Identification and Characterization of T-Cell Epitopes Deduced from RGS5, a Novel Broadly Expressed Tumor Antigen

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Abstract

Purpose: Identification of tumor-associated antigens and advances in tumor immunology resulted in the development of vaccination strategies to treat patients with malignant diseases. In a novel experimental approach that combined comparative mRNA expression analysis of defined cell types with the characterization of MHC ligands by mass spectrometry, we found that regulator of G protein signaling 5 (RGS5) is extensively up-regulated in a broad variety of malignant cells, and we identified two HLA-A2− and HLA-A3− binding peptides derived from the RGS5 protein. Interestingly, RGS5 was recently shown to be involved in tumor angiogenesis.

Experimental Design: We used monocyte-derived dendritic cells pulsed with these novel antigenic peptides or transfected with RGS5-mRNA for the in vitro induction of CTLs, generated from healthy donors, to analyze the presentation of RGS5 -deduced epitopes by malignant cells.

Results: The generated CTL lines elicited an antigen-specific and HLA-restricted cytolytic activity against tumor cells endogenously expressing the RGS5 protein. Furthermore, we were able to induce RGS5-specific CTLs using peripheral blood mononuclear cells from a patient with acute myeloid leukemia capable of recognizing the autologous leukemic blasts while sparing nonmalignant cells.

Conclusions: These results indicate that the RGS5 peptides represent interesting candidates for the development of cancer vaccines designed to target malignant cells and tumor vessels.

The identification of relevant tumor-associated antigens (TAA) capable of mediating tumor cell elimination is a central issue in the development of immunotherapeutic strategies to treat malignant diseases.

Novel experimental approaches that are currently applied combine the information of all transcribed genes (“transcriptome”) of defined cell types with the analysis of MHC ligands by mass spectrometry for the identification of tumor antigens (1–5). In a first step, comparative expression profiling of tumor tissues and the corresponding normal tissue by DNA oligonucleotide microarray technology is done for the identification of genes selectively expressed or overexpressed in the tumor cells. This is followed by isolation and characterization of MHC class I ligands identified in the malignant tissue by mass spectrometry–based peptide sequencing. Newly identified MHC ligands encoded by genes identified by the microarray technique are considered as potential targets in immunotherapeutic strategies. By applying this approach to renal cell carcinoma (RCC) tissues, RGS5 was found to be extensively up-regulated (1).

G protein–linked receptors form the largest family of cell surface receptors and are found in all eukaryotes. G proteins are attached to the cytoplasmic face of the cellular plasma membrane, where they serve as relay molecules, functionally coupling the receptors to enzymes or ion channels in this membrane. In the inactivated state, G proteins exist as heterotrimers consisting of α, β, and γ subunits. The α subunit binds guanosine 5’-diphosphate, which is replaced by GTP upon stimulation by an activated receptor. This exchange causes the G protein trimer to dissociate into an α subunit and a βγ dimer. Both subunits transduce signals to a variety of G protein effectors, including adenylyl cyclase, voltage-sensitive Ca2+ and K+ channels, phosphatidylinositol 3-kinase, phospholipases C-β and A2, cyclic guanosine 3’,5’-monophosphate phosphodiesterase, and, indirectly, mitogen-activated protein kinase (6–8).

The key function of regulators of G protein signaling (RGS) is to bind to G protein α subunits and to stimulate their intrinsic GTPase activity. The hydrolysis of GTP to guanosine 5’-diphosphate thereby is accelerated, and the inactivate heterotrimer is more rapidly restored. Thus, RGS proteins inhibit the biological activity of G proteins (9–11).

Seki et al. isolated RGS5 from a neuroblastoma cDNA library (12). The amino acid sequence deduced from the cDNA possessed all consensus motifs of the RGS domain and showed closest homology to mouse RGS5. It was ascertained that RGS5 attenuates angiotensin II−, endothelin-1−, and

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platelet-derived growth factor–induced ERK-2 phosphorylation (13–15).

Interestingly, it was recently shown that RGS5 is overexpressed in pericytes of newly developing tumor vessels, indicating that RGS5 plays an important role during tumor angiogenesis (16–18). We found RGS5 expressed in a broad variety of malignant cells. Thus, targeting RGS5 may affect both tumor cells and tumor vessels.

In the present study, we examined the possible function of this molecule as a novel TAA using antigen-specific CTLs that were generated by in vitro priming with monocyte-derived dendritic cells as antigen-presenting cells. These dendritic cells were either loaded with RGS5 peptides or electroporated with pure full-length RGS5 in vitro transcript (IVT) that coded for the entire RGS5 protein.

We show here that the CTLs generated from several healthy donors and patients with acute myeloid leukemia (AML) elicited an antigen-specific and HLA-A2– or HLA-A3–restricted cytolytic activity against tumor cells endogenously expressing the RGS5 protein, including RCCs, breast cancer, melanoma, multiple myeloma cells, ovarian carcinoma, and primary autologous blasts from an AML patient.

Materials and Methods

**Tumor cell lines.** MCF-7 (breast cancer, RGS5+, HLA-A2+); A498, MZI1774, and MZI257 (RCC cell lines, RGS5+, HLA-A2+; kindly provided by Prof. A. Knuth, Zurich, Switzerland); U266 (multiple myeloma, RGS5+, HLA-A2+); HCT116 (colon cancer, RGS5+, HLA-A2+); Mel1479 (malignant melanoma, RGS5+, HLA-A3+; kindly provided by Prof. G. Papelcz, Tübingen, Germany); SKOV-3 (ovarian cell line, RGS5+, HLA-A3+; kindly provided by O.J. Finn, Pittsburgh, PA); Caki-1 (RCC, RGS5+); CROFT (EBV-immortalized B-cell line, HLA-A2+; kindly provided by O.J. Finn); SD-1 (human peripheral blood lymphoblastoid cells, RGS5+); THP-1 (RGS5+, human acute monocytic leukemia); NT-2 (NTERA-2, pluripotent, embryonal carcinoma cells, RGS5+); Kasumi-1 (AML, RGS5+); Weri-RE-1 (human retinoblastoma, RGS5+); HL-60 (human AML, RGS5+).

**Generation of dendritic cells from adherent peripheral blood mononuclear cells.** Dendritic cells were generated from peripheral blood mononuclear cells as described previously (19, 20). 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 × 107 per 3 mL per well) into six-well plates (Corning) in serum-free VIVO 20 medium (Bio Whittaker). After 2 h of incubation at 37°C/5% CO2, non-adherent cells were removed and cryopreserved at -80°C to be used later for cell isolation or re-stimulations. 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 2nd day starting at the 1st day of culture to generate immature dendritic cells. Maturation was induced at day 6 of culture by adding tumor necrosis factor-α (R&D Systems; 10 ng/mL) for 24 h.

**Synthetic peptides.** RGS5 A*02: LAALPHSCL, RGS5 A*03: GLASFSDKFLK (1), HER-2/neu (E75) A*02: KIFGSLAILF, BCR-ABL A*02: SSSKLQRPV, and BCR-ABL A*03/A*11: KQSSKLQRQV (21) were synthesized in an automated peptide synthesizer EPS221 (Abimed) following the fluoroen-9-ylmethoxy carbonyl/i-butyir strategy and analyzed by high-performance liquid chromatography (Varian star, Zinsser Analytics) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (future, GSG).

**Gene expression analysis by high-density oligonucleotide microarrays.** Frozen fragments of tumors RCC0044, RCC0068, RCC0070, RCC0073, RCC0075, RCC0098, and RCC0103 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, RCC0068, RCC0070, RCC0073, RCC0075, RCC0098, and RCC0103 was done by Affymetrix Human Genome U133A oligonucleotide microarrays (Affymetrix). For all other samples, HG-U133 Plus 2.0 was 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 protocol.3

Briefly, double-stranded cDNA was synthesized from 5 to 8 μg total RNA, using SuperScript RTI (Invitrogen) and the oligo-dT-77 primer (MWG Biotech) as described in the manual. In vitro transcription was done with the BioArray High Yield RNA Transcription 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-phycocerythrin and biotinylated anti-streptavidin 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. Relative expression values were calculated from the signal log ratios given by the software, and the normal kidney sample was arbitrarily set as 1.

**RNA isolation for reverse transcription-PCR.** Total RNA was isolated from tumor cell lysates using RNeasy Mini anion-exchange spin columns (Qiagen) according to the protocol for isolation of total 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-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 RGS5 and the β2-microglobulin cDNA, the PCR temperature profiles were as follows: 5-min pretreatment at 94°C and 32 or 25 cycles, respectively, at 94°C for 30 s, annealing for 30 s and 72°C for 60 s with a final extension at 72°C for 7 min. The annealing temperatures for β2-microglobulin and RGS5 were 55°C and 60°C, respectively. Primer sequences were deduced from published cDNA sequences (RGS5 accession no. NM_003617): β2-microglobulin, 5′-GGTTTCTATCCATCCGACAT-3′ and 5′-GATGCTGTTATCATGTTCGGA-3′; RGS5, 5′-AGATGCGGCTGAAAGCCAAA-3′ and 5′-TCAGGTCATGGTTTCTTC-3′. Reverse transcription-PCR (RT-PCR) reactions (10.0 μl) were electrophoresed through a 3.0% agarose gel electrophoresis for integrity and stored under UV light. The semiquantitative estimation of RGS5 expression was done by normalization of the density volumes of ethidium bromide stained PCR products, RCC50 PCR products to the corresponding β2-microglobulin PCR products (RGS5 PCR product [Σ (pixel, gray values)]/β2-microglobulin [Σ (pixel, gray values)]) using a CCD camera and gel analysis system (BioDocAnalyze video, Whatman Biometra).

**Generation of RGS5-IVT and EGFP-IVT.** A HindIII/XhoI-RGS5 fragment was excised from the plasmid pDNA3.1/V5-His-TOPO (generously provided by Y. Liang, Allergan, Inc., Irvine, CA) and

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subcloned into the modified plasmid pSP64 Poly(A) (Promega) harboring a new single NdeI restriction site (generously provided by V.F. van Tendeloo, Antwerp University Hospital, University of Antwerp, Edegem, Belgium) in front of a synthetic polyadenylic acid tail that allowed in vitro transcription under the control of an SP6 promoter. The plasmid was linearized behind the polyadenylic acid tail by NdeI restriction enzyme digestion and in vitro transcribed with the SP6 mMESSAGE mMACHINE kit (Ambion) according to the protocol provided by the manufacturer. Purification of IVTs was done with RNaseasy Mini anion-exchange spin columns (Qiagen) according to the RNA cleanup protocol provided by the manufacturer. EGFP-IVT was generated as described previously (22). Quantity and purity of IVTs were determined by UV spectrophotometry. The IVTs were routinely checked by formaldehydeagarose gel electrophoresis for size and integrity and stored at -80°C in small aliquots.

**RNA electroporation of dendritic cells.** Electroporation of dendritic cells with RNA was done as described previously (23–25). Briefly, on day 7 of culture, immature dendritic cells were harvested, washed twice with X-VIVO 20 medium (Bio Whittaker), and resuspended to a final concentration of 2 × 10⁶ cells per milliliter. Subsequently, 200 µL of the cell suspension were mixed with 10 µg of RNA and electroporated in a 4-mm cuvette using an EasyJet Plus unit (EquiBio/Peqlab). The physical variables were voltage of 300 V, capacitance of 150 µF, and resistance of 1,540 Ω. After electroporation, cells were immediately transferred into RPMI 1640 with glutamax (Life Technologies/Invitrogen) supplemented with 10% heat-inactivated FCS, 2 mmol/L L-glutamine, 50 µmol/L 2-mercaptoethanol, and antibiotics and returned to the incubator.

**Induction of RGS5-specific CTLs using peptide-pulsed dendritic cells.** For CTL induction, 5 × 10⁴ dendritic cells were transfected with 50 µg/mL synthetic peptide for 2 h, washed, and incubated with 2.5 × 10³ autologous PBMCs in RPMI. After 7 days 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 ⁵¹Cr-label release assay.

**CTL induction using dendritic cells transfected with RNA.** Dendritic cells were electroporated with different sources of RNA as described above. After transfection, dendritic cells were incubated for 24 h in RPMI 1640 medium supplemented with granulocyte macrophage colony-stimulating factor and interleukin-4. For induction of specific CTLs, 5 × 10⁵ electroporated dendritic cells were washed and incubated with 3.0 × 10⁶ PBMCs. After 7 days of culture, cells were restimulated with RNA-electroporated dendritic cells, and 2.0 ng/mL interleukin 2 (R&D Systems) was added on days 1, 3, and 5. The cytolytic activity of induced CTLs was analyzed on day 5 after the last restimulation in a standard ⁵¹Cr-label release assay.

**Standard ⁵¹Cr-release assays (CTL assays).** CTL assays were done as described previously (19). In brief, target cells were transfected with RNA or pulse with 50.0 µg/mL synthetic peptide for 2 h and labeled with ⁵¹Cr sulfuric acid in X-VIVO 20 (Bio Whittaker) medium for 1 h at 37°C. Target cells (1.0 × 10⁵) 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 beta-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 (Bio Whittaker) 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 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 RGS5 peptide to block lysis of tumor cell lines. The spontaneous release of dendritic cells as target cells was 15% to 20% throughout all experiments.

### Results

**Complementary analysis of gene expression in RCC and analysis of tumor-associated peptides presented on RCC.** We did high-density oligonucleotide microarray analysis to assess the expression of RGS5-mRNA in human RCCs compared with the corresponding normal renal tissues. RGS5 was found to be overexpressed in 9 of 17 tumor samples analyzed (data not shown; ref. 1). The liquid chromatography/mass spectrometry–based peptide sequencing of HLA ligands, extracted from surgically removed RCC specimens, yielded ~100 different peptides per patient. We identified two HLA-A2– or HLA-A3–restricted peptides derived from the novel antigen RGS5 (see Materials and Methods; ref. 1).

**Expression analysis of RGS5 by RT-PCR.** To assess the possible use of RGS5 as a broadly applicable target antigen for the development of vaccination therapies, RT-PCR analysis was done on human primary leukemic cells and established tumor cell lines. As shown in Fig. 1, RGS5-mRNA expression was found in all tested human tumor cell lines (A) as well as in several primary chronic lymphocytic leukemia, AML, three of five chronic myelogenous leukemia, and four of five acute lymphoblastic leukemia tumor samples analyzed (B). The semiquantitative estimation of RGS5 expression revealed that the mRNA levels in dendritic cells were lower as in various cell lines and AML blasts (C).

Thus, these experiments indicate that RGS5 is expressed in a broad variety of human malignancies.

**Induction of RGS5-specific CTLs using peptide-pulsed dendritic cells.** According to the expression analysis, RGS5 might represent a possible universal target antigen for the development of vaccination therapies (Fig. 1).

To analyze the presentation of RGS5-derived T-cell epitopes, we induced RGS5-specific CTLs in vitro using dendritic cells derived from adherent PBMCs of HLA-A2+/HLA-A3+ healthy donors. These dendritic cells were pulsed with either the HLA-A2– or HLA-A3–binding antigenic peptide and used as antigen-pulsing cells. The cytotoxicity of the in vitro induced CTLs was assessed in a standard ⁵¹Cr-release assay. As shown in Fig. 2A, the CTL line obtained after several weekly restimulations with dendritic cells loaded with the cognate HLA-A2 peptide showed HLA-A2 peptide–specific killing: T cells only recognized dendritic cells loaded with the respective RGS5-A2-peptide, whereas they did not lyse target cells pulsed with an irrelevant HLA-A2–binding peptide (HER-2/neu, E75), confirming the specificity of the cytolytic activity. Additionally, we included autologous dendritic cells electroporated with an excess of pure RGS5-IVT as targets in the cytotoxicity assay. As indicated in Fig. 2A, these cells were efficiently lysed, whereas dendritic cells electroporated with irrelevant EGFP-IVT were
Corresponding results were obtained for the HLA-A3–binding RGS5 peptide: the CTLs recognized dendritic cells loaded with the cognate peptide but spared dendritic cells pulsed with an irrelevant HLA-A3–binding BCR-ABL peptide. Accordingly, the CTLs did lyse dendritic cells electroporated with RGS5-IVT but not dendritic cells transfected with irrelevant EGFP-IVT (Fig. 2B).

To further characterize the effector functions of the RGS5-specific CTLs, we analyzed the secretion of cytokines using an enzyme-linked immunosorbent assay. Stimulation of the CTLs with the cognate peptide resulted in an antigen-specific production of IFN-γ as shown in Fig. 2C.

These findings indicate that dendritic cells loaded with RGS5-derived peptides can induce specific CTLs that recognize cells presenting RGS5 epitopes in an antigen-specific and HLA-A–restricted manner. Furthermore, dendritic cells electroporated with an excess of pure full-length RGS5-IVT present RGS5-derived peptides on the cell surface, which are recognized by RGS5-specific CTLs. The fact that despite their RGS5-mRNA expression, dendritic cells are not recognized by the CTLs per se is probably due to the lesser expression of the antigen compared with various cell lines or AML blasts (Fig. 1C).

**RGS5-specific CTLs induced by peptide-pulsed dendritic cells can lyse tumor cells of epithelial and hematologic origin.** In the next set of experiments, we evaluated the ability of the in vitro induced RGS5-specific CTLs to lyse tumor cells endogenously expressing RGS5. Therefore, dendritic cells generated from an HLA-A2+/HLA-A3+ healthy donor were pulsed with either the HLA-A2– or HLA-A3–binding peptide and used for CTL induction in vitro. In the first set of experiments, CTLs were induced by dendritic cells pulsed with the HLA-A2 peptide. The RGS5- and HLA-A2–expressing cell lines A498 (RCC), MCF-7 (breast cancer), and U266 (multiple myeloma) were used as target cells in a standard 51Cr-release assay. The RGS5-specific CTLs were able to efficiently lyse malignant cells expressing both HLA-A2 and RGS5 (Fig. 3A). In contrast, we detected no lysis of the HLA-A3+ ovarian cancer cell line SKOV-3, showing...
that the presentation of RGS5 peptides in context of HLA-A2 molecules on the tumor cells is necessary for the efficient lysis of target cells. The in vitro induced CTLs did not recognize K-562 cells, indicating that the cytotoxic activity was not natural killer cell mediated.

Corresponding results were obtained for CTLs induced with the HLA-A3–binding RGS5 peptide: reciprocally, the HLA-A3* SKOV-3 cells were recognized by the induced CTLs, whereas the HLA-A2–expressing cell line A498 was spared (Fig. 3B).

In cold target inhibition assays, PBMCs or dendritic cells loaded with either the HLA-A2– or HLA-A3–binding RGS5 peptide could inhibit the recognition of HLA-matched tumor cells, thus confirming that the in vitro induced CTLs are specific
for the respective RGS5 epitope. As shown in Fig. 3C, PBMCs loaded with the HLA-A2–binding RGS5 peptide efficiently inhibited the lysis of the HLA-A2+ A498 cells. In contrast, PBMCs loaded with an irrelevant peptide (HER-2/neu) could not inhibit the recognition of tumor cell–derived RGS5 epitopes.

Similar results were obtained for the HLA-A3+ cell lines SKOV-3 and Mel1479: dendritic cells pulsed with the HLA-A3–binding RGS5 peptide efficiently inhibited the lysis of the tumor cells, whereas dendritic cells loaded with irrelevant control peptide (BCR-ABL-A3) could not inhibit the lysis of both cell lines (Fig. 3D and E, respectively).

Furthermore, the specific lysis of the target cells could be blocked using a monoclonal antibody directed against HLA class I molecules, indicating that the elicited T-cell responses were HLA class I restricted. Consequently, IgG or the antibody directed against HLA class II molecules did not inhibit the lysis of the tumor cells (Fig. 3C and D, respectively).

These data indicate that dendritic cells pulsed with RGS5-derived peptides can induce RGS5–specific class I–restricted CTLs that recognize tumor cells endogenously expressing RGS5 in an antigen-specific and HLA-A–restricted manner in vitro.

**Induction of RGS5–specific CTLs using dendritic cells electroporated with RGS5-IVT.** In the next set of experiments, we sought to analyze the feasibility to induce RGS5–specific CTLs by dendritic cells electroporated with an excess of pure full-length RGS5-IVT that coded for the entire RGS5 protein. Therefore, monocyte-derived dendritic cells were generated from the PBMCs of a healthy HLA-A2+/HLA-A3+ donor and transfected with RGS5-IVT. Dendritic cells loaded with either the RGS5-HLA-A2 or RGS5-HLA-A3 peptide were used as target cells. As shown in the standard 51Cr-release assay, the induced CTLs did lyse cells loaded with RGS5 peptide, presented either by HLA-A2 or HLA-A3 molecules, whereas dendritic cells loaded with an irrelevant BCR-ABL peptide, both HLA-A2 and HLA-A3, were spared (Fig. 4A).

To further confirm and extend our findings, we used various tumor cell lines as targets in a standard 51Cr-release assay using CTLs as described above that were induced from an HLA-A2+/HLA-A3+ donor. As shown in Fig. 4B, the malignant cells were efficiently lysed by the CTLs. In contrast, the in vitro induced T cells did not recognize K-562 cells, indicating that the cytotoxic activity was not natural killer cell mediated.

These data indicate that dendritic cells electroporated with an excess of pure full-length RGS5-IVT can result in the presentation of RGS5–derived peptides on the cell surface that can induce RGS5–specific CTLs that recognize RGS5+ target cells in an antigen-specific manner in vitro.

**RGS5–specific CTLs can lyse autologous AML blasts.** The long-term objective of our study is the development of a dendritic cell–based adoptive immunotherapy for patients with malignant diseases. Thus, from our previous experiments, it was apparent that RGS5–specific CTLs recognize and efficiently lyse tumor cells of various origins. Furthermore, expression analysis showed that all AML blood samples analyzed by RT-PCR exhibited RGS5 expression (Fig. 1). In a last series of experiments, we therefore wanted to test whether our previous findings could be applied to AML blasts in an autologous setting. To achieve this, we generated monocyte-derived dendritic cells from an HLA-A2+/HLA-A3+ AML M2 patient in complete remission after chemotherapy and used them as antigen-presenting cells for CTL induction after pulsing with either the RGS5-HLA-A2 or RGS5-HLA-A3 peptide. As target cells, we included autologous dendritic cells loaded with the respective peptides. As shown in Fig. 5A, CTLs induced with the HLA-A2–binding RGS5 peptide recognized dendritic cells loaded with the respective HLA-A2 peptide but spared dendritic cells pulsed with HLA-A3 peptide or with an irrelevant control peptide (BCR-ABL-A2). Additionally, we included the autologous AML blasts frozen at the time of diagnosis and normal PBMCs and used them as targets in the standard 51Cr-release assay. As shown in Fig. 5A, these cells were efficiently lysed by the CTLs specific for the RGS5-HLA-A2 peptide, thus confirming the RGS5 expression analysis by RT-PCR. Importantly, the normal autologous PBMCs were not recognized by the CTLs. Natural killer cell–mediated cytotoxic activity was excluded by K-562 cells, which were not recognized by the in vitro induced T cells. Corresponding results were obtained when the HLA-A3 peptide was used for CTL induction: the autologous dendritic cells pulsed with the RGS5-A3-peptide as well as the autologous AML blasts were efficiently lysed, whereas the autologous normal PBMCs and dendritic cells pulsed with either the RGS5-A2- or BCR-ABL-A3-peptide were spared (Fig. 5B).

To further confirm and extend our findings, we repeated these experiments using the CTLs as above and dendritic cells

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**Fig. 4.** Induction of RGS5–specific CTLs using dendritic cells electroporated with RGS5-IVT. Immature monocyte-derived dendritic cells generated from healthy donors (HLA-A2+/HLA-A3+) were electroporated with pure RGS5-IVT and used as antigen-presenting cells for the induction of antigen-specific CTLs. The cytolytic activity of the generated CTLs was determined after several weekly re-stimulations in a standard 51Cr-release assay. A, dendritic cells pulsed with the HLA-A2– or HLA-A3–binding RGS5 peptide were used as target cells. Dendritic cells pulsed with an irrelevant HLA-A2– or HLA-A3–binding BCR-ABL–derived peptide served as controls. B, tumor cell lines A498, MCF-7, U266, and Mel1479 were used as targets in a standard 51Cr-release assay. K-562 cells were included to determine the natural killing cell activity.
electroporated with either pure RGS5-IVT or total RNA, isolated from the autologous AML blasts at the time of diagnosis as targets in a standard \(^{51}\)Cr-release assay. As shown in Fig. 5C, these cells were efficiently lysed by the CTLs, whereas dendritic cells electroporated with total RNA purified from the normal autologous PBMCs or EGFP-IVT as a control were spared. These findings could be confirmed by using the RGS5-HLA-A3 peptide for CTL induction because dendritic cells transfected with RGS5-IVT or AML-RNA were recognized by the induced CTLs as above, whereas all control target cells were spared (Fig. 5D).

These data show that dendritic cells generated from an AML patient in complete remission and pulsed with RGS5-derived peptides can induce RGS5-specific CTLs that recognize autologous AML blast cells endogenously expressing RGS5 in an antigen-specific manner in vitro.

**Discussion**

During the last decade, great efforts have been made to develop immunotherapeutic approaches for the treatment of malignant diseases as an alternative 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 TAA-specific CTLs or monoclonal antibodies 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 TAs as target structures that allow the specific attack of malignant cells. In recent years, a large number of tumor-specific antigens and TAs have been identified, which can be recognized by T cells (26).

Dendritic cells are the most powerful professional antigen-presenting cells, with the ability to initiate and maintain primary immune responses. Recently, several procedures to generate a large number of dendritic cells as adjuvants from circulating precursors, including peripheral blood monocytes and CD34+ progenitor cells, have been developed for clinical use to treat patients with infections and malignant diseases. Hence, numerous attempts to optimize delivery of tumor antigens to dendritic cells, as well as routes and schedules of delivery, are currently ongoing.
administration to cancer patients, are currently being analyzed in clinical trials (27–30).

Comparative analysis using gene expression profiles of normal kidney and RCCs by differential display approach, quantitative RT-PCR, or DNA microarray technology revealed that RGS5 is highly overexpressed in some malignant RCC samples. Moreover, using RT-PCR analysis, expression of RGS5 was found in several human tumor cell lines, indicating that this gene is expressed in a broad variety of human malignancies.

G protein–coupled receptors regulate a multitude of cellular processes. They consist of three subunits: α, β, and γ. The binding of a ligand to its receptor leads to a conformational change of the receptor and the cytosolic-bound G protein by what guanosine 5’-diphosphate is replaced with GTP. This exchange leads to the dissociation of the α and βγ subunits, which both serve as second messengers. RGS proteins activate the GTPase of the α subunit; therefore, the restoration of the inactive trimeric αβγ molecule. In this way, RGS proteins terminate the receptor signaling. Because G proteins and RGS are involved in many cellular signaling cascades, the overexpression of RGS5 in malignant tissues, ascertained in this work, suggests an etiologic relation to the cancerous phenotype.

We identified HLA-A2– or HLA-A3–binding peptides derived from the RGS5 protein in tumors of RCC patients by applying an integrated functional genomics approach. This procedure combined comparative expression profiling of tumor samples with the corresponding autologous normal tissue using DNA microarray technology to find genes overexpressed in the malignant cells with mass spectrometry to identify MHC class I ligands derived from these selectively expressed or overexpressed antigens.

Subsequently, we analyzed the possible use of the identified peptides as T-cell epitopes that can induce antigen-specific CTLs and mediate tumor cell lysis. To accomplish this, we used PBMCs from HLA-A2– or HLA-A3–positive donors in an in vitro immunization protocol. CTL induction was carried out using monocyte-derived dendritic cells pulsed with the RGS5 peptides or transfected with in vitro transcribed RGS5-RNA as antigen-presenting cells. After several rounds of re-stimulations, cultures were tested for their lytic activity against target cells pulsed with the cognate peptides or human tumor cell lines expressing RGS5. Additionally, dendritic cells electroporated with various species of RNA were included as target cells. The in vitro induced peptide-specific CTLs were not only able to lyse target cells pulsed with the antigenic peptide but also recognized tumor cells endogenously expressing the RGS5 protein in an antigen-specific and HLA-restricted manner, including RCC, malignant melanoma, breast cancer, and multiple myeloma cells. The specificity of the lytic activity was confirmed by the addition of a monoclonal antibody blocking the HLA molecules or by performing cold target inhibition assays.

To further analyze the specificity of the elicited CTL responses and presentation of identified peptides, we used dendritic cells that were electroporated with an excess of pure full-length RGS5-IVT as target cells in standard 51Cr-release assays. The in vitro generated CTLs efficiently lysed peptide-pulsed dendritic cells and the dendritic cells transfected with antigenic RNA, showing that the peptides used for CTL induction are also processed and presented on transfection of RGS5-IVT.

In previous studies, we could show that transfection of dendritic cells with specific or whole-cell RNA is able to induce potent antigen- and tumor-specific T-cell responses directed against multiple epitopes. This technique does not require the definition of the TAA or HLA haplotype of the patients and has the potential of a broad clinical application (24, 31–36). According to this, we were successful in the induction of RGS5-specific CTLs by dendritic cells electroporated with pure full-length RGS5-IVT. Furthermore, these CTLs recognized the two novel peptides, confirming the presentation of the identified epitopes.

Finally, we tested the ability of the identified RGS5 peptides to elicit CTL responses in patients with malignant diseases. Therefore, CTL lines were generated from the PBMCs of a patient with AML in complete remission by in vitro immunization with peptide-pulsed autologous dendritic cells. These peptide-specific CTLs were used as effectors against 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 PBMCs as well as autologous dendritic cells from this patient. These results showed that RGS5-specific CTLs can be generated in patients with malignant diseases able to recognize primary autologous tumor cells. Interestingly, expression analysis of RGS5 revealed that it can hardly be detected in monocytes or granulocytes. However, RGS5 transcription could be verified in malignant myeloid cells, indicating that it might be involved or induced during malignant transformation.

Furthermore, we found RGS5-mRNA in monocyte-derived dendritic cells, suggesting that it could play a role in the process of differentiation of dendritic cells from monocytes. In contrast to malignant cells, RGS5-specific CTLs, while lysing tumor cells expressing RGS5, did not recognize RGS5-positive dendritic cells. They were, however, able to lyse dendritic cells pulsed with the antigenic peptide or transfected with RNA coding for RGS5. These observations suggest that the TAA RGS5 is only recognized as non-self if overexpressed in the respective cells, whereas the basal expression in normal tissue is not sufficient to elicit an immunoresponse.

In conclusion, our study describes the identification of novel broadly expressed T-cell epitopes deduced from the RGS5 protein that represent interesting candidates to be applied in immunotherapies of human malignancies and extend the number of possible CTL epitopes that can be used for the design of cancer vaccines.

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References
RGS5 Is a Novel Broadly Expressed Tumor Antigen

Identification and Characterization of T-Cell Epitopes Deduced from RGS5, a Novel Broadly Expressed Tumor Antigen

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