Matrilysin (*MMP-7*) Is a Novel Broadly Expressed Tumor Antigen Recognized by Antigen-Specific T Cells

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Abstract

Purpose: A prerequisite for the development of vaccination strategies is the identification and characterization of relevant tumor-associated antigen. Using microarray and reverse transcription-PCR analysis, we found matrix metalloproteinase (*MMP*)-*7* to be extensively up-regulated in renal cell carcinomas and expressed in a broad variety of malignant cells. *MMP-7* can promote cancer invasion and angiogenesis by proteolytic cleavage of extracellular matrix and basement membrane proteins, thus making it a promising target in the context of immunotherapies.

Experimental Design: To analyze the possible use of *MMP-7* as a tumor-associated antigen, specific CTLs were induced using monocyte-derived dendritic cells electroporated with *MMP-7*-mRNA. In addition, to better characterize the fine specificity of these CTLs, *MMP-7* MHC class I ligands were isolated and characterized in renal cell carcinoma tissue, which overexpressed *MMP-7*, by mass spectrometry–based peptide sequencing. Using this approach, we identified a novel HLA-A3–binding antigenic *MMP-7* peptide. CTLs generated from healthy donors by *in vitro* priming with dendritic cells, pulsed with the novel peptide, were used as effectors in ^{51}Cr-release assays.

Results: The induced CTLs elicited an antigen-specific and HLA-restricted cytolytic activity against tumor cells endogenously expressing the *MMP-7* protein. Furthermore, we were able to induce *MMP-7*–specific CTLs using peripheral blood mononuclear cells from a patient with acute lymphoblastic leukemia capable of recognizing the autologous leukemic blasts while sparing nonmalignant cells.

Conclusions: Our study describes the identification of a novel broadly expressed T-cell epitope derived from the *MMP-7* protein that represents an interesting candidate to be applied in immunotherapies of human malignancies targeting both tumor cells and neovascularization.

Identification and targeting of antigens involved in malignant transformation, survival advantage of tumor cells, or their metastatic spread might represent a promising approach in the development of immunotherapeutic strategies to treat malignant diseases.

Using microarray analysis of primary renal cell carcinoma (RCC) tissues, we recently found that matrix metalloproteinase (*MMP*)-*7* is extensively up-regulated in malignant samples (1). MMPs are a family of structural-related zinc-dependent endopeptidases that degrade macromolecules of the extracellular matrix. Degradation of connective tissue is involved in many physiologic processes, such as embryogenesis, postpartum uterine involution, bone turnover, tissue repair, and angiogenesis as well as pathologic processes such as arthritis, decubitus ulcer, and tumor invasion. The gene family of the human MMPs comprises at least 19 members. They are divided into four classes, either in respect of their domain structure and preferred substrate (e.g., collagenases and gelatinases) or due to their localization such as the class of the membrane-bound MMPs (membrane type, MT1; ref. 2). With the exception of stromelysin 3 (*MMP-3*) all free MMPs are secreted as inactive proenzymes and are activated by enzymatic cleavage of the propeptide domain (3, 4). After their secretion and activation, they are inhibited by the tissue inhibitors of metalloproteinases (5, 6). The balance between the amount of activated MMP and tissue inhibitors of metalloproteinases determines the effective MMP activity (7).

Matrilysin (*MMP-7*) was discovered in rats in 1980 by Sellers and Woessner (8), and the human homologous gene was cloned in 1988 (9). Unlike most MMPs, it is constitutively expressed by many epithelial cell types, often ductal epithelium of adult exocrine glands in skin, salivary glands, pancreas, liver, and breast, and by glandular epithelium of the intestine and reproductive organs (10, 11). It is also found in the lumenal surface of dysplastic glands in the early-stage human colorectal tumors.

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Note: Y. Yokoyama and F. Grünebach contributed equally to this work.

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MMP-7 has a wide substrate specificity and can promote cancer invasion by proteolytic cleavage of extracellular matrix and basement membrane proteins, such as fibronectin, collagen type IV, laminin, elastin, entactin, osteopontin, and cartilage proteoglycan aggregates. Furthermore, this enzyme activates other MMPs, such as pro*MMP-2* and pro*MMP-9*, to facilitate tumor invasion and seems to mediate the proteolytic processing of tumor necrosis factor precursor and urokinase plasminogen activator (12–15). *MMP-7* knockout mice have reduced intestinal tumorigenesis (16). In line with this finding, it was reported that *MMP-7* expression in humans is correlated with invasiveness of cancer tissues of the esophagus, stomach, colon, liver, and pancreas (17–22). Furthermore, several studies showed that *MMP-7* is overexpressed in a broad variety of malignancies making this antigen an interesting candidate for immunotherapeutic approaches (23).

Angiogenesis is a critical step in cancer progression to enlarge and invade surrounding tissues (24, 25). Previous studies showed that several MMPs are expressed in vascular endothelial cells adjacent to tumor cells, suggesting their involvement in these processes by forming openings within the extracellular matrix through which newly generated capillaries can intrude and extend. *MMP-7* has been shown to accelerate the proliferation of human umbilical vein endothelial cells in a dose-dependent manner *in vitro* (26). In another study, angiogenesis at the site at which human colon cancer cells were implanted in a mice model could be inhibited by a *MMP-7*–specific antisense oligonucleotide (27). Taken together, these findings suggest that this enzyme directly promotes angiogenesis, at least in part, through proliferative action on vascular endothelial cells. Thus, targeting *MMP-7* may affect both tumor cells and tumor angiogenesis. Consequently, we initially examined the possible function of this molecule as a novel tumor-associated antigen using antigen-specific CTLs induced *in vitro* by monocyte-derived dendritic cells (DC) that have been electroporated with synthetic mRNA coding for *MMP-7* as antigen-presenting cells (APC). We show that these CTLs elicited an antigen-specific and HLA class I–restricted cytolytic activity against tumor cells endogenously expressing the *MMP-7* protein. Thereupon, we isolated and characterized *MMP-7* MHC class I ligands in RCC tissue that overexpressed *MMP-7*, by mass spectrometry–based peptide sequencing (1, 28–31). Using these peptides, we were able to induce CTLs *in vitro* that elicited an antigen-specific and HLA-A3–restricted cytolytic activity against tumor cells expressing *MMP-7*. In a last series of experiments, we show that *MMP-7*–specific CTLs induced by DCs transfected with *MMP-7*–*in vitro* transcript (IVT) efficiently lysed autologous blasts from acute lymphoblastic leukemia (ALL) patients.

In conclusion, our study describes the identification of a novel broadly expressed T-cell epitope derived from the *MMP-7* protein that represents an interesting candidate to be applied in immunotherapies of human malignancies capable of targeting neovascularization and malignant cells in developing tumors.

Materials and Methods

Tumor cell lines. All cells were cultured in RP10 medium (RPMI 1640 with glutamax I, supplemented with 10% inactivated FCS and antibiotics; Invitrogen). The cell lines used were A498, MZ1774, MZ1257 (RCC cell lines, HLA-A2$^+$, kindly provided by Prof. A. Knuth, UniversitätsSpital Zürich, Zürich, Switzerland), CAKI-2 (RCC, HLA-A1$^+$), U266 (multiple myeloma, HLA-A2$^+$), SKOV-3 (ovarian cell line, HLA-A3$^+$, kindly provided by O. J. Finn, University of Pittsburgh. Pittsburgh, PA), CROFT (EBV-immortalized B-cell line, HLA-A2$^+$; kindly provided by O. J. Finn), THP-1 (human acute monocytic leukemia), NT-2 (NTERA-2, pleuripotent, embryonal carcinoma cells), KASUMI-1 (acute myeloid leukemia), WERI-RB-1 (human retinoblastoma), HL-60 (human acute myeloid leukemia), and K-562 (human chronic myeloid leukemia, HLA-A2$^+$).

Generation of DCs from adherent peripheral blood mononuclear cells. DCs were generated from peripheral blood monocytes as described previously (32, 33). In brief, peripheral blood mononuclear cells (PBMC) were isolated by Ficoll/Paque (Biochrom) density gradient centrifugation of blood obtained from buffy coats of healthy volunteers from the blood bank of the University of Tübingen. Cells were seeded (1×10^8 cells/10 mL per flask) into 275-mL tissue culture flask (Becton Dickinson) in serum-free X-VIVO 20 medium (BioWhittaker). After 1.5 h of incubation at 37°C/5% CO_2, nonadherent cells were removed and cryopreserved at -80°C to be used later for cell isolation or restimulations. Human recombinant granulocyte macrophage-colony stimulating factor (100 ng/mL; Leukine Liquid Sargramostim; Berlex) and interleukin-4 (20 ng/mL; R&D Systems) were added every second day starting at the first day of culture to generate immature DCs. Maturation was induced at day 6 of culture by adding 2.0 μg/mL R848 (TLR7/8L; InvivoGen) for 24 h.

Synthetic peptides. *MMP-7* A*03: SLFPNSPKWTSK, *RGS5* A*03: GLASFKSFLK (1), were synthesized in an automated peptide synthesizer EPS221 (Abimed) after the F-moc/tBu strategy and analyzed by high performance liquid chromatography (Varian Star; Zinsser Analytics) and MALDI-TOF mass spectrometry (future; GSG).

Gene expression analysis by high-density oligonucleotide microarrays. Frozen fragments of tumors RCC0044, 0068, 0070, 0073, 0075, 0098, and 0103 were homogenized with mortar and pestle under liquid nitrogen. Total RNA was prepared from these samples using TRIzol (Invitrogen) according to the manufacturer's protocol, followed by a cleanup with RNeasy (QIAGEN). Quality and quantity were assessed on an Agilent 2100 Bioanalyzer (Agilent) using the RNA 6000 Pico LabChip kit (Agilent). Gene expression analysis of RNA samples from RCC0044, 0068, 0070, 0073, 0075, 0098, and 0103 were done by Affymetrix Human Genome U133A oligonucleotide microarrays (Affymetrix). For all other samples, HG-U133 Plus 2.0 were used. The same normal kidney sample was hybridized to both array types to achieve comparability (data not shown). All steps were carried out according to the Affymetrix manual.[4]

Briefly, double-stranded cDNA was synthesized from 5 to 8 μg of total RNA, using SuperScript RTII (Invitrogen) and the oligo-dT-T7 primer (MWG Biotech) as described in the manual. *In vitro* transcription was done with the BioArray High Yield RNA Transcript Labeling kit (ENZO Diagnostics, Inc.) for the U133A arrays or with the GeneChip IVT Labeling kit (Affymetrix) for the U133 Plus 2.0 arrays, followed by cRNA fragmentation, hybridization, and staining with streptavidin-phycoerythrin and biotinylated antistreptavidin antibody (Molecular Probes). Images were scanned with the Agilent 2500A GeneArray Scanner (U133A) or the Affymetrix GeneChip Scanner 3000 (U133 Plus 2.0), and data were analyzed with the MAS 5.0 (U133A) or GCOS (U133 Plus 2.0) software (Affymetrix), using default settings for all variables. Pairwise comparisons were calculated using the respective normal kidney array as baseline. For normalization, 100 housekeeping genes provided by Affymetrix were used.[5] Relative expression values were calculated from the signal log ratios given by the software and the normal kidney sample was arbitrarily set as one.

RNA isolation for reverse transcriptipn-PCR. Total RNA was isolated from tumor cell Iysates using RNeasy Mini anion-exchange spin columns (QIAGEN) according to the protocol for isolation of total

[4] http://www.affymetrix.com/support/technical/manual/expression.manual.affx
[5] http://www.affymetrix.com/support/technical/mask.files.affx

RNA from animal cells provided by the manufacturer. Quantity and purity of RNA was determined by UV spectrophotometry. RNA samples were routinely checked by formaldehyde/agarose gel electrophoresis for integrity and stored at -80°C in small aliquots.

Reverse transcription-PCR. Up to 5.0 μg total RNA was subjected to a 20-μL cDNA synthesis reaction (Transcriptor First Strand cDNA Synthesis kit; Roche) using random primers. cDNA (1.0 μL) was used in a 50.0-μL PCR amplification reaction. To control the integrity of the RNA and the efficiency of the cDNA synthesis, 1.0 μL cDNA was amplified by an intron-spanning primer pair for the *β2-microglobulin* gene. For the *MMP-7* and the *β2-microglobulin* cDNA, the PCR temperature profiles were as follows: 5 min pretreatment at 94°C and 25 (*β2-microglobulin*) or 32 (*MMP-7*) cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s with a final extension at 72°C for 7 min. Primer sequences were deduced from published cDNA sequences. *β2-microglobulin*, 5′-GGGTTTCATCCATCCGACAT-3′ and 5′-GATGCTGCTTACATGTCTCGA-3′; *MMP-7*, 5′-GAGTGCCAGATGTTGCAGAA-3′ and 5′-AAATGCAGGGGGATCTCTTT-3′. Reverse transcription-PCR reactions (10.0 μL) were electrophoresed through a 3.0% agarose gel and stained with ethidium bromide for visualization under UV light.

Generation of* MMP-7– *and enhanced green fluorescent protein*– *in vitro transcripts. A 1.1-kb *MMP-7* cDNA fragment was excised by *Eco*RI restriction enzyme digestion from plasmid pLXSN-MAT.WT (generously provided by L. M. Matrisian, Vanderbilt University, Nashville, USA) and subcloned into pcDNA3.1 (Invitrogen), which allowed *in vitro* transcription under the control of a T7 promoter. Plasmid, harboring the *MMP-7* insert in the correct orientation, was linearized behind the polyadenylate tail by *Bam*HI restriction enzyme digestion and *in vitro* transcribed using the T7 mMESSAGE mMACHINE kit (Ambion) according to the protocol provided by the manufacturer. Purification of IVTs was done with RNeasy Mini anion-exchange spin columns (QIAGEN) according to the RNA cleanup protocol provided by the manufacturer. Enhanced green fluorescent protein (*EGFP*)–IVT was generated as described previously (34). Quantity and purity of IVTs were determined by UV spectrophotometry. The IVTs were routinely checked by formaldehyde/agarose gel electrophoresis for size and integrity and stored at -80°C in small aliquots.

RNA electroporation of DCs. Electroporation of DCs with RNA was done as described previously (35–37). Briefly, on day 7 of culture, mature DCs were harvested, washed twice with X-VIVO 20 medium, and resuspended to a final concentration of 1×10^7 cells/mL. Subsequently, 200 μL of the cell suspension were mixed with 10 μg of RNA and electroporated in a 4-mm cuvette using an Easyject Plus unit (EquiBio/Peqlab). The physical variables were as follows: voltage of 300 V, capacitance of 150 μF, and resistance of 1540 Ω. After electroporation, cells were immediately transferred into RP10 medium supplemented with interleukin-4, granulocyte macrophage colony-stimulating factor, and tumor necrosis factor-α and returned to the incubator.

CTL induction using DCs transfected with RNA. DCs were electroporated with different sources of RNA as described above. After transfection, DCs were incubated for 24 h in serum-free X-VIVO 20 (BioWhittaker) medium supplemented with granulocyte macrophage-colony stimulating factor, interleukin-4, and tumor necrosis factor-α. For T-cell induction, 5.0×10^5 electroporated DCs were incubated with 3.0×10^6 autologous PBMCs in X-VIVO 20 (BioWhittaker). After 7 d of culture, cells were restimulated with RNA-electroporated DCs, and 2.0 ng/mL interleukin 2 (R&D Systems) was added on day 1, 3, and 5. The cytolytic activity of induced CTLs was analyzed on day 5 after the last restimulation in a standard ^{51}Cr-release assay.

Induction of* MMP-7–*specific CTLs using peptide-pulsed DCs. For CTL induction, 5×10^5 DCs were pulsed with 50 μg/mL synthetic peptide for 2 h, washed, and incubated with 3.0×10^6 autologous PBMCs in RP10 medium. After 7 d of culture, cells were restimulated with autologous peptide-pulsed PBMCs, and 1.0 ng/mL human recombinant interleukin-2 (Genzyme) was added on days 1, 3, and 5. The cytolytic activity of induced CTL was analyzed on day 5 after the last restimulation in a standard ^{51}Cr-labeled release assay.

Standard ^{51}Cr-release assays (CTL assays). CTL assays were done as described previously (38). In brief, target cells were transfected with RNA or pulsed with 50.0 μg/mL synthetic peptide for 2 h and labeled with ^{51}Cr sodium chromate in X-VIVO 20 medium for 1 h at 37°C. Target cells (1.0×10^4) were transferred to a well of a round-bottomed 96-well plate. Varying numbers of CTLs were added to a final volume of 200.0 μL and incubated for 4 h at 37°C. At the end of the assay, supernatants (50.0 μL per well) were harvested and counted in a β-plate counter. The percentage of specific lysis was calculated as: 100 × (experimental release - spontaneous release)/(maximal release - spontaneous release). Spontaneous and maximal releases were determined in the presence of either X-VIVO 20 medium or 2% Triton X-100, respectively. Inhibition of HLA class I molecules was achieved by incubating target cells for 1 h before the assay with the monoclonal antibody (mAb) W6/32 (20.0 μg/mL) directed against HLA class I molecules. Antigen specificity of cell lysis was further determined in a cold target inhibition assay by analyzing the capacity of unlabeled PBMCs pulsed with *MMP-7* peptide to block lysis of tumor cell lines.

IFN-γ ELISPOT assay. *MMP-7*–specific CTLs generated *in vitro* using autologous DCs pulsed with the *MMP-7* peptide (2×10^5 cells per well) were incubated at a concentration of 2×10^5 cells per well in an anti-human IFN-γ antibody-coated (mAb 1-D1K; 10 μg/mL; Mabtech AB) 96-well plate with autologous PBMCs pulsed for 1 h with the *MMP-7* or *RGS5* peptide as a control. For the detection of spots, a biotin-labeled antihuman IFN-γ antibody (mAb 7-B6-1-Biotin; 2 μg/mL; Mabtech AB) was used. Spots were counted after 40 to 44 h of incubation using an automated ELISPOT reader (IMMUNOSPOT ANALYZER; CTL Analyzers LLC).

Results

Expression analysis of MMP-7. Recently, we found *MMP-7* to be overexpressed in RCC (1). To assess the possible use of *MMP-7* as a target antigen for the development of vaccination therapies, reverse transcription-PCR analysis was done on human primary ALL cells and established tumor cell lines. As shown in Fig. 1, *MMP-7* expression was found in CAKI-2, MZ1257, SKOV-3, CROFT, NT-2 (faint), A498, and HL-60 tumor cell lines as well as in blood samples of patients with ALL.

Induction of* MMP-7–*specific CTLs using DCs electroporated with* MMP-7-*IVT. In the first series of experiments, we sought to analyze the feasibility to induce *MMP-7*–specific CTLs. Therefore, monocyte-derived DCs were generated from the PBMCs of a healthy HLA-A-A3$^+$ donor, electroporated with an excess of pure full-length *MMP-7*-IVT that coded for the entire *MMP-7*-protein and used as APCs for the *in vitro* induction of antigen-specific CTLs. DCs electroporated with the respective

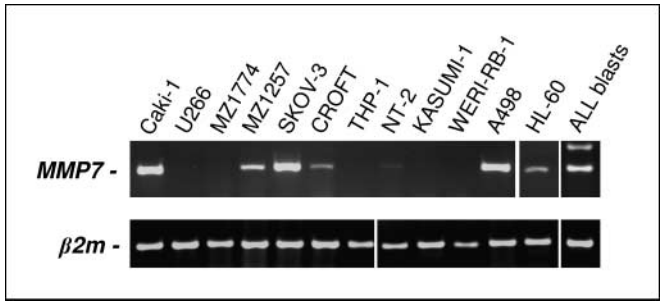

Fig. 1. Expression analysis of *MMP-7*. Reverse transcription-PCR using *MMP-7*–specific primers was done to analyze the mRNA expression of *MMP-7* in human tumor cell lines and primary ALL blasts. *β2-microglobulin* (*β2m*)-specific primers were used as controls.

MMP-7-IVT were used as target cells in the standard ^{51}Cr-release assays. As shown in Fig. 2A, these CTLs did lyse cells electroporated with *MMP-7*-IVT, whereas DCs transfected with irrelevant *EGFP*-IVT were spared.

To further confirm and extend our findings, we included the *MMP-7* – expressing HLA-A3$^+$ tumor cell line SKOV-3 as target in a standard ^{51}Cr-release assays using CTLs as above that were induced from an HLA-A3$^+$ donor. As shown in Fig. 2B, the malignant cells were efficiently lysed by the CTLs. In contrast, the *in vitro* induced T cells did not recognize the HLA-A2$^+$ cell line A498 that expresses *MMP-7*. K-562 cells were also spared, indicating that the cytotoxic activity was not natural killer cell mediated. Furthermore, the specific lysis of the target cells could be blocked using a mAb directed against HLA class I molecules demonstrating that the elicited T-cell responses were HLA class I restricted. Consequently, the antibody directed against HLA class II molecules did not inhibit the lysis of the tumor cells (Fig. 2B).

These data indicate that DCs electroporated with an excess of pure full-length *MMP-7*-IVT can result in the presentation of *MMP-7* – derived peptides on the cell surface that can induce *MMP-7* – specific CTLs that recognize *MMP-7* – positive target cells in an antigen-specific manner *in vitro*.

Induction of MMP-7 – specific CTLs using peptide-pulsed DCs. In the next set of experiments, we wanted to identify HLA class I – presented *MMP-7* peptides that are recognized by our CTL lines. To accomplish this, peptides were isolated from surgically removed RCC specimens that overexpressed *MMP-7* according to standard protocols (39) using the HLA class I – specific antibody W6/32. By applying mass spectrometry – based peptide sequencing of the HLA ligands, a HLA-A3 – binding peptide derived from the novel antigen *MMP-7* (HLA-A*03 ligand SLFPNSPKWTSK) was identified (1).

To analyze the immunogenicity of the newly identified *MMP-7* – derived peptide with respect to its ability to induce T-cell responses, we induced *MMP-7* – specific CTLs *in vitro*. DCs derived from adherent PBMCs of HLA-A3$^+$ healthy donors were pulsed with the HLA-A3 – binding antigenic peptide and used as APCs. The cytotoxicity of the induced CTLs was assessed in standard ^{51}Cr-release assays. As shown in Fig. 3A, the CTL line obtained after several weekly restimulations showed antigen-specific killing: T cells only recognized autologous DCs loaded with the cognate *MMP-7*-A3 peptide, whereas they did not lyse target cells pulsed with an irrelevant HLA-A3 – binding *RGS5* peptide, confirming the specificity of the cytolytic activity. Additionally, we included autologous DCs electroporated with *MMP-7*-IVT as targets in the cytotoxicity assay. As shown in Fig. 3A, these cells were efficiently lysed, whereas DCs electroporated with irrelevant *EGFP*-IVT were spared.

These findings indicate that DCs loaded with *MMP-7* – derived peptides can induce specific CTLs that recognize cells presenting *MMP-7* epitopes in an antigen-specific and HLA-A – restricted manner. Furthermore, DCs electroporated with an excess of pure full-length *MMP-7*-IVT can result in the presentation of the newly identified *MMP-7* – derived peptide on the cell surface, thus confirming the processing and presentation of the HLA-A3 – binding peptide.

In the next set of experiments, we evaluated the ability of the *in vitro* induced *MMP-7* – specific CTLs generated with HLA-A3 – binding peptide to lyse tumor cells endogenously expressing *MMP-7*. The *MMP-7* – and HLA-A3 – expressing cell line SKOV-3 was used as target in a standard ^{51}Cr-release assay. The *MMP-7* – specific CTLs were able to efficiently lyse malignant cells expressing both HLA-A3 and *MMP-7* (Fig. 3B). In contrast, we detected no lysis of the HLA-A2$^+$ and *MMP-7*$^+$ cell line A498, demonstrating that the CTLs were HLA-A3 restricted. The

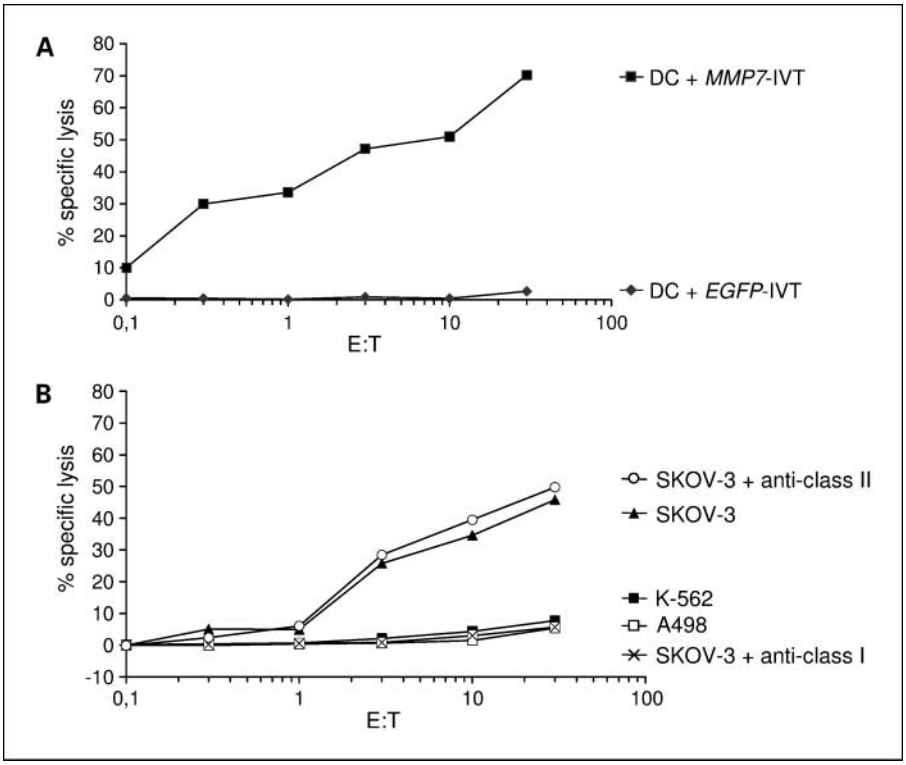

Fig. 2. Induction of *MMP-7* – specific CTLs using DCs electroporated with *MMP-7*-IVT. Immature monocyte-derived DCs generated from healthy donors (HLA-A3$^+$) were electroporated with pure *MMP-7*-IVT and used as APCs for the induction of antigen-specific CTLs. The cytolytic activity of the generated CTLs was determined after several weekly restimulations in a standard ^{51}Cr-release assay. *A,* DCs transfected with the cognate *MMP-7*-IVT were used as target cells. DCs electroporated with an irrelevant EGFP-IVT served as controls. *B,* tumor cell lines A498 and SKOV-3 were used as targets in a standard ^{51}Cr release assay. K-562 cells were included to determine the natural killer cell activity. Inhibition of HLA class I or class II was done by incubating target cells before the assay with anti-HLA class I or II antibodies. The data shown is representative of three independent experiments.

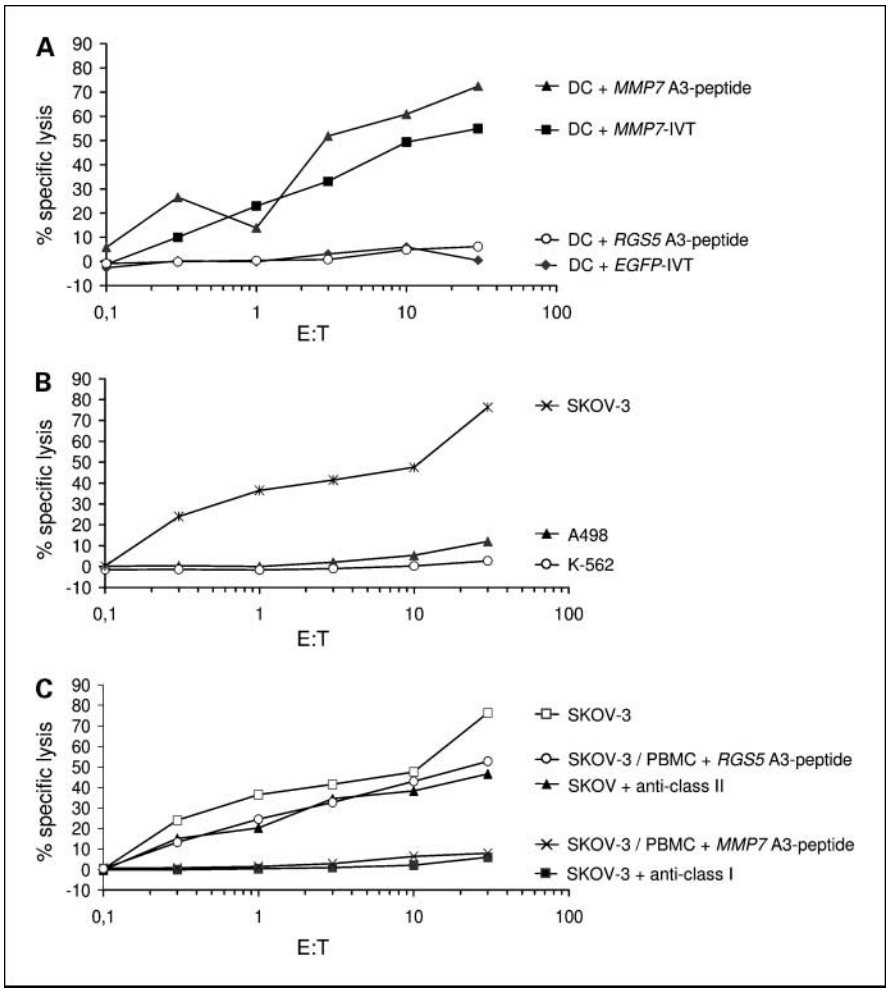

Fig. 3. Induction of *MMP-7* – specific CTLs using peptide-pulsed DCs. Immature monocyte-derived DCs generated from healthy donors (HLA-A3$^+$) were pulsed with synthetic *MMP-7* peptide and used as APCs for the induction of antigen-specific CTLs. The cytolytic activity of the generated CTLs was determined after several weekly restimulations in a standard ^{51}Cr-release assay. *A,* DCs loaded with the HLA-A3 – binding *MMP-7* peptide were used for CTL induction. DCs pulsed with the cognate peptide as well as DCs electroporated with pure *MMP-7*-IVT were used as target cells. DCs pulsed with an irrelevant HLA-A3 – binding RGS5-derived peptide and DCs electroporated with EGFP-IVT were included as a controls. *B,* A498 cells as well as cell line SKOV-3 were used as targets. K-562 cells were included to determine the natural killer cell activity. *C,* SKOV-3 cells were used as targets. Inhibition of HLA class I was done by incubating target cells before the assay with anti-HLA class I antibody. For cold target inhibition experiments, unlabeled PBMCs loaded with HLA-A3 – binding *MMP-7* peptide or with irrelevant HLA-A3 – binding RGS5-derived peptide as a control were added to ^{51}Cr-labeled SKOV-3 cells at the cold/labeled target ratio of 20:1. The data shown is representative of three independent experiments.

in vitro induced CTLs did also not recognize K-562 cells, indicating that the cytotoxic activity was not natural killer cell mediated.

To further confirm the antigen specificity, we did cold target inhibition assays. PBMCs loaded with the HLA-A3 – binding *MMP-7* peptide but not with the HLA-A3 – binding *RGS5* peptide could inhibit the recognition of HLA-matched SKOV-3 tumor cells, thus confirming that the *in vitro* induced CTLs are specific for the respective *MMP-7* epitope (Fig. 3C). Furthermore, the specific lysis of the target cells could be blocked using a mAb directed against HLA class I molecules, indicating that the elicited T-cell responses were HLA class I restricted (Fig. 3C).

These data indicate that DCs pulsed with *MMP-7* – derived peptides can induce *MMP-7* – specific class I – restricted CTLs that recognize tumor cells endogenously expressing *MMP-7* in an antigen-specific and HLA-A – restricted manner *in vitro*.

MMP-7 – *specific CTLs induced by DCs electroporated with MMP-7-IVT can lyse target cells pulsed with MMP-7 peptide.* To further prove the presentation of the identified peptide, we next wanted to test whether *MMP-7* – specific CTLs induced by DCs electroporated with pure full-length *MMP-7*-IVT are capable of recognizing target cells pulsed with the newly identified *MMP-7* peptides. As shown in Fig. 4, the induced CTLs did efficiently lyse DCs loaded with the *MMP-7* A3 peptide, whereas cells pulsed with the irrelevant *RGS5*-A3 peptide as a control were spared. Additionally, the induced CTLs did lyse cells electroporated with *MMP-7*-IVT, whereas DCs transfected with irrelevant *EGFP*-IVT were spared.

Induction of MMP-7 – specific CTLs from a patient with RCC. Using IFN-γ ELISPOT Assays, we found in 2 of 5 analyzed HLA-A3 – positive patients with metastatic RCC *MMP-7* peptide – specific T cells in peripheral blood with a frequency of 25.5 in 5×10^6 PBMCs and 39.5 in 10^6 PBMCs, respectively.

In our next experiments, we wanted to test the feasibility to induce *MMP-7* – specific CTLs in patients with RCC. Therefore, we generated monocyte-derived DCs from an HLA-A3$^+$ RCC patient with metastatic disease and used them as APCs for CTL induction after pulsing with the HLA-A3 – binding *MMP-7* peptide. As presented in Fig. 5A, the induced CTLs did efficiently lyse the HLA-A3$^+$ SKOV-3 tumor cell line. The specific lysis of these target cells could be blocked using a mAb directed against HLA class I molecules. As a control, we additionally included the HLA-A2$^+$ tumor cell line A498 and the HLA-A1$^+$ cell line CAKI-2. These cell lines were spared by the CTLs, confirming their HLA-A3$^+$ restriction.

In addition, as shown in Fig. 5B, autologous DCs loaded with *MMP-7* peptide or *MMP-7*-IVT were efficiently lysed by the induced CTLs, whereas DCs pulsed with irrelevant *RGS5* peptide or *EGFP*-IVT were spared.

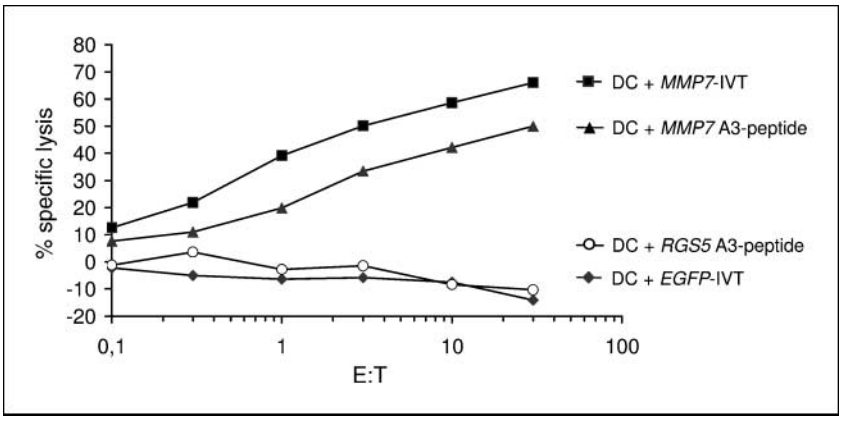

Fig. 4. Induction of *MMP-7* – specific CTLs using DCs electroporated with *MMP-7*-IVT. Immature monocyte-derived DCs generated from healthy donors (HLA-A3$^+$) were electroporated with pure *MMP-7*-IVT and used as APCs for the induction of antigen-specific CTLs. The cytolytic activity of the generated CTLs was determined after several weekly restimulations in a standard ^{51}Cr-release assay. DCs pulsed with the HLA-A3 – binding *MMP-7* peptide were used as target cells. DCs pulsed with an irrelevant HLA-A3 – binding RGS5-derived peptide as well as DCs electroporated with EGFP-IVT served as controls. The data shown is representative of three independent experiments.

Taken together, these experiments indicate the feasibility to induce *MMP-7* – specific CTLs from RCC patients that efficiently recognize and lyse tumor cells endogenously expressing *MMP-7*.

MMP-7 – *specific CTLs induced by DCs electroporated with* MMP-7-*IVT can lyse autologous ALL-blasts.* In the last series of experiments, we analyzed, whether our previous findings could be applied in an autologous setting. To achieve this, we generated monocyte-derived DCs from an HLA-A2$^+$/-A3$^+$ ALL patient in complete remission after chemotherapy and used them as APCs for CTL induction after electroporation with full-length *MMP-7*-IVT. As target cells, we included autologous DCs loaded with the respective peptide or transfected with *MMP-7*-IVT. As shown in Fig. 6A, these cells were efficiently lysed, whereas cells pulsed with an irrelevant *RGS5* peptide or electroporated with *EGFP*-IVT were spared.

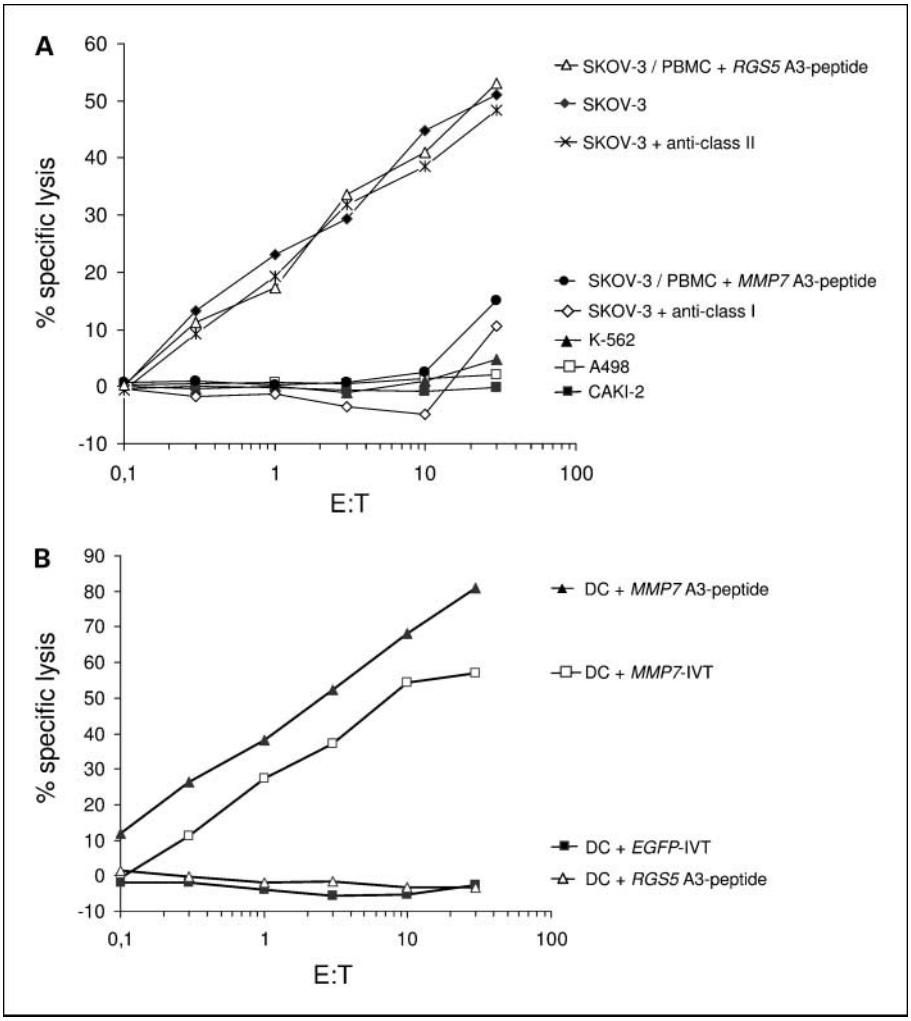

Fig. 5. Induction of *MMP-7* – specific CTLs in patients with RCC. Monocyte-derived DCs were generated from an HLA-A3$^+$ RCC patient with metastatic disease and used as APCs for CTL induction after pulsing with the HLA-A3 – binding *MMP-7* peptide. *A,* the HLA-A3$^+$ SKOV-3 or CAKI-2 tumor cell lines were used as targets in a standard ^{51}Cr-release assay. The specific lysis of these target cells could be blocked using a mAb directed against HLA class I molecules. For cold target inhibition experiments, unlabeled PBMCs loaded with HLA-A3 – binding *MMP-7* peptide or with irrelevant HLA-A3 – binding RGS5-derived peptide as a control were added to ^{51}Cr-labeled SKOV-3 cells at the cold/labeled target ratio of 20:1. *B,* autologous DCs loaded with *MMP-7* peptide or-IVT were included as targets in a standard ^{51}Cr-release assay. The data shown is representative of three independent experiments.

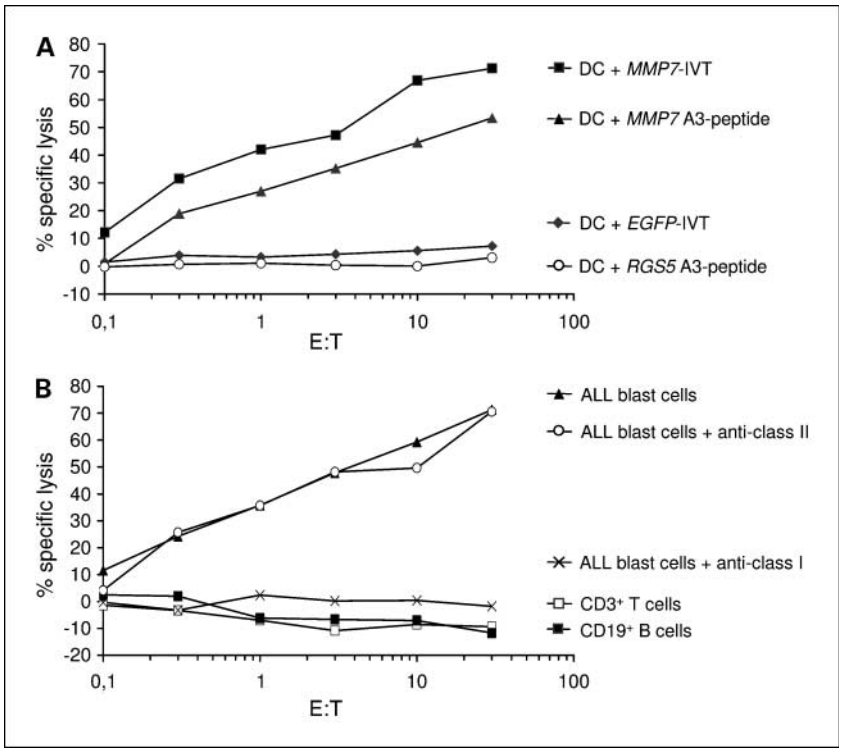

Fig. 6. *MMP-7* – specific CTLs can lyse autologous ALL-blasts. Monocyte-derived DCs from an HLA-A2$^+$/-A3$^+$ ALL patient in complete remission were generated under serum-free conditions and used as APCs for CTL induction after transfecting with *MMP-7*-IVT. *A,* DCs pulsed with the HLA-A3 – binding *MMP-7* peptide or electroporated with the cognate *MMP-7*-IVT were used as target cells. DCs pulsed with an irrelevant HLA-A3 – binding RGS5-derived peptide served as controls in the standard ^{51}Cr-release assay. *B,* autologous ALL blasts frozen at the time of diagnosis and normal purified CD3$^+$ T and CD19$^+$ B cells were included as targets. Inhibition of HLA class I or class II was done by incubating target cells before the assay with anti-HLA class I or II antibodies. The data shown is representative of three independent experiments.

Additionally, we included the autologous ALL blasts frozen at the time of diagnosis and used them as targets in standard ^{51}Cr-release assays. These cells were efficiently lysed by the CTLs specific for *MMP-7*, confirming the expression analysis by reverse transcription-PCR. Importantly, the normal autologous purified CD3$^+$ T and CD19$^+$ B cells (i.e., the normal lymphoid counterparts of the ALL blasts) were not recognized by the CTLs (Fig. 6B).

Discussion

During the last decade, great efforts have been made to develop immunotherapeutic approaches for the treatment of malignant diseases as an additional strategy to chemotherapy and radiotherapy. A prerequisite for this purpose is the understanding of the molecular pathways required for induction and maintenance of the immunosurveillance and immunoresponse and the development of methodologies to generate tumor-specific antigen and/or tumor-associated antigen – specific CTLs or mAbs as immunologic therapeutic agents. According to this, the basic requirement for these approaches is the availability of well-defined and characterized tumor-specific antigens and/or tumor-associated antigens as target structures that allow the specific attack of malignant cells. In recent years, a large number of tumor-specific antigens and tumor-associated antigens have been identified, which can be recognized by T cells (40).

DCs are recognized as the most powerful professional APCs, with the ability to initiate primary immune responses *in vivo*. Several procedures to generate large number of DCs as adjuvant from circulating precursors, including peripheral blood monocytes and CD34$^+$ progenitor cells, have been developed for clinical use. Hence, numerous attempts to optimize delivery of tumor antigens to DCs, as well as routes and schedules of administration to cancer patients, are currently being evaluated in clinical trials. Some of them showed promising results with the induction of tumor regression in a proportion of patients (41, 42).

The *putative metalloproteinase I* (*PUMP1*) gene was identified through studies of collagenase-related connective tissue – degrading metalloproteinases produced by human tumors. It was later called *matrilysin* or *MMP-7*. MMPs are enzymes implicated in normal and pathologic tissue – remodeling processes. *MMP-7* was shown to be involved in the invasiveness and progression of cancer cells. Generalized, *MMP-7* is expressed in many malignancies, thus representing an interesting target antigen for immunotherapeutic approaches. The high-density oligonucleotide microarray analysis revealed *MMP-7* to be overexpressed in 4 of 11 RCC tumor samples analyzed (1), and we found *MMP-7* expression in a broad variety of tumor cells, including renal and ovarian cell carcinomas, B-cell lines, embryonal carcinoma, acute myeloid leukemia, and primary ALL blasts.

Interestingly, MMPs can be produced by endothelial cells (43, 44). Endothelial synthesis of MMPs seems to have opposite effects on tumor angiogenesis, on the one hand, facilitating extracellular matrix degradation and new blood vessel formation (45) and, on the other hand, blocking angiogenesis by producing inhibitors of endothelial cell growth, including angiostatin (46). Taken together, targeting this molecule in tumors should not only affect the malignant cells but also inhibit neoangiogensis that accompanies tumor progression.

To generate *MMP-7* – specific CTLs, we first used PBMCs from HLA-A3$^+$ healthy donors in an *in vitro* immunization protocol. CTL induction was carried out using monocyte-derived DCs that were electroporated with an excess of pure full-length *MMP-7*-IVT. The *in vitro* generated CTLs efficiently lysed DCs

transfected with cognate antigenic RNA in ^{51}Cr-release assays, demonstrating the feasibility of these approach and immunogenicity of *MMP-7* – derived peptides processed by the DCs. To determine the fine specificity of our HLA-A3 – restricted CTL line, peptides were isolated and analyzed by mass spectrometry from primary tumor samples of patients with RCC overexpressing *MMP-7*. Using this approach, a HLA-A3 – restricted peptide derived from the novel antigen *MMP-7* was identified. This peptide was analyzed as a means to induce T-cell responses that mediate tumor cell lysis. The *in vitro* induced *MMP-7*-specific CTLs using DCs electroporated with IVT-RNA or *MMP-7* peptide were not only able to lyse target cells pulsed with the antigenic peptide but also recognized tumor cells endogenously expressing the *MMP-7* protein in an antigen-specific and HLA-restricted manner. The specificity of the lytic activity was confirmed by the addition of a mAb blocking the HLA molecules or by performing cold target inhibition assays.

Finally, we tested the ability of the identified *MMP-7* peptides to elicit CTL responses in patients with malignant diseases. Therefore, CTL lines were generated from the PBMCs of a patient with metastatic RCC or ALL in complete remission. These *MMP-7* – specific CTLs were used as effectors against autologous DCs, HLA-matched tumor cells, or the autologous malignant cells frozen at the time of diagnosis. The *in vitro* induced CTLs efficiently lysed the autologous leukemic cells in a MHC-restricted and antigen-specific manner but spared normal B and T cells from this patient. These results showed that *MMP-7* – specific CTLs can be generated in patients with malignant diseases able to recognize primary autologous tumor cells.

In conclusion, our study describes the identification of novel broadly expressed tumor-associated antigen and a T-cell epitope derived from the *MMP-7* protein that represents interesting candidate to be applied in immunotherapies of human malignancies.

Disclosure of Potential Conflicts of Interest

P. Brossart and H.-G. Rammensee have an ownership interest with Immatics Biotechnologies (Tübingen, Germany).

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