

MMP11: A Novel Target Antigen for Cancer Immunotherapy

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Abstract **Purpose:** Matrix metalloproteinases (MMP) are zinc-dependent endopeptidases that mediate numerous physiologic and pathologic processes, including matrix degradation, tissue remodeling, inflammation, and tumor metastasis. To develop a vaccine targeting stromal antigens expressed by cancer-associated fibroblasts, we focused on MMP11 (or stromelysin 3). MMP11 expression correlates with aggressive profile and invasiveness of different types of carcinoma.

Experimental Design: To show the efficacy of a vaccine targeting MMP11, we constructed a series of plasmid DNA vectors expressing murine MMP11. Mice were vaccinated by i.m. injection followed by *in vivo* DNA electroporation. A chemically induced, MMP11-overexpressing colon cancer model was established and characterized. Antibody and T-cell responses were determined, and immunoreactive epitopes were characterized. To analyze the possible use of MMP11 as tumor-associated antigen in cancer patients, HLA-A2.1 transgenic mice (HHD) were used to identify reactive epitopes as tools to assess immunogenicity in humans.

Results: Using microarray, we confirmed the overexpression of MMP11 mRNA in a large panel of human tumor samples. MMP11 vaccine induced cell mediated and antibody immune response and exerted significant antitumoral protection in mice with colon cancer in prophylactic and therapeutic settings. HHD transgenic mice were vaccinated with a plasmid encoding human MMP11, and a HLA-A2.1-restricted epitope (hMMP₂₃₇) was identified. hMMP₂₃₇ was shown to be immunogenic in human peripheral blood mononuclear cells (PBMC) by *in vitro* priming.

Conclusion: Our study describes the identification of MMP11 as a novel broadly expressed tumor associated antigen as target candidate for cancer immunotherapy.

Solid tumors are composed of malignant cells and variety of different nonmalignant cells, defined as tumor stroma and composed of endothelial cells, fibroblasts, and inflammatory cells that support tumor growth. Stromal cells contribute 20% to 50% of the tumor mass but may account up to 90% in several carcinomas. The tumor microenvironment can influence the stromal cells by promoting angiogenesis, recruitment of reactive stromal fibroblasts, lymphoid and phagocytic infiltrates, production of proteolytic enzymes, and modifying extracellular matrix, thus enabling tumor progression (1). Unlike cancer cells, stromal cells are genetically more stable and differ from their normal counterparts for the up-regulation or induction of different classes of proteins (2, 3) that can be target antigens for immunotherapy. Stromal antigens are also expressed by a broad spectrum of solid tumors; thus, therapies designed to target tumor stroma are not restricted to a selected tumor type.

Matrix metalloproteinases (MMP) are important components of tumor stroma. They regulate and shape tumor microenvironment (4), and their expression and activation are increased in almost all human cancers compared with normal tissue (5–7). MMP11 was isolated as a breast cancer associated gene and is expressed in most invasive primary carcinomas, in a number of their metastases, and more rarely in sarcomas and other nonepithelial malignancies (8). MMP11 is almost absent in normal adult organs.

The role of MMP11 in cancer progression has been shown by several preclinical observations: its expression promotes tumor take in mice (9), homing of malignant epithelial cells (10), cancer progression by remodeling extracellular matrix (11), and antiapoptotic and antinecrotic effect on tumor cells (12, 13). MMP11 deficiency increases tumor-free survival and modulates local or distant invasion (14); knockdown of MMP11 mRNA in gastric cancer cells suppresses tumor growth *in vitro* and *in vivo* (6) and inhibits spread of murine hepatocarcinoma cells to lymph nodes (15). Levels of MMP11 expression may be used to identify patients at greatest risk for cancer recurrence, in breast carcinoma, pancreatic tumors (16), and colon cancer (17).

1,2-Dimethylhydrazine or its metabolite, azoxymethane, induce colonic tumors in numerous species of animals (18) through induction of methyl adducts to DNA bases, point mutations, micronuclei, sister chromatid exchanges, and apoptosis in the colon (19), as well as an increase in cellular proliferation of colonic epithelial cells (20), which is a characteristic of human colon cancer. In susceptible mouse strains, 1,2-dimethylhydrazine-induced carcinogenesis

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Translational Relevance

This study describes the rationale and the use of matrix metalloproteinase (MMP) 11 as a novel target of cancer for immunotherapy. MMP11 is shown to be overexpressed in a variety of human malignancies by microarray, including colon cancer. Optimized MMP11 genetic vaccine delivered via DNA electroporation can impair tumor growth in mice with colon lesions, and this effect is associated with antigen-specific immunity. As a novel research and potential clinical tool, we identify and characterize an immunogenic epitope within human MMP11 by means of HLA-A2.1 transgenic mice. This epitope can generate an immune response in *in vitro* stimulated human PBMCs. Because of MMP11 expression in several tumor types and the notion that targeting tumor stroma by T cells can represent an important alternative approach to the effective control of tumor growth, MMP11 vaccine can provide a new therapeutic opportunity in clinical trials for cancer patients.

progression in colon tissue goes through different stages: (a) aberrant crypt formation, (b) adenoma, (c) polyp, and (d) adenocarcinoma (21).

We have investigated the use of MMP11 as target for cancer immunotherapy. We used i.m. injection of plasmid DNA encoding mouse MMP11 derivatives, followed by DNA electroporation as vaccine platform and 1,2-dimethylhydrazine-induced MMP11-overexpressing colon cancer as therapeutic preclinical model. *In vitro* priming was used to assess if it was possible to elicit an immune response against this antigen in humans.

Materials and Methods

Microarray analysis. Total RNA from human matched normal and tumor samples was isolated with RNazol B. cRNA was generated by *in vitro* transcription using T7 RNA polymerase on 5 µg of total RNA and labeled with Cy5 or Cy3 (Cy-Dye, Amersham Pharmacia Biotech). Five µg of labeled RNA from each sample were cohybridized with 5 µg of a normal reference pool, consisting of an equal amount of cRNA from the pool of normal tissue samples extracted from the same patients used to extract the tumors. Labeled cRNAs were fragmented to an average size of 50 to 100 nucleotides by heating the samples to 60°C with 10 mmol/L of zinc chloride and then adding a hybridization buffer containing 1 mol/L NaCl, 0.5% sodium sarcosine, 50 mmol/L MES (pH 6.5), and formamide to a final concentration of 30%. The final volume was 3 mL at 40°C.

RNA samples were hybridized on a Human 25K array containing 23,720 unique probes for ~21,000 human genes. The probes were 60 bp in length and were selected based on the oligonucleotide probe design program (22). After hybridization, slides were washed and scanned using a confocal laser scanner (Agilent Technologies). The intensities obtained after scanning were quantified, background corrected, and normalized. The Rosetta error model (23) was applied to dye-swapped pairs of hybridizations. Expression changes between each tumor and the corresponding adjacent normal sample were quantified as \log_{10} of the expression ratio tumor/normal. The statistical significance of the expression change in each tumor type was assessed using the Student's *t* test. In all tumor types, MMP11 was found up-regulated, with a *P* value lower than 10^{-5} .

Plasmid vectors. The wild-type mouse MMP11 sequence (GenBank:U17385) was cloned by reverse transcription-PCR. Total RNA was isolated from NIH-3T3 cells using Qiagen RNeasy kit, and cDNA was synthesized using SuperScript Onestep reverse transcription-PCR (Invitrogen). The sequence of the primers was as follows: 5'-CCCCGGGGCGGATGGCACGGGGCCGCTGTC-3' and 5'-GTCAG(AC)GGAAAGT(AG)TTGG CAGGCTCAGCACAG-3'. reverse transcription-PCR amplification was done under the following conditions: 45°C for 30 min, 94°C for 2 min for cDNA synthesis, 94°C for 15 s, 58°C for 30 s, 68°C for 2 min for 40 cycles, and extension for 2 min at 72°C. An amplification product of about 1,630 bp was obtained and cloned into pCR2.1 vector. Sequence analysis revealed complete match with published mouse MMP11 cDNA and was subcloned in plasmid pV1J-nsB.

Mouse and human MMP11opt cDNAs were synthesized by oligonucleotide assembly by Genart and cloned into pCR-script (Stratagene). The cDNAs were then subcloned in pV1J-nsA. pV1J-MMP11/LTBopt was constructed by PCR amplification of MMP11opt cDNA and cloning in plasmid pV1J-LTBopt, which contains the B subunit of *Escherichia coli* heat-labile enterotoxin (LTB) cDNA encompassing nucleotide 64 to 375 (amino acid 21 to 125; ref. 24).

Mouse immunization. All animal studies were approved by the institutional animal care and use committee of Istituto di Ricerca di Biologia Molecolare. Eight-week-old BALB/c mice were purchased from Charles River Italy. HHD transgenic mice were provided by Dr. Lemonnier (Institute Pasteur, Paris). Groups of mice were subjected to DNA electroporation in the quadriceps muscle, as described (25). Mice were subjected to injections of 50 µg plasmid DNA per injection.

1,2-Dimethylhydrazine-induced colon carcinogenesis. Mice were treated with 1,2-dimethylhydrazine, purchased from Sigma (Cat. D16,180-2). Animals were injected i.p. once a week for 6 wk with 1,2-dimethylhydrazine at 20 mg/kg. The carcinogen was dissolved in Tris-HCl and buffered with 1N NaOH (pH 6.5). For 1,2-dimethylhydrazine/dexin sulfate sodium treatment, mice were given 1,2-dimethylhydrazine i.p. once at 10 mg/kg. Starting 1 wk after the injection, animals were given 2% (w/v) DSS in drinking water for 7 d.

Peptides. Lyophilized MMP11 peptides were purchased from JPT Peptide Technologies GmbH and resuspended in DMSO at 40 mg/mL. Pools of 15 amino acid peptides overlapping by 11 residues were assembled as described previously (26). N-term and C-term pools consisted of 60 and 61 peptides, respectively. The final concentration of each peptide in the pools was 0.5 mg/mL. Subpools were composed of 36 peptides each, mixed as cross-matrices.

Ex vivo immune response. Interferon (IFN) γ enzyme-linked immunospot assay was carried out with mouse splenocytes and MMP11-specific peptides, as described (27). For intracellular staining, interferon- γ production by stimulated T cells was measured as described (28). Briefly, 1 to 2 million mouse PBMCs or splenocytes were incubated overnight with 5 to 6 µg/mL of MMP11 peptide pools or of the mMMP₃₉₆ CD8⁺ epitope of mouse MMP11 (396VWGPEKNKI₄₀₄; H-2K^d restricted). For HHD mice, human MMP11 pools or hMMP₂₃₇ (237YTFRYPLSL₂₄₅) were used. BrefeldinA (1 µg/mL; BD Sciences; Pharmingen) and DMSO were used as positive and negative controls, respectively.

Cytotoxic assay. Mice splenocytes at 1×10^7 cells/mL were restimulated for a week with 10 µg CD8⁺-specific MMP11 peptide or pool and 10 U of recombinant human interleukin 2 (R&D Systems) for 7 d. Target cells p815 (ATCC; TIB64) or HeLa-HHD cells were labeled with Na³¹CrO₄ (Amersham Pharmacia Biotech) and pulsed with the specific peptide for 2 h. The percent of lysis was calculated as $100 \times [(\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})]$, wherein the spontaneous and maximum release refer to the counts in medium or 1% Triton X-100 of target cells alone, respectively.

Detection of antibodies. Induction of anti-MMP11 antibodies was monitored by Western blot with whole cell lysates of HeLa transfected with pV1J-mMMP11. As control, rat sera diluted 1:1,000 were used to

detect protein band. As secondary detecting antibody an anti-rat immunoglobulin G (whole molecule) peroxidase was used (Sigma; A9037). Anti-mMMP11 polyclonal sera were generated by immunizing four rats with pV1J-mMMP11. Rats were immunized by DNA electroporation with 200 µg of DNA in tibialis muscle every other week (29). Two weeks after the last immunization, rats were bled and sera were assayed for the presence of antibodies against mMMP11.

Whole-mount intestine analysis. Lesions quantification of the upper and lower intestine was done after whole mount fixation and methylene blue staining (30, 31). Lesion scoring was done by two independent researchers and one pathologist, the tumors volume was calculated by Zeiss Axiovision Image processing software.

Histology and immunohistochemistry (IHC). Tumor samples from mice were formalin fixed and paraffin embedded. After H&E staining, samples were processed for IHC (32). A rabbit polyclonal antibody (BioVision) was used at 1:500 dilution. As secondary antibody anti-rabbit immunoglobulin G (Sigma) was used. As amplification system, the biotin-streptavidin avidin-biotin complex method (Vectastain ABC Kit) was used. The IHC signal was detected by nickel-enhanced diaminobenzidine (DAB Peroxidase Substrate Kit). Vectastain and DAB were both purchased from Vector.

T-cell in vitro priming. Human PBMCs were obtained from buffy coats collected from HLA-A*0201⁺ healthy donors by Ficoll-Paque density gradient centrifugation (Pharmacia Biotech), as described (33). Dendrite cells were pulsed for 2 h at 37°C with 5 µg/mL hMMP₂₃₇ peptide, irradiated at 3,000 rad, and cocultured in 24-well plates with autologous PBMCs (at 1:4 DC:PBMC ratio) in RPMI 1640 medium supplemented with 10% human serum. After 24 h, interleukin 2 was added at 60 IU/mL (Proleukin; Chiron). T-cell lines were expanded in RPMI 1640 supplemented with 10% of human serum, 900 IU/mL interleukin 2 (Proleukin), and 1 µg/mL phytohemagglutinin (Sigma-Aldrich). PE-HLA-A*0201 tetramers carrying hMMP₂₃₇ peptide or carcinoembryonic antigen₆₀₅ peptide (Beckman Coulter) were used according to manufacturer's directions. The granzyme B enzyme-linked immunospot assay was done using a commercially available kit (Becton Dickinson). Human TAP-deficient T2 cells pulsed with 5 µg/mL of hMMP₂₃₇ or irrelevant peptide carcinoembryonic antigen₆₀₅, were used as stimulator cells. Effector cells (1×10^4) and peptide-prepulsed T2 cells (1×10^4) were seeded into 96-well polyvinylidene fluoride-backed microplates coated with monoclonal antibody specific for granzyme B. After incubation at 37°C for 4.5 h, cells were removed and plates were processed according to the manufacturer's instructions.

Statistical analysis. Statistical analysis was done by Student's *t* test for unpaired samples. $P < 0.05$ was considered as statistically significant.

Results

MMP11 is overexpressed in several human cancer types. To establish whether MMP11 is a relevant target in human cancer and extend previous published observations, MMP11 expression was assessed by microarray analysis of gastric, kidney, colon, lung, and breast cancer samples. The relative abundance of MMP11 mRNA in tumor samples was compared with the matched normal tissues to verify the differential gene expression within the same patient/tissue. Expression of MMP11 resulted significantly higher in all types of tumors compared with the normal adjacent section (Student's *t* test P value $< 10^{-5}$; Fig. 1). These data show that MMP11 mRNA is widely overexpressed in tumors and should be evaluated as a target for cancer therapy.

MMP11 is immunogenic in mice. To identify the best immunogen for vaccination, we tested three different forms of murine MMP11: wild type (MMP11_{wt}), codon optimized (MMP11_{opt}), and fused to the LTB (MMP11-LTB_{opt}). Codon

optimization of cDNA has been shown to enhance expression and the induction of the immune response (27, 34, 35), whereas fusion to LTB increases antigen immunogenicity (24). To abrogate enzymatic activity, a point mutation was introduced in the latter two versions, changing glutamic acid to alanine at position 220 (11).

BALB/c mice were immunized with four DNA electroporation, 1 week apart. Two weeks after the last injection, mice were euthanized and the immune response against MMP11 was measured by intracellular staining for interferon-γ using peptides encompassing the N-term or the C-term of the entire protein. Most of the reactivity was detected in the C-term pool of peptides (data not shown). Wild-type and optimized version of MMP11 broke tolerance, but a significant difference was observed between MMP11_{wt} and MMP11_{opt} ($P = 0.03$; Fig. 2A). Similarly, MMP11-LTB_{opt} elicited a greater CD8⁺ T-cell response than MMP11_{opt} ($P = 0.01$). A CD4⁺-specific response against LTB was measured in mice vaccinated with MMP11-LTB_{opt}, thus suggesting that fusion with LTB can provide CD4⁺ help (Fig. 2B).

To measure antibody response, sera from immunized mice were analyzed by Western blotting using cell extracts from HeLa cells transfected with pV1J-MMP11_{wt}. Detection of a band corresponding to MMP11 molecular mass indicated the presence of antibodies against MMP11. No significant difference was observed between MMP11_{wt}- and MMP11-LTB_{opt}-immunized mice (Fig. 2C).

These data show that it is possible to elicit a cell-mediated and antibody response against MMP11 in mice. MMP11-LTB_{opt} was selected as the immunogen to be used for vaccination studies in therapeutic models.

MMP11 is overexpressed in 1,2-dimethylhydrazine-induced murine colon cancer. To identify a suitable preclinical model to study the effect of MMP11 vaccination on tumor development, expression of the antigen was measured in a model of chemical-induced colon carcinogenesis. BALB/c mice received six weekly injections of 1,2-dimethylhydrazine and were euthanized 5 weeks after the last injection; at this stage, aberrant crypts and adenomas are present in the colon. Intestinal tissue was snap frozen and analyzed by IHC using an antibody against mouse MMP11. In untreated mice, IHC analysis detected expression of MMP11 at the base of normal crypts, limited to colonic stem cells (Fig. 3A). Strong and diffused expression was detected in aberrant crypt and adenoma formations (Fig. 3B and C). To characterize the kinetics of MMP11 expression in the 1,2-dimethylhydrazine model, treated mice were euthanized at different time points starting from the fifth week after the last injection. Lysates from the whole colon tissue were analyzed by Western blot for the expression of MMP11. Expression increased over time and was directly related to tumor progression (Fig. 3D). Similar results were also obtained in a second mouse strain (A/J mice; data not shown).

These data indicate that MMP11 expression is associated with colon cancer progression and that 1,2-dimethylhydrazine-induced carcinogenesis is a suitable model for anti-MMP11 therapy and vaccination.

MMP11 vaccine in 1,2-dimethylhydrazine-colon cancer model. The observation that 1,2-dimethylhydrazine does not interfere with the efficacy of genetic vaccination (36) prompted us to test the MMP11 vaccine in this model. BALB/c mice were treated

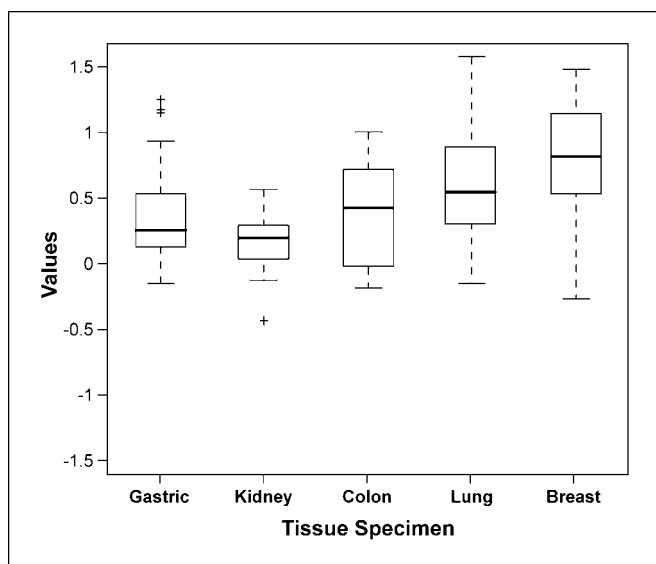


Fig. 1. MMP11 is overexpressed in a variety of tumor types. RNA from tumor and matched normal tissues was obtained from 40 colorectal, 72 gastric, 133 breast, 139 lung, and 124 kidney samples. Box plots, tumor versus normal expression values of MMP11 in five different tumor types: gastric, kidney, colon, lung, and breast. Each value is calculated as the \log_{10} ratio of the MMP11 expression in the tumor versus the adjacent normal tissue. The boxes refer to a specific tumor type and delimit the first, the second (median), and the third quartile of the distribution of tumor/normal ratios. To each box is associated a "whisker" (dotted line), which delimits the interquartile range * 1.5 distances from the first and the third quartiles. Any data point that lies above or below the whisker is considered an outlier. Outliers are represented as single data points (crosses).

with 1,2-dimethylhydrazine; one group was left untreated, and a second group was immunized with pV1J-MMP11-LTB_{opt}, as shown in Fig. 4. A third group was vaccinated with pV1J-NS3A, encoding the NS3 protein of hepatitis C virus. Three weeks after the last immunization, a CD8⁺ T-cell response to the C terminus of MMP11 was detected in mice injected with pV1J-MMP11-LTB_{opt}. Similarly, a strong CD8⁺ and CD4⁺ T-cell response against NS3 was found in mice vaccinated with pV1J-NS3A (Fig. 4A).

Splenocytes from mice vaccinated with pV1J-MMP11-LTB_{opt} were analyzed by enzyme-linked immunospot with single peptides covering mouse MMP11. Two overlapping 15-mer peptides (MMP99, ₃₉₃AALVWGPEKNKIYFF₄₀₇, and MMP100, ₃₉₇WGPEKNKIYFFRGGD₄₁₁) in the C-term peptide pool contained the H2-K^d-restricted epitope, mMMP₃₉₆ (₃₉₆VWGPEKNKI₄₀₄), identified in the sequence of the overlapping 11 residues (data not shown). The immune response against mMMP₃₉₆ was tested by intracellular staining, and T cells showed strong antigen-specific secretion of interferon- γ (Fig. 4B). In addition, stimulated effector cells were fully functional and able to lyse target cells loaded with mMMP₃₉₆ (Fig. 4C).

At the same time point, the colon of each mouse was analyzed for the presence of aberrant crypt formation, adenomas, polyps, and adenocarcinomas. MMP11-vaccinated mice showed significant reduction of 1,2-dimethylhydrazine-induced formations at all stages (Fig. 5A), whereas pV1J-NS3A was unable to confer any protective effect (data not shown).

To assess the effect of MMP11 vaccine on the progression of late-stage colon adenocarcinoma, mice were boosted with three DNA electroporation (week 21, 25, 27) and euthanized at week

30 (Fig. 5B). At this point, the vaccinated group had a lower number of tumors at diverse differentiation stages: 80% of the control mice presented poorly differentiated adenocarcinoma, whereas only 5% of the vaccinated mice had tumors with this

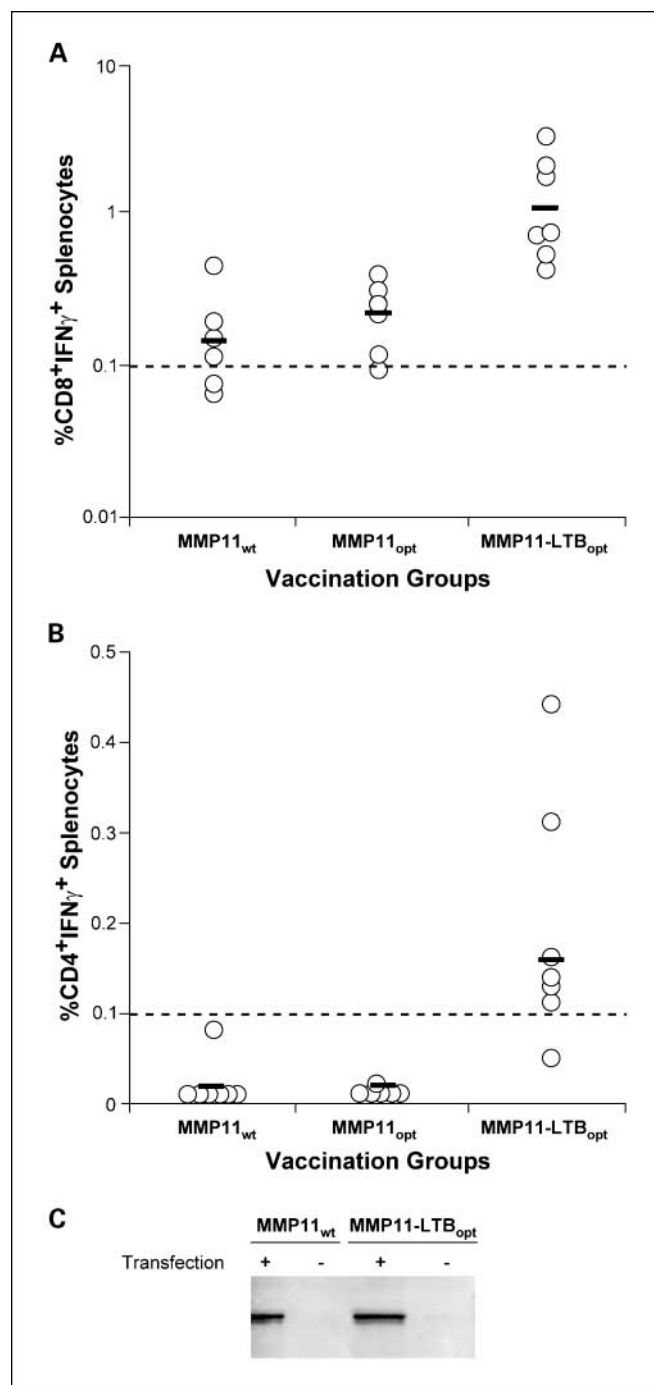


Fig. 2. Immune response elicited by MMP11_{wt}, MMP11_{opt}, and MMP11-LTB_{opt}. Six BALB/c mice were immunized with four weekly injections by DNA electroporation. **A**, T-cell immune response was measured by intracellular staining using peptides covering the C-term of MMP11 protein. Empty circles, percentage of CD8⁺IFN- γ ⁺ for each single mouse. The geometric mean of the group is shown (black dash). The assay background (DMSO) was set to 0.01%. Frequencies superior to 0.1% were considered significant. **B**, cell-mediated immune response against LTB. Empty circles, percentage of CD4⁺IFN- γ ⁺ for each single mouse. Black dash, geometric mean of the group. **C**, antibody response elicited by pV1J-MMP11_{wt} and pV1J-MMP11-LTB_{opt}. Presence of antibodies was measured by Western blot. A 50-kDa band corresponding to MMP11 molecular mass was detected.

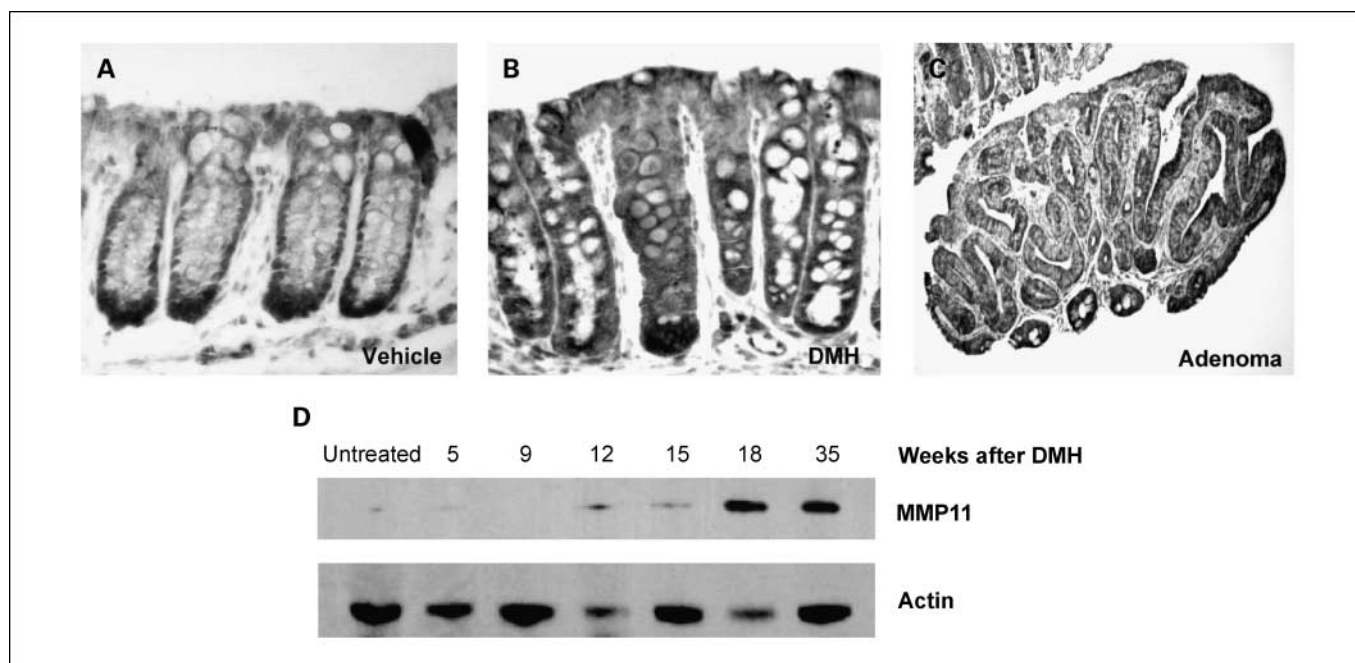


Fig. 3. MMP11 is overexpressed in mouse colon adenomas induced by 1,2-dimethylhydrazine. BALB/c mice were treated with six i.p. injections of 1,2-dimethylhydrazine. Five weeks later, colon tissue was analyzed by IHC and Western blotting. *A*, IHC of colon tissue from untreated mice. *B*, colon tissue from a 1,2-dimethylhydrazine treated mouse. *C*, IHC of a colon adenoma. *D*, Western blot of colon tissue homogenates. Mice were sacrificed at the indicated time points after the last treatment.

phenotype (Fig. 5B). Conversely, the vaccinated and the control group showed 95% and 20% of moderately differentiated carcinomas (G2), respectively.

To verify whether MMP11 vaccine exerts a therapeutic effect in advanced stages of cancerous lesions, BALB/c mice were treated with a single dose of 1,2-dimethylhydrazine/DSS and vaccinated with pV1J-MMP11-LTB_{opt}, starting the treatment at week 3 (regimen A), 6 (regimen B), or 9 (regimen C) after tumor induction (Fig. 5C). DSS was added at the tumor induction phase because the combination is more effective in inducing inflammation and colon carcinogenesis (37). Two weeks after the last immunization, a CD8⁺ immune response to MMP11 was measured by intracellular staining in all vaccinated mice that ranged between 0.5% and 7% (not shown). At week 18, mice vaccinated with pV1J-MMP11-LTB_{opt} showed significant reduction of adenomas and no difference in efficacy among the three regimens was observed (Fig. 5C). No effect was obtained in control or NS3 vaccinated mice (not shown). These data indicate that MMP11 is an optimal target for active specific immunotherapy and MMP11 vaccine interferes with tumor progression.

Identification and immunogenicity of a HLA-A2.1-restricted human MMP11 epitope. To identify human MHC class I epitopes within human MMP11, HHD transgenic mice were vaccinated by DNA electroporation with pV1J-hMMP11_{opt} encoding the codon-optimized, catalytically inactive form of the human proteinase. A strong CD8⁺-specific immune response was detected in the N-term and C-term peptide pools (Fig. 6A). Deconvolution of peptide subpools by intracellular staining identified two overlapping peptides (hMMP60, ₂₃₃MSAFYTFRYPLSLSP₂₄₇, and hMMP61, ₂₃₇YTFRYPLSLPDDCR₂₅₁) present in N-term and C-term peptide pools, respectively, and containing the epitope hMMP₂₃₇, ₂₃₇YTFRYPLSL₂₄₅, responsible for the overall reactivity (Fig. 6A).

To verify whether hMMP₂₃₇ was processed and presented by HLA-matched cells overexpressing hMMP11, HeLa-HHD cells were transfected with pV1J-hMMP11_{opt} and incubated with T cells obtained from vaccinated HHD mice. The effectors deriving from most of the treated mice were able to lyse the hMMP11-transfected or hMMP₂₃₇-loaded HeLa-HHD but not pV1J-EGFP transfected cells (Fig. 6B), indicating that the epitope is processed and presented on MHC I.

To determine whether MMP11-specific CD8⁺ response could be induced in humans, monocyte-derived DCs were generated from PBMCs of a healthy HLA-A2.1⁺ donor, loaded with hMMP₂₃₇ peptide and used as APCs for the *in vitro* induction of antigen-specific CTLs. After three amplification passages, the T-cell lines were characterized by tetramer staining. Two (14.3%) of 14 lines were tetramer positive (Fig. 6C). To assess if the amplified clones were functional, cells were stimulated with HLA-A2.1⁺ hMMP₂₃₇-loaded B-lymphoblastoid cell lines (BLCL) and analyzed by intracellular staining for interferon- γ . T-cell lines showed significant cytokine secretion upon stimulation (Fig. 6C) and showed a cytolytic phenotype because they were capable of releasing granzyme B, as detected by enzyme-linked immunospot (Fig. 6D). These preliminary data suggest that functional MMP11-specific CD8⁺ T cells can be induced in humans.

Discussion

The therapeutic potential of targeting tumor stroma has been shown in several preclinical and clinical studies. T cells represent an important alternative approach to the effective control of tumor growth, particularly in the absence of direct targeting of cancer cells (38). Cellular targets of active immune interventions include cancer-associated fibroblasts, infiltrating macrophages/histiocytes, and tumor endothelial cells. Antigens as carbonic anhydrase IX or fibroblast activation protein (FAP)

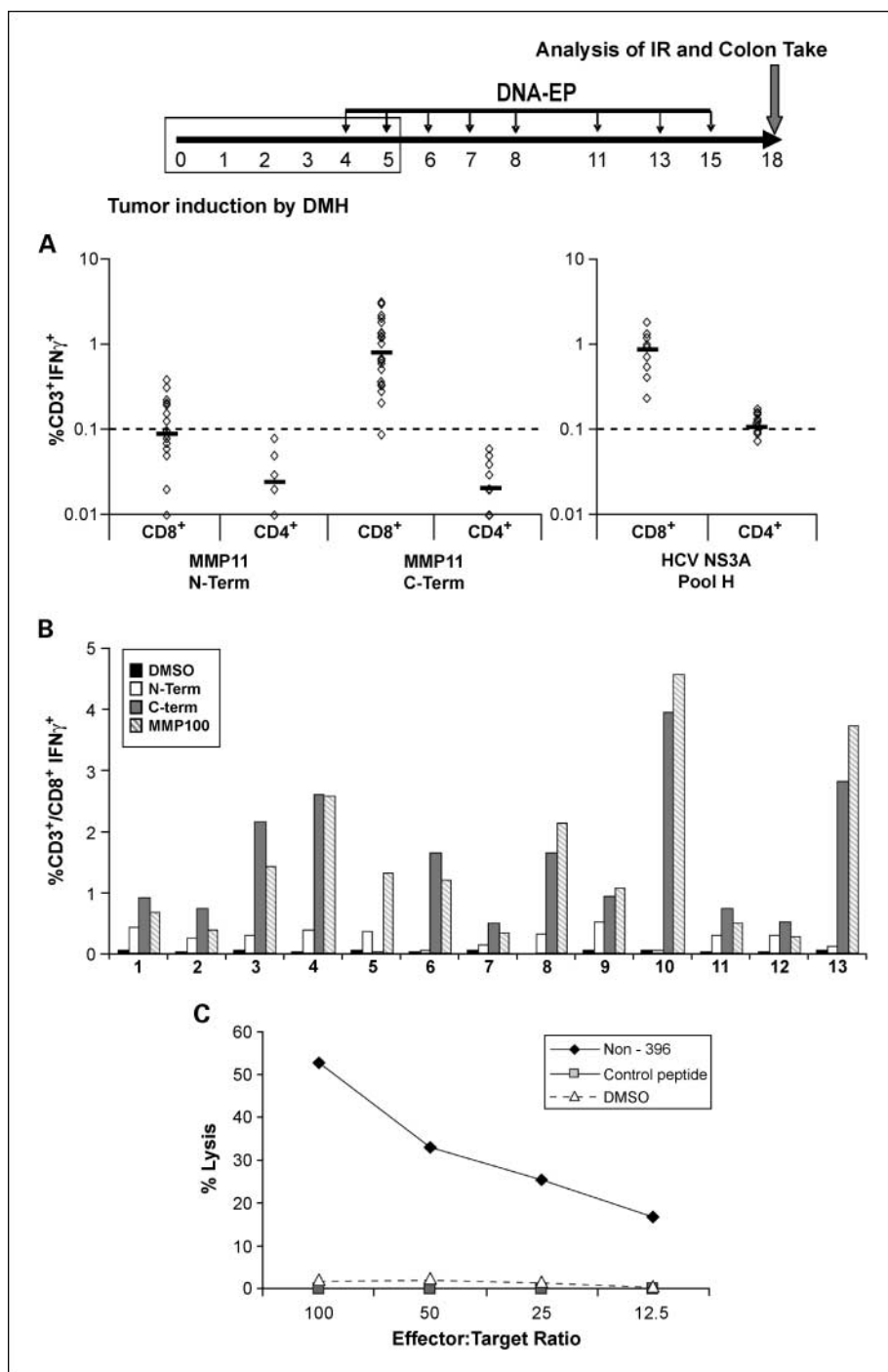
α suggest that vaccination against stromal antigens is a feasible approach for anticancer therapy (reviewed in ref. 39).

MMP11 is an ideal self-antigen for immunotherapy. It is differentially expressed in tumor versus normal tissue (Fig. 1), although it is unclear if it is expressed in cancer cells or in the supporting stroma. Vaccination against MMP11 exerts anti-tumor effects. In addition, the immunogenic potency of this antigen is enhanced by DNA electroporation and by the use of codon-optimized cDNA fused to immunomodulatory sequences of LTB (24). Therefore, it is not surprising that pV1J-

MMP11-LTB_{opt} is the most efficient among the different MMP11 derivatives in eliciting an immune response to the target antigen (Fig. 2).

The therapeutic potency of MMP11 vaccination was tested in the 1,2-dimethylhydrazine tumor model. This model was selected in view of its similarities with human colon adenocarcinoma. As detected in human colon cancer, 1,2-dimethylhydrazine-induced carcinogenesis progresses through different steps, from aberrant crypt formation, to adenoma, polyp, and adenocarcinoma (21). MMP11 was expressed in the aberrant

Fig. 4. Immune response in 1,2-dimethylhydrazine-treated mice. BALB/c mice were treated with six i.p. injections of 1,2-dimethylhydrazine and received eight DNA electroporation with pV1J-MMP11-LTB_{opt}, as shown. **A**, immune response was measured by intracellular staining. Diamonds, percentages of CD8⁺IFN- γ ⁺ and CD4⁺IFN- γ ⁺ splenocytes for each single mouse. Left, response against N-term and C-term peptide pools covering mouse MMP11. Right, response against HCV NS3A (pool H) in control group vaccinated with pV1J-NS3A. Black dash, geometric mean of the group. **B**, identification of mMMP₃₄₆ epitope. The immune response in a group of 13 vaccinated mice was measured with N-term, C-term, and the 15-mer peptide containing epitope MMP100. **C**, CTL assay. Effectors cells were stimulated for 7 d with mMMP₃₄₆. p815 Cells loaded with or without mMMP346 were used as target. An unrelated peptide from ratHER2/neu was used as negative control peptide.



crypt formation and adenoma, and its expression increased during cancer progression (Fig. 3). Thus, the 1,2-dimethylhydrazine-induced carcinogenesis is an interesting model to study the effect of MMP11 vaccination in the initiation/progression of colon cancer.

Mice treated with pV1J-MMP11LTB_{opt} at the time of tumor induction with 1,2-dimethylhydrazine showed a significant

reduction of tumor formation at all precancerous stages (Fig. 5A). Vaccination affected tumor progression because it interfered with the number of lesions and the differentiation stage of colon adenocarcinoma at late time points (Fig. 5B). The effect was evident also in therapeutic setting (Fig. 5C). These data indicate that MMP11 expression is intimately connected with tumor progression and suggest that it may play a role in

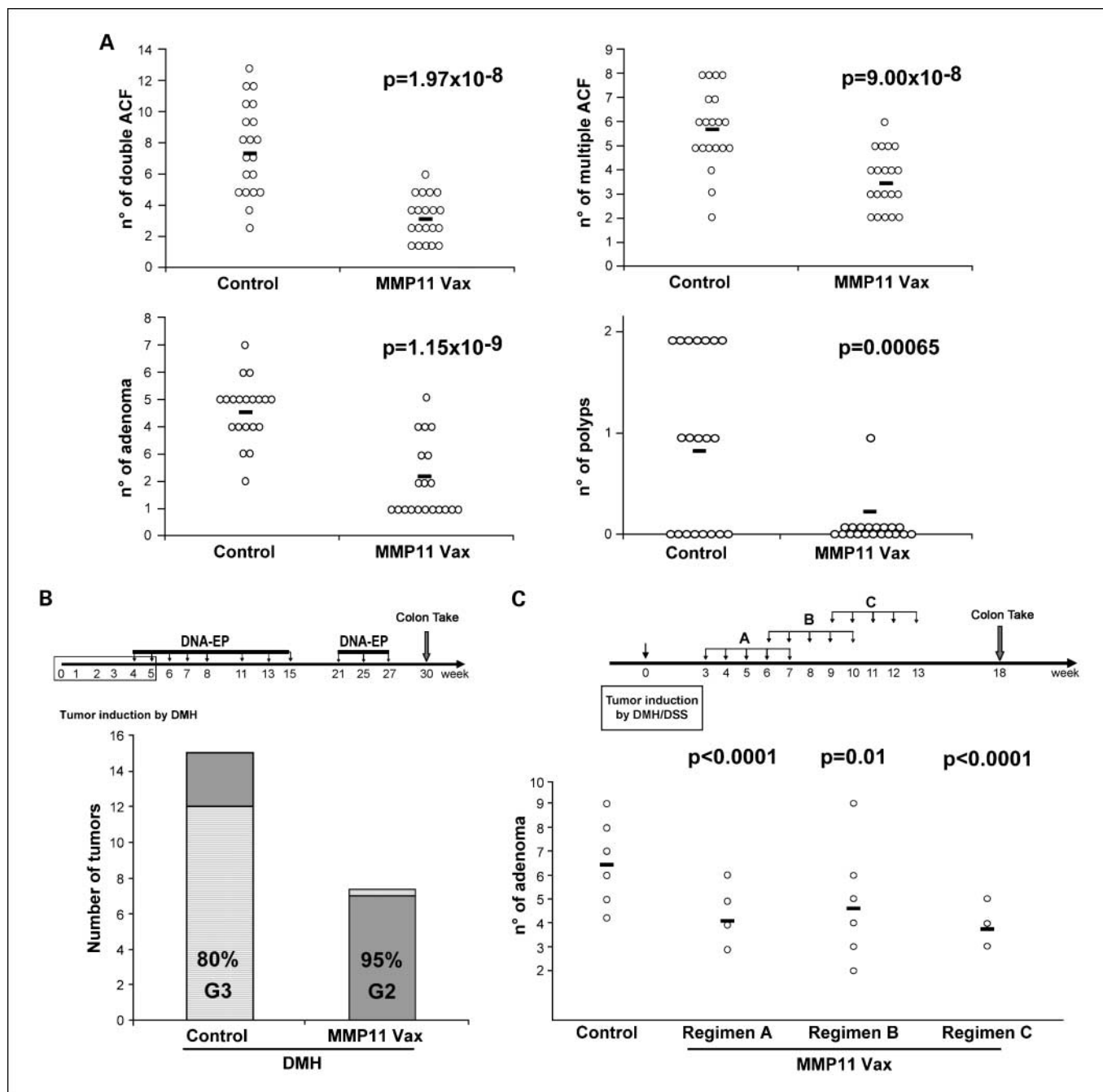


Fig. 5. Therapeutic efficacy of MMP11 vaccine in mice treated with 1,2-dimethylhydrazine. BALB/c mice were treated as described in Fig. 4. *A*, seven to eight weeks after the last 1,2-dimethylhydrazine injection, mice were sacrificed and colon analyzed for aberrant crypt formation, polyps, and adenomas. Empty circles, number of formations per mouse; black dashes, geometric mean of the group. Statistical analysis is indicated. *B*, BALB/c mice were treated with six i.p. injections of 1,2-dimethylhydrazine and vaccinated with a total of 11 DNA electroporation. Mice were sacrificed at treatment week 30, and evaluation of gross anatomic colon lesions was done. Adenocarcinomas were classified as G1, G2, and G3. G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated. *C*, BALB/c mice were treated with 1,2-dimethylhydrazine/DSS, as described. Three groups of 20 mice were vaccinated with five DNA electroporation, starting at week 3 (regimen A), 6 (regimen B), or 9 (regimen C). As control, a group of 10 mice was vaccinated with pV1J-NS3A according to regimen A. At week 18, 7 to 16 mice were sacrificed and colon tissue was analyzed for the presence of adenomas.

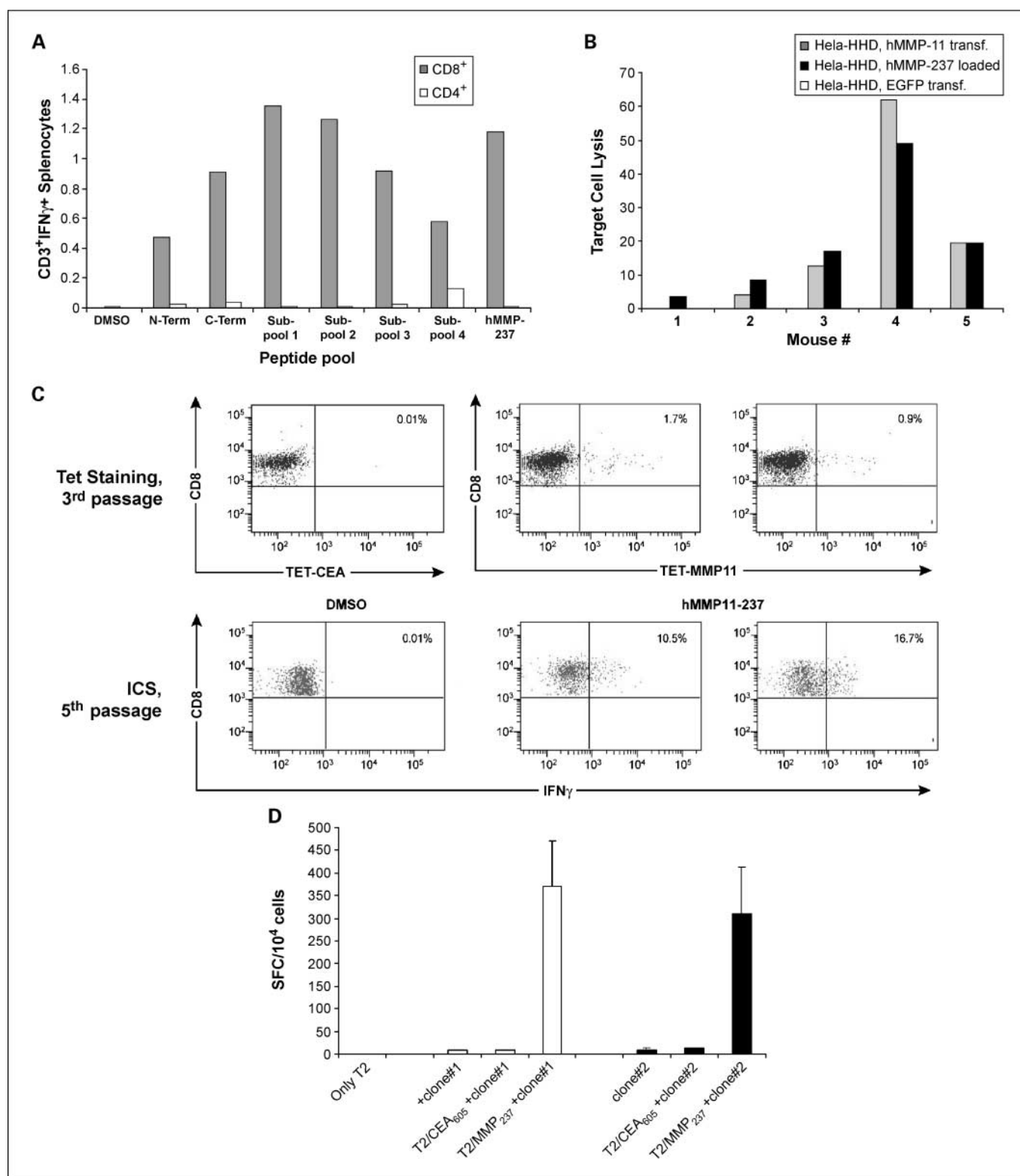


Fig. 6. Identification of hMMP₂₃₇ and *in vitro* priming with human PBMCs. **A**, identification of hMMP₂₃₇. HHD mice were vaccinated twice with pV1J-hMMP11 2 wk apart. Fifteen days later, splenocytes were analyzed by intracellular staining with the indicated pool of peptides. Each pool contains either hMMP-60 or hMMP-6115-mer. The whole response was attributable to hMMP₂₃₇ nonamer. **B**, hMMP₂₃₇ is processed and presented. HeLa-HHD cells were transfected with 0.5 μ g pV1J-hMMP11opt or pV1J-EGFP and a standard 4-h ⁵¹Cr release assay was done. As positive control, cells were loaded with hMMP₂₃₇ peptide. **C**, *in vitro* priming. T-cell lines were generated by priming PBMCs from a HLA-A2.1⁺ healthy donor with autologous DCs loaded with hMMP₂₃₇. After three or five passages, lines were analyzed by tetramer (Tet-MMP11) or intracellular staining, respectively. Staining with a tetramer specific for carcinoembryonic antigen (Tet – carcinoembryonic antigen) or incubation with DMSO represented the negative controls of the assays, respectively. **D**, T-cell lines secrete granzyme B. The two T-cell lines were tested against T2 cells prepulsed with hMMP₂₃₇ or carcinoembryonic antigen₆₀₅ control peptide (5 μ g/mL). Then, granzyme B production by peptide-induced CTLs was measured using enzyme-linked immunospot assay. Each sample was set with quadruplicate wells.

tumor invasion. In addition, they show that immunologic targeting of MMP11 can be extremely efficient in controlling disease progression.

We identified a H-2K^d-restricted CD8⁺ epitope (mMMP₃₄₆), capable of inducing CTL in tumor-bearing mice. A strong interferon- γ /cytotoxic cell-mediated and antibody response was elicited by MMP11 vaccine. Although it is possible to envisage how cellular and antibody response to MMP11 can control tumor progression, additional studies are needed to fully elucidate how tumor growth is impaired by vaccination with pV1J-MMP11-LTB_{opt}.

A novel T-cell epitope derived from human MMP11 was identified. hMMP₂₃₇ was identified by vaccination of HHD mice with pV1JhMMP11-LTB_{opt}; this approach allows a rapid evaluation of epitopes that can efficiently bind to HLA-A2.1 and are correctly processed by the proteasome/TAP machinery. As shown by *in vitro* priming with human PBMCs, hMMP₂₃₇ is immunogenic (Fig. 6C) and activated CTLs secrete granzyme B, a key mediator of target cell death via the granule-mediated pathway (Fig. 6D). Thus, hMMP₂₃₇ is an interesting candidate for cancer immunotherapy.

Targeting of MMPs has been suggested in several studies. The antitumoral effects of a vaccine against MMP2 have been reported (40). MMP-2-specific autoantibodies were detected in sera of mice immunized with a tumor cell vaccine expressing chicken MMP2. Vaccination prolonged survival of cancer-

bearing mice, and the antitumor activity was dependent on CD4⁺ and CD8⁺ T cells. Recently, MMP7 was identified as a novel broadly expressed tumor-associated antigen and a T-cell epitope derived from this protein was proposed as candidate for vaccine development (41). These observations further show that MMPs are valid candidates for antigen-specific immunotherapy.

This study confirmed that MMP11 is overexpressed in different tumors compared with normal tissues in patients and showed for the first time that MMP11 vaccine is able to induce an immune response and to confer a significant antitumor protection in a preclinical colon cancer model. Thus, our data support the use of MMP11 as a potential candidate for cancer immunotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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