conditions. Mean values for macrophage infiltration were 21 ± 6 cells per $\times 200$ field in the tumor sections of mice treated with the IgG fraction of the 225D9.2⁺-TT antiserum and 19 ± 7 or 19 ± 6 cells in the tumor sections of mice treated with the IgG fraction of the TT

antiserum or saline solution, respectively. The differences were not statistically significant.

Discussion

The induction of tumor-protective immunity against malignant melanoma by using the HMW-MAA as a target is a promising strategy in dermato-oncology. Thus, different therapeutic approaches targeting HMW-MAA including anti-idiotypic antibodies were already under investigation (4, 5). We have identified previously a linear HMW-MAA peptide mimotope, which induced a humoral anti-HMW-MAA IgG response in rabbits (8). The efficacy of the mAb 225.25S, being the template for the linear peptide mimotope, was shown by reduction of primary tumor growth in a melanoma SCID mouse xenograft model (7). Here, we extend our findings reporting the *in vivo* efficacy of passively administered rabbit antibodies induced by the HMW-MAA peptide mimotope in reducing tumor growth in a melanoma SCID mouse xenograft model.

A prerequisite for an effective mimotope vaccine is its ability to induce antibodies specifically recognizing the mimotope and cross-reacting with the original antigen (13). We showed that our HMW-MAA mimotope vaccine elicited an antibody response in rabbits that specifically reacted with the mimotope 225D9.2⁺ (Fig. 1). Previously, we showed that a humoral response induced by the mimotope vaccine 225D9.2⁺-TT specifically recognized the original antigen, the HMW-MAA (8). These findings strengthen the hypothesis that mimotopes represent structural equivalents of epitopes on antigens and are able to induce humoral immune responses directed against these epitopes. Mimotopes have been successfully isolated and shown to elicit antibodies against the nominal antigens and/or trigger biological effects against tumors (9, 14–17) but also in autoimmune diseases (18, 19), infectious diseases (20–22), and allergy (23).

Based on our *in vitro* results, the purified antibodies were tested in an *in vivo* model for their antitumor activity. The proof of concept of the efficacy of antibodies induced by a HMW-MAA mimotope was investigated in a SCID mouse melanoma xenograft model; as of date, no transgenic HMW-MAA model is available. A prophylactic and a therapeutic approach revealed that tumor growth was inhibited following application of the IgG fraction of the 225D9.2⁺-TT antiserum (Fig. 3A and B). In the tumor prevention experiments, we observed a significant reduction of tumor growth by 40% compared with the mice treated with the IgG fraction of the TT antiserum and by 62% to sham-treated mice. In the therapeutic experiments, the reduction of tumor growth reached 40% and 47% compared with the sham-treated mice and the mice treated with the IgG fraction of the TT antiserum, respectively. Interestingly, these results are comparable with the degree of tumor growth reduction we observed using the anti-HMW-MAA mAb 225.28S in similar experiments (7). A similar result was obtained with mimotopes isolated with a mAb that binds a 47- to 50-kDa membrane determinant on Daudi cells (24). Immunization of mice with the mimotopes prevented tumor development in lung of mice inoculated with B16 melanoma to a similar extent as in treatment of the mice with the mAb.

Identification of a method for inducing strong antibody responses to HMW-MAA would provide means for stimulating progress toward the development of an effective vaccine against HMW-MAA-expressing melanomas. As cancer vaccines that

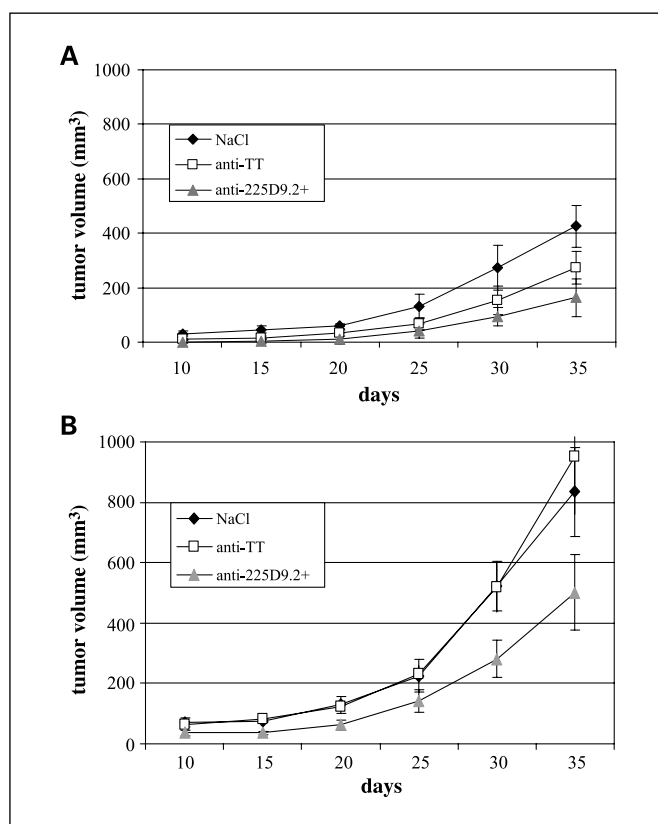


Fig. 3. Treatment with the IgG fraction of the 225D9.2⁺-TT antiserum inhibited tumor growth in a SCID mouse melanoma xenotransplant model. Mice were treated with saline solution (NaCl), the IgG fraction of the TT antiserum (anti-TT), or the IgG fraction of the 225D9.2⁺-TT antiserum (anti-225D9.2⁺). Tumor volumes were measured and are illustrated as mean $\pm$ SD for the tumor prevention experiments (A) and the therapeutic experiments (B).

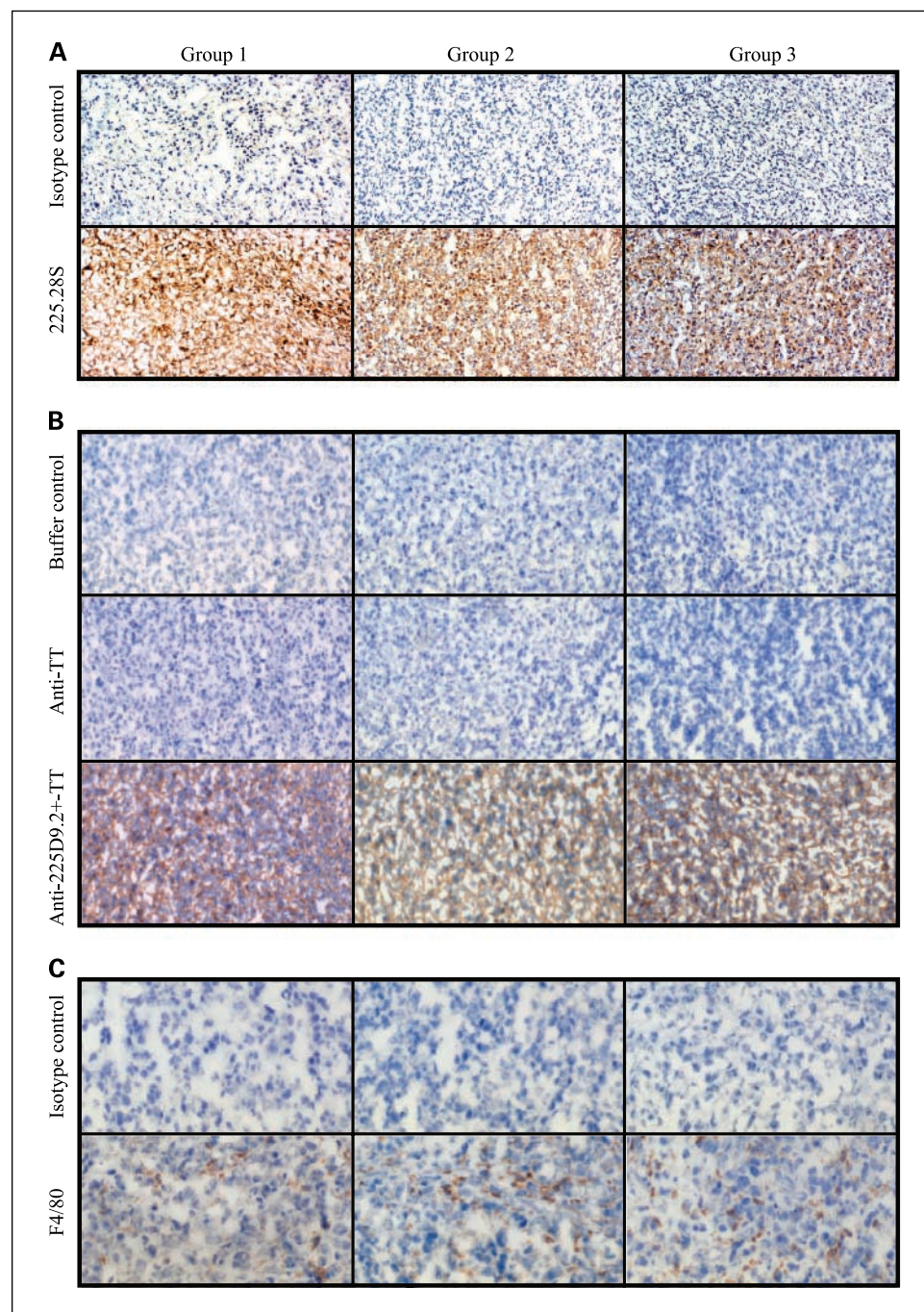


Fig. 4. Immunohistochemistry. HMW-MAA staining was done with (A) the mAb 225.28S or an isotype control, (B) PBS buffer, biotinylated IgG fraction of the TT antiserum (anti-TT), or the IgG fraction of the 225D9.2⁺-TT antiserum (anti-225D9.2⁺-TT) in tumor tissues of sham-treated mice (group 1), mice treated with the IgG fraction of the TT antiserum (group 2), or mice treated with the IgG fraction of the 225D9.2⁺-TT antiserum (group 3). For macrophage staining (C), tumor sections of the three groups were stained with F4/80 antibody or an isotype control.

elicit primarily long-lasting antibody responses will likely be most effective in the minimal residual disease setting, the stage when tumor cells are few and dispersed, the induction of HMW-MAA-specific antibodies by the mimotope vaccine may be beneficial for melanoma patients. The significant slowing down of tumor growth in the therapeutic approach indicates that such a vaccine might also lead to prolonged survival times of patients with end-stage disease.

Tumor growth was inhibited partially but not completely in our model. This finding does not reflect an isolated growth of a melanoma cell subpopulation, which does not express HMW-MAA, because we have shown expression of HMW-MAA in all

melanoma tumor samples to be equal (Fig. 4A). The staining of HMW-MAA was possible in all tumor lesions by the IgG fraction of the 225D9.2⁺-TT antiserum. This indicates that cross-reactivity of the anti-225D9.2⁺-TT antibodies with the HMW-MAA was also achieved *in vivo*. As shown previously *in vitro*, we expected antibody-dependent cell cytotoxicity as the mechanism responsible for tumor growth inhibition. Surprisingly, we did not detect a difference in infiltration of macrophages in all tumor samples (Fig. 4B). This observation does not completely exclude antibody-dependent cell cytotoxicity as a possible mechanism *in vivo* but indicates additional mechanisms involved in tumor growth inhibition. In view of the role of the HMW-MAA in

interactions of melanoma cells with extracellular matrix components and the ability of anti-HMW-MAA antibodies to inhibit these interactions in *in vitro* assays, the mechanism we favor to account for the inhibition of melanoma cell growth in SCID mice is the disruption of interactions of melanoma cells with extracellular matrix components as shown by Luo et al. (25).

In summary, we describe the antitumor efficacy of antibodies derived from immunization of rabbits with the HMW-MAA peptide mimotope 225D9.2⁺ in a melanoma SCID mouse xenograft model. The therapeutic effect might be further improved by using the mimotope as a vaccine in a transgenic HMW-MAA model, as in our tumor prevention experiments the

high number of 1×10^6 melanoma cells was used for tumor establishment. Based on our results, we suggest that the 225D9.2⁺ mimotope used as a vaccine is capable to launch functional antitumor immune reactions *in vivo*. The findings described here provide important groundwork for the further development of this immunotherapeutic approach for the treatment of malignant melanoma.

Disclosure of Potential Conflicts of Interest

H. Pehamberger and O. Scheiner: stakeholders of BioLife Science, Vienna, Austria.

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