Identification of HLA-A*0201-Restricted T Cell Epitopes Derived from the Novel Overexpressed Tumor Antigen Calcium-Activated Chloride Channel 2

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Vaccination against tumor Ags may become a promising treatment modality especially in cancer types where other therapeutic approaches fail. However, diversity of tumors requires that a multitude of Ags become available. Differential expression in normal vs cancerous tissues, both at the mRNA and the protein level, may identify Ag candidates. We have previously compared transcripts from squamous cell lung cancer and normal lung tissue using differential display analysis, and found a transcript that was overexpressed in malignant cells and was identical with the calcium-activated chloride channel 2 (*CLCA2*) gene. We have now selected HLA-A2-restricted peptides from CLCA2, and have generated T cell lines against the CLCA2-derived KLLGNCLPTV, LLGNCLPTV, and SLQALKVTV peptides using in vitro priming. Specificity of T cells was ascertained in ELISPOT assays. The primed T cells also recognized allogeneic tumor cells in an Ag-specific and HLA-restricted fashion. Moreover, peptide LLGN CLPTV was also independently recognized by CD8$^+$ T cells expanded from pancreatic carcinoma/T cell cocultures. CLCA2-specific CD8$^+$ T cells were absent from the peripheral blood of healthy donors. These data indicate that an immune response can be induced against CLCA2, which thus may become an important Ag for anti-tumor vaccination approaches. ***The Journal of Immunology,*** **2002, 169: 540–547.**

The immune system has long been implicated in controlling tumor cells, and evidence collected in the last decade indicates that CD8$^+$ T cells that recognize peptide fragments derived from tumor-associated Ags (TAA)[2] in an HLA-restricted fashion play a preeminent role in this process (1, 2). Such cytotoxic T cells isolated from tumor patients and cultured in vitro were used as detection reagents to identify TAAs with great success in the past (3). Tumor Ags discovered with this approach were in many cases indeed unique to the tumor (4, 5), or were expressed only at immunoprivileged sites in addition to tumors. It is conceivable that upon vaccination, the immune system recognizes these Ags as foreign, and mounts a response. However, the very same approach also identified molecules that were also shared by normal somatic cells and were merely overexpressed in tumors (6–8). These examples indicated that overexpression of Ags may in certain cases suffice for the immune system to discriminate between malignant and normal cells (9, 10).

Diversity of tumors and Ag-loss escape mechanisms require that a multitude of Ags is used for vaccination (11). This need for novel Ags cannot be satisfied with the CTL-expression cloning method alone. Recent advances in high-throughput expression analysis (12) made it possible to explore the genetic differences between normal and malignant cells on a large scale. The identification of LAGE-1 was an important example which showed that such genetically based approaches can indeed yield valuable candidate tumor Ags (13). However, in contrast to the CTL-based approach, immunologic recognition of overexpressed products is not granted a priori. CD8$^+$ T cells that would recognize self-Ags can be eliminated by thymic selection or silenced upon peripheral interactions. In vitro priming is an established method to assess whether the T cell repertoire of normal donors still contains cells that can respond to such putative Ags, as it was successfully shown, e.g., for telomerase (14) or for a number of melanoma Ags (15, 16).

We have compared transcripts from squamous cell lung cancer and normal lung tissue using differential display analysis, and a gene that was newly expressed in malignant cells was found to be identical to the Ca-activated chloride channel 2 (CLCA2) (U. König and W. Sommergruber, manuscript in preparation). Subsequent screens verified that expression of CLCA2 was consistently high on several cancer cell types, including squamous cell lung carcinoma, pancreatic carcinoma, esophagus carcinoma, whereas expression was low or undetectable in most normal tissues, such as heart, prostate, brain cortex, kidney, and others.

We have addressed the question of whether CLCA2 could be a useful immunogen using in vitro priming in combination with ELISPOT detection of reactive T cells. We have successfully generated reactive CD8$^+$ T lines from multiple donors against HLA-A2-restricted epitopes. These T cells also recognized allogeneic tumor cells in an Ag-specific and HLA-restricted fashion. Moreover, the most active CLCA2-derived epitope, LLGNCLPTV, was also recognized by CD8$^+$ T cells expanded independently from pancreatic carcinoma/T cell cocultures. These data identify CLCA2 as a potentially useful novel Ag for anti-tumor vaccination approaches.

Materials and Methods

Cell lines

APCs and CD8$^+$ cells were obtained from HLA-A*02011-positive healthy donors. Tumor cell lines MZ-PC-1, MZ-PC-2 (both pancreatic carcinomas; Ref. 17), MZ-LC-16 (lung carcinoma; a kind gift of Drs. S. Horn and W.

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[2] Abbreviations used in this paper: TAA, tumor-associated Ag; CLCA2, calcium-activated chloride channel 2; DC, dendritic cells; IVP, in vitro priming.

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Moersig, Department of Cardiothoracic and Vascular Surgery, University of Mainz, Mainz, Germany), SK29-MEL-1 (melanoma; Ref. 18), and COGA12 (colon cancer; a kind gift of Dr. E. Wagner, Boehringer Ingelheim Austria, Vienna, Austria) were maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mmol/L L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. All cell culture reagents were purchased from Life Technologies (Gaithersburg, MD).

Peptides

Peptides were synthesized using automated solid phase techniques, purified by reversed phase-HPLC and their structures were verified by mass spectrometry. In addition to the peptides listed in Table I, the HLA-A2 binding peptide YMDGTMSQV (tyrosinase 369–377; Ref. 18) and the HLA-A1 binding peptide EADPTGHSY (MAGE-1 161–169; Ref. 3) were tested. Peptides were obtained in lyophilized form, dissolved in 100% DMSO at 40 mg/ml and a stock solution of 2 mg/ml was prepared by further dilution in PBS. The first four letters of the peptide sequences are used as shorthand identification throughout the manuscript.

Ag-presenting cells

For the generation of dendritic cells (DCs; Refs. 19 and 20), PBMCs were isolated by density gradient centrifugation on Lymphoprep gradients (Nycomed, Oslo, Norway) and washed four times with Dulbecco's buffered saline. $CD8^+$ T cells were isolated using positive immunomagnetic MicroBead selection (Miltenyi Biotec, Bergisch Gladbach, Germany) and cryopreserved until used. The remaining cells were resuspended in AIM-V (Life Technologies) supplemented with 1% autologous plasma at a density of 5×10^6/ml and incubated in 6-well plates (Costar, Cambridge, MA; 3 ml each well) for 60 min (37°C, 5% CO_2). After complete removal of the supernatant, the adherent cells were cultured in AIM-V supplemented with 1% autologous plasma, 1000 U/ml human rIL-4 and 1000 U/ml human rGM-CSF (BD PharMingen, San Diego, CA) in 6-well plates. On days 3 and 5, one-third of the supernatant was replaced by fresh DC medium. On day 6, part of the nonadherent cells showing an immature phenotype in flow cytometric analysis (lack of CD83 expression) was rinsed off and cryopreserved. The adherent cells were resuspended in AIM-V supplemented with 1% autologous plasma, 10 ng/ml IL-1β (BD PharMingen), 10 ng/ml IL-6 (BD PharMingen), 10 ng/ml TNF-α (Boehringer Ingelheim Austria), and 1 μg/ml PGE_2 (Sigma-Aldrich, St. Louis, MO) at a density of 1×10^6 cells/ml. On day 9, DCs of mature phenotype (positive for CD83) were harvested and cryopreserved.

In vitro priming (IVP)

DCs from day 9 were thawed, loaded with various peptides at 100 μg/ml for 4 h at 37°C, and irradiated at 3300 rad. Cells were washed and resuspended in AIM-V supplemented with 5% AB serum (PAA Laboratories, Parker Ford, PA), 10 ng/ml IL-6, and 10 ng/ml IL-12 (both obtained from BD PharMingen) at a density of 1×10^5/ml. For each peptide, 3×10^6 cryopreserved $CD8^+$ cells were added to 1×10^5 pulsed, irradiated DCs per well in a 24-well plate. After 7 days, $CD8^+$ (1×10^6/well) cells were restimulated with irradiated peptide-pulsed DCs and cultured in AIM-V supplemented with 5% AB serum, 100 U/ml IL-2 (Proleukin; Chiron, Emeryville, CA) and 10 ng/ml IL-7 (BD PharMingen; Ref. 21).

ELISPOT assay for IFN-γ

ELISPOT assays were performed as previously described (22) using capture mAb anti-human IFN-γ (1-D1K; Mabtech, Stockholm, Sweden) and detection biotinylated mAb anti-human IFN-γ (7-B6-1; Mabtech). Ten days after the last stimulation, 1×10^4 IVP-$CD8^+$ cells were incubated with 75,000 nonirradiated peptide-pulsed T2 stimulator cells. Control wells contained IVP-$CD8^+$ cells alone or IVP-$CD8^+$ cells in the presence of unloaded T2 cells. For HLA Ab-blocking experiments, tumor cell lines were preincubated with either W6/32 (anti-HLA-I; Ref. 23) or MA2.1 (anti-HLA-A2/-B17; Ref. 24) or B1.23.2 (anti-HLA-A/B; Ref. 25); a kind gift from Dr. F. Lemonnier (Institut Pasteur, Paris, France) at 0.17–0.3 mg/ml for 1 h at 4°C, and then used as stimulator cells in the ELISPOT assay. For transfection experiments, transiently transfected 293-EBNA cells (Invitrogen, San Diego, CA) were used as stimulators. Spot numbers were automatically determined with the use of a computer-assisted video image analyzer (Zeiss-Kontron, Jena, Germany).

Cytotoxicity assays

Cultured IVP-$CD8^+$ T cells were tested for cytotoxic activity in a 2 h-europium release assay. Target cells (peptide pulsed DCs or T2 cells and tumor cells) were labeled according to the manufacturer's recommendation. Briefly, 2×10^6 targets were incubated with fluorescence-enhancing ligand (BATDA; Wallac Oy, Turku, Finland) at 37°C, extensively washed with PBS and used at different E:T ratios (5000 targets/well). After 2-h incubation time at 37°C, supernatants were mixed with europium solution (Wallac Oy), and incubated at room temperature for 15 min. Finally, fluorescence was measured in a time-resolved fluorometer (1420 Victor; Wallac Oy). Percentage of specific release was calculated as follows: ((mean experimental release − mean spontaneous release)/(mean maximum release − mean spontaneous release)) × 100, where spontaneous release represents counts in supernatants from wells containing target cells in medium only, and maximum release represents counts in supernatants from wells containing target cells in medium supplemented with 1% NP40.

Table I. *CLCA2-derived peptides selected for HLA-A2 binding analysis*

Peptide	Position	Parker Score	Tübingen Score	Motifator Score	T2 Binding	Reactive $CD8^+$ Obtained
CNLKFVTLL	11		17	44	−	
IMFMQSLSSV	240	726.7	27	36	++	0/1
FMQSLSSVV	242	94.7	20	54	−	
NLQNQMCSL	264	49.1	22	45	−	
SLVQAGDKV	303	34.0	24	41	−	0/1
LVQAGDKVV	304		17	60	−	
KMAEADRLL	321	44.2	23	35	−	
LQQAAEFYL	331		12	20	−	0/1
QIVEIHTFV	341	215.6	20	26	+	0/1
EIRAQLHQI	359		18	44	−	
LLVSYLPTTV	375	118.2	24	43	−	
KGFEVVEKL	398		20	37	−	
LVTSGDDKL	417		19	48	−	
KLLGNCLPTV	424	2071.6	20	52	+++	6/6
LLGNCLPTV	425	271.9	29	28	+++	6/6
ELSRLTGGL	455		21	42	−	
QLESTGENV	496		19	35	−	
SLQALKVTV	585	69.5	27	45	++	4/6
ILNATVTATV	635	118.2	22	17	+	
LLDDGAGADV	656	193.9	25	34	−	
QMNAPRKSV	722	50.2	21	37	−	
KIIDLEAVKV	768	137.2	26	2	−	
IIDLEAVKV	769		24	3	−	
NIAQAPLFI	880		19	39	−	
GVLTAMGLI	905		15	43	−	

Plasmids and transfection

In the mammalian expression vector pVAX1 (Invitrogen), first, a pair of oligonucleotides was inserted that encode the adenovirus E3 19k-derived insertion sequence (26). A second pair of complementary oligonucleotides encoding for the epitopes was then inserted downstream of the E3 sequence. The recombinant vectors encode for the following products: IC NLKFVTLL (vector pE3_ICNL); KLLGNCLPTV (pE3_K/LLGN); SLQALKVTV (pE3_SLQA). The plasmids pCDNA3_HLAA2 and pCDNA3_tyrosinase have been described elsewhere (18). The FuGENE 6 lipid transfection reagent (Roche Diagnostic Systems, Somerville, NJ) was used at DNA-lipid compound ratios and incubation conditions suggested by the manufacturer. Briefly, 293-EBNA cells were seeded in 6-well plates the day before transfection; on the next day, cells were washed free of serum and the DNA-liposomal complex was added in RPMI 1640 without serum. After a 4-h incubation, FCS was added to a final concentration of 10%.

Results

*Selection of high-affinity HLA-A*0201-binding peptides from the CLCA2 Ag*

Three different computer programs were used to identify potential HLA-A*02011-binding epitopes from the 943-aa long reading frame of CLCA2. Two of them, available via Internet from the National Center for Biotechnology Information (http://bimas.dcrt.nih.gov/molbio/hla_bind/) and from the University of Tübingen (http://www.uni-tuebingen.de/uni/kxi/) (27, 28), predict binding affinity and stability based on matrices derived from known epitopes. These were complemented by our own approach, in which the context in which a certain epitope was embedded was closely analyzed. Whether a certain epitope was likely to be processed and displayed or not was predicted. This forecast was then given more weight when peptides were selected. Twenty-five peptides listed in Table I were thus selected; some of them were qualified as promising candidates from all three prediction methods, but also others favored by only one algorithm were included. Peptides were synthesized and tested for their binding activities toward the restricting *HLA-A*02011* allele by a T2 stabilization assay (29). The tyrosinase-derived peptide YMDGTMSQV served as positive control and the HLA-A1-binding peptide EADPTGHSY (MAGE-1) served as negative control. Four CLCA2 peptides showed concentration-dependent stabilization (Fig. 1): IMFM (position 240), KLLG (424), LLGN (425), and SLQA (585). The peptides ILNA (635) and QIVE (341) had minimally promoted HLA-A2 stabilization and were thus qualified as weak binders (Table I).

In vitro priming with high-affinity peptides resulted in specific CD8$^+$ T cell lines

Published data suggest a direct relationship between the HLA binding affinity of a peptide and its immunogenicity (30). Thus only peptides that bound above background to HLA-A*02011 in the T2 stabilization assay were tested in in vitro priming assays. CD8$^+$ T cells were magnetically purified from healthy donors and cocultures with in vitro-maturated peptide-loaded autologous DCs were set up. Only three of seven of the CLCA2-derived peptides, KLLGN, LLGN, and SLQA, initiated expansions of CD8$^+$T cells in these initial cultures (Table I, *last column*). Most notably, two of the effective peptides, LLGN and KLLG, displayed the highest binding affinity. On day 10 after the third restimulation, the specificity of IVP responder T cells was tested in ELISPOT assays (Fig. 2). In vitro priming using the peptides KLLGN, LLGN, and SLQA along with the positive control peptide YMDG was then repeated with cells obtained from several other healthy donors, and tested in a similar way on a broad panel of target peptides (Fig. 3). Although there were considerable differences among donors, we were able to expand LLGN- and KLLG-specific CD8$^+$ T cells without exception in all cases. Moreover, these LLGN- and KLLG-specific cells were cross-reacting (Fig. 3, two *upper rows*). Reactivity of CD8$^+$ T cells primed with SLQA was less apparent, but their specificity was still significant. Raising YMDG-specific CD8$^+$ T cells was successful in three of four donors (Fig. 3).

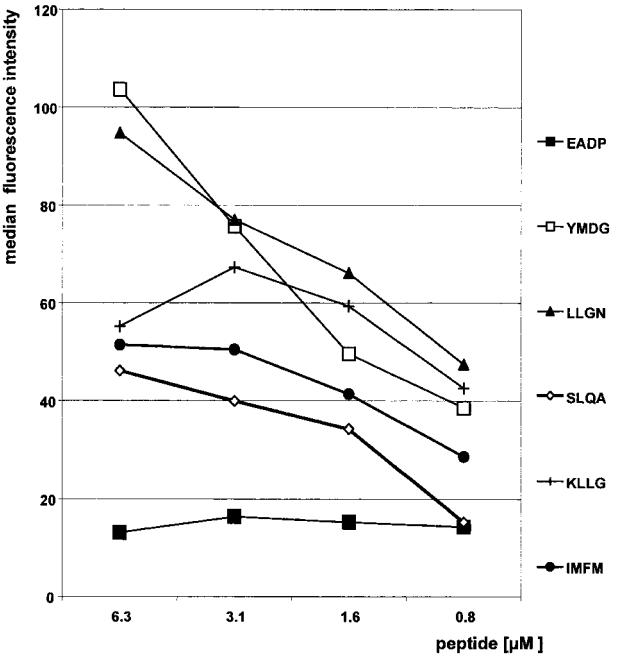

FIGURE 1. Stabilization of HLA-A2 on the surface of T2 cells by peptides. T2 cells were loaded with peptides at concentrations indicated on the *x*-axis overnight in serum-free medium. HLA-A2 expression was determined by FACS staining with the mAb PA2.1, and the median fluorescence intensity was calculated.

Titration of the relevant peptide epitopes confirmed specificity of the cultivated cell lines. T2 cells were coated with various peptide concentrations, starting with 100 μM and followed by 10-fold dilutions, and tested in ELISPOT with the corresponding T cell lines. Although titration differed between peptides and CD8$^+$ T cell lines, in all cases the reactivity could be saturated, and could be diluted down to nearly background values (Fig. 4). Thus, reactivity is dependent on the formation of the correct (peptide + HLA) complex.

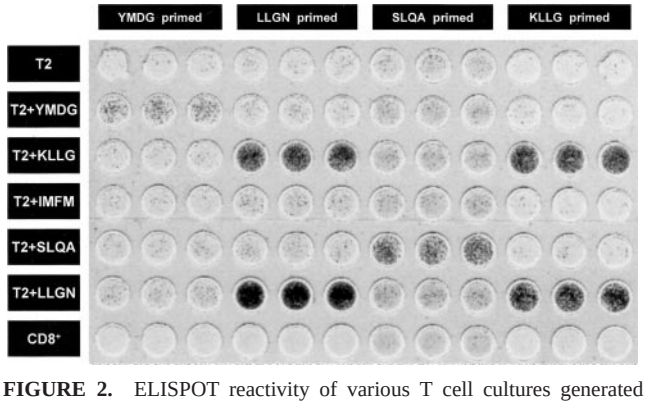

FIGURE 2. ELISPOT reactivity of various T cell cultures generated from a single donor. After three rounds of stimulation, 10,000 T cells from donor 2 that were primed with the peptides YMDG, LLGN, SLQA, and KLLG were tested (in triplicate columns) against T2 cells loaded with different peptides (in rows). Unloaded T2 cells (*first row*) and CD8$^+$ T cells (*seventh row*) served as negative controls.

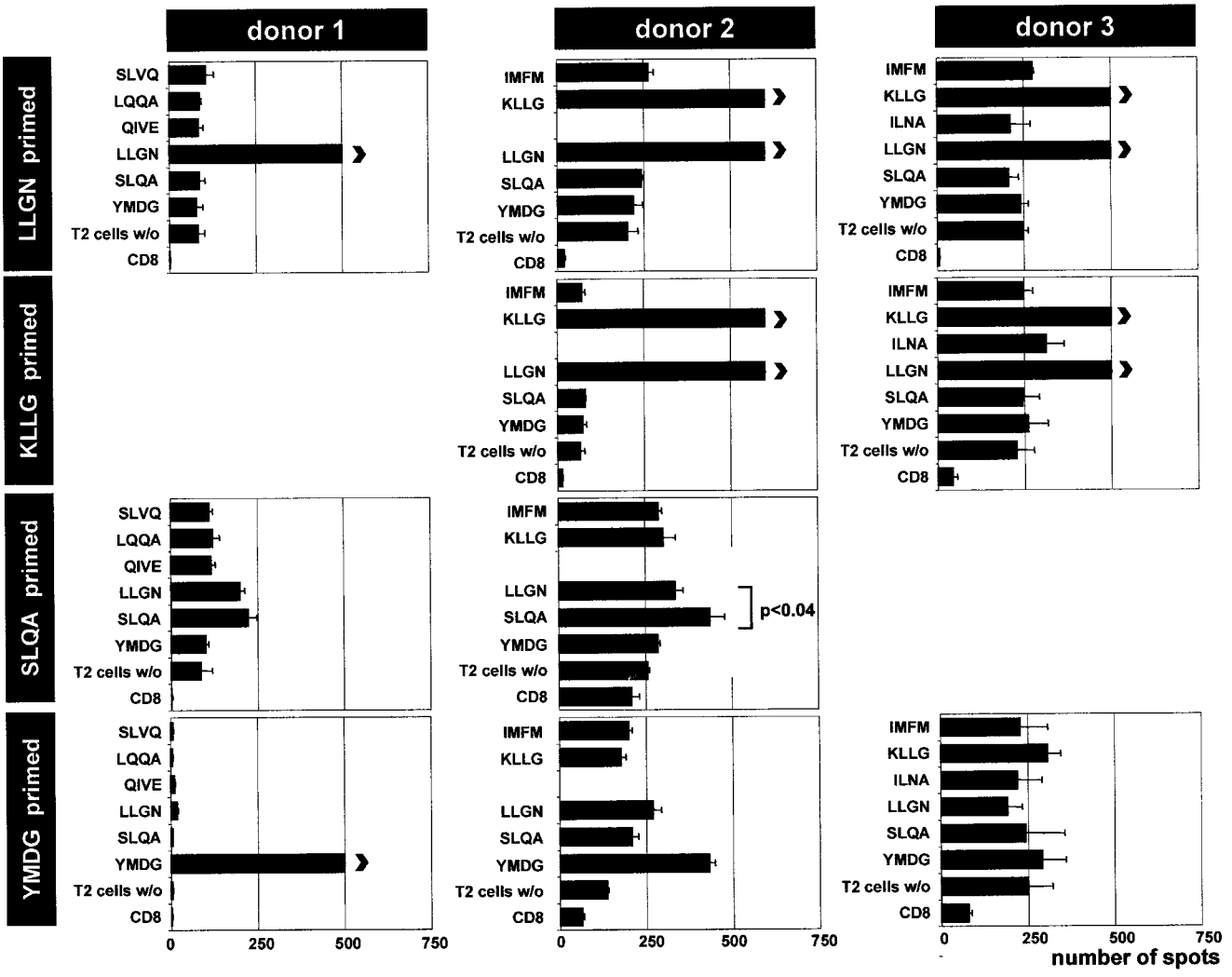

FIGURE 3. ELISPOT reactivity of in vitro-primed T cells. T cells from three different donors (each donor shown in a separate column) were stimulated three times with LLGN, KLLG, SLQA, and YMDG (each shown in separate rows). Ten-thousand cells were then tested against T2 cells loaded with various peptides. Spots determined from triplicate cultures are shown as bars; the $>$ symbol indicates that the number of spots exceeded the top counting limit. For the SLQA-stimulated cells of donor 2, $p < 0.04$ reflects a significant difference between recognition of SLQA- and LLGN-loaded targets.

T2 cells that were loaded with the three different peptides at the optimum 10 μM concentration were also tested as stimulators in the presence of blocking mAbs (Fig. 5). The pan-anti-class I mAb W6/32 decreased spot numbers down to background. The HLA-A2-specific mAb MA2.1 blocked recognition of SLQA and KLLG-primed cells completely, and of LLGN-primed cells substantially. These results indicated that the in vitro-primed effector T cells were class I HLA-A2-restricted.

To determine whether endogenously translated and processed products are also recognized by the reactive $CD8^+$ T cells, cotransfection experiments were performed. Unfortunately, vector-mediated overexpression of full-length CACL2 protein appeared to be toxic (data not shown). Also larger fragments which necessarily have one or more hydrophobic membrane-spanning domains were not expressed at detectable levels after transfection. We have thus generated a set of vectors that direct the expression of small CACL2-fragments fused to the adenoviral E3 insertion sequence. These vectors were cotransfected with the pCDNA3_HLA-A2 plasmid into 293-EBNA cells. Transfectants were then tested as stimulators in ELISPOT. SLQA-primed effectors recognized exclusively the product of the pE3_SLQA plasmid (Fig. 6*a*), whereas LLGN-primed effectors recognized only the product of the pE3_K/LLGN vector (Fig. 6*b*).

We have also examined whether ex vivo $CD8^+$ T lymphocytes isolated from peripheral blood of healthy HLA-A2 positive donors could recognize CLCA2 peptides. ELISPOT assays were performed using K562-A*02011 cells loaded with all the peptides listed in Table I as stimulators. Although the background was below 30 spots/100, 000 $CD8^+$ cells in all cases, and below 10 spots/100,000 $CD8^+$ cells in four of seven tests, peptide-specific $CD8^+$ T cells were not detected ex vivo in any of the healthy donors (data not shown).

CLCA2-specific $CD8^+$ T cells recognize allogeneic tumor cell lines

Next, we examined whether peptide-specific T cell lines could recognize selected tumor cell lines. K562 cells expressed HLA-A*02011 after stable transfection. SK29-MEL-1 and MZ-PC-2 carried the *HLA-A*02011* allele as determined by DNA sequencing. MZ-PC-1 and MZ-LC-16 were serologically HLA-A2-positive. HLA-A2 surface expression was verified by FACS staining. Expression of CLCA2 was verified by RT-PCR. These tumor cell lines were tested in ELISPOT assays for recognition. For the most productive T cell-allogeneic tumor interactions, specificity was tested by performing the assays in the presence of blocking mAbs specific for certain HLA alleles.

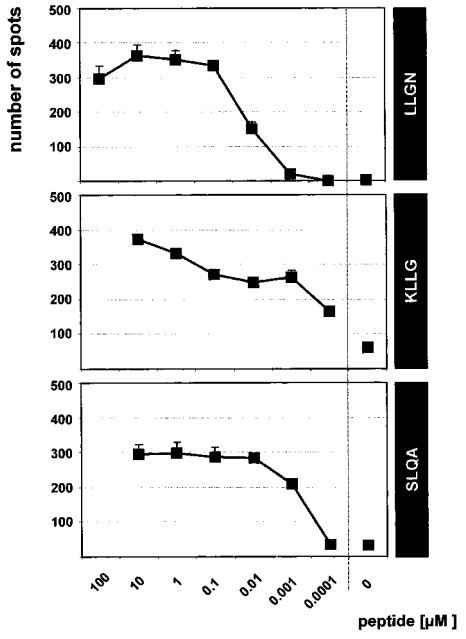

FIGURE 4. Titration of peptides with T2 cells. T2 cells were loaded with various concentrations of LLGN (*upper panel*), KLLG (*middle panel*), and SLQA (*lower panel*) peptides, as indicated on the *x*-axis (in micromoles). ELISPOT was performed in duplicates using 10,000 responder cells from donor 2.

We found that each T cell line can recognize one or more allogeneic tumor cell lines with varying efficiency (Fig. 7). Also each tumor cell line that expressed both HLA-A2 and CLCA2 was recognized by at least one T cell line. Stimulator cells, that lack one of the two such as K562-A*02011 (no CLCA2 expression) or

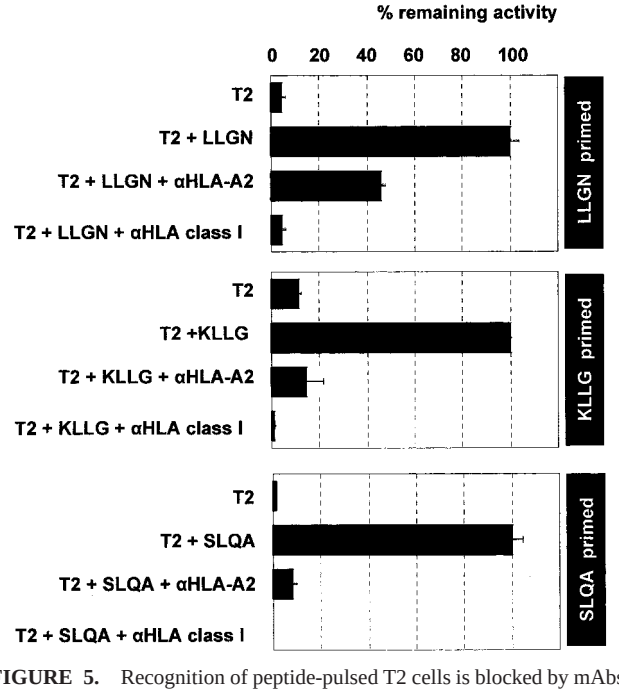

FIGURE 5. Recognition of peptide-pulsed T2 cells is blocked by mAbs. In vitro-primed T cells (3,000 cells/well) were stimulated with 75,000 T2 cells pulsed with three different peptides (LLGN, KLLG, SLQA). Where indicated, the anti-pan-HLA Ab W6/32 or the anti-HLA-A2 mAb MA2.1 was added. SLQA-primed cells were from donor 2, whereas LLGN- and KLLG-primed cells were from donor 4.

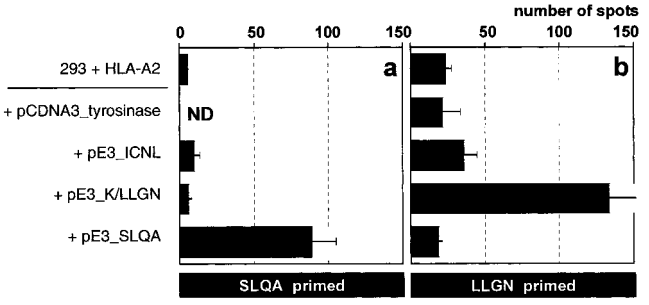

FIGURE 6. In vitro-primed T cells recognize cotransfectants. The plasmid pCDNA3_HLAA2 was transfected alone or in combination with either pE3_ICNL, pE3_K/LLGN, pE3_SLQA, or pCDNA3_tyrosinase plasmids into 293-EBNA cells. Transfectants (50, 000) and 10,000 primed $CD8^+$ T cells were cocultured. Production of IFN-γ was determined by overnight ELISPOT assay. Both SLQA- (*a*) and LLGN-(*b*) reactive cells were from donor 2.

COGA12 (HLA-A2-negative), induced only background reactivity. Recognition of the MZ-LC-16 lung carcinoma line by SLQA-specific T cells was blocked by an HLA-A2-specific mAb (striped bar, Fig. 7*a*) but not by an Ab directed against the *HLA B* and *C* alleles (data not shown). Blockade with HLA-A2 mAb was again only partial when LLGN-specific T cells were tested on the two pancreatic carcinoma lines MZ-PC-1 and MZ-PC-2 (striped bars, Fig. 7*c*). The pan-class I mAb W6/32 blocked recognition in all combinations (data not shown). These data indicated that tumor cells were recognized in an HLA-A2-restricted fashion.

Assessing specific lytic activity of the primed T cells is also helpful to establish the relevance of the selected epitope peptides. The data show that IVP-$CD8^+$ cells were highly lytic against autologous DCs, which were pulsed with the corresponding epitope peptides (Fig. 8). Highest reactivity was obtained by LLGN-primed T cells, and these cells were then also tested against peptide-pulsed T2 target cells and against the HLA-$A2^+$ allogeneic MZ-PC-2 and MZ-LC-16 tumor cells. LLGN-primed cells showed potent activity against pulsed T2 cells (Fig. 9). Their reactivity against the tumor cell lines was markedly lower, but lytic activity of the primed $CD8^+$ T cells consistently and reproducibly increased with increasing E:T ratios.

Epitope LLGN is a dominant Ag on the MZ-PC-2 pancreatic carcinoma line

In search of novel tumor Ags also other methods besides the genetic approach that yielded CLCA2 were tried. The MZ-PC-2 pancreatic carcinoma line had been stably transfected to express CD80. Cocultures of this cell line with completely or partially matched allogeneic $CD8^+$ T cells were then performed. Successfully expanded T cell cultures that originated from such experiments were then also tested for recognition of T2 cells loaded with peptides that were derived from various putative pancreas tumor Ag candidates. The tested peptides included predicted epitopes of CLCA2. Compared with the average spot number induced by these peptides, reactivity against LLGN was higher at a statistical significance of $p < 0.001$ (Fig. 10).

Discussion

Ideal tumor Ags are expected to be "nonself", being expressed exclusively on tumor cells, but not on normal tissues. Tumor Ags that belong to the viral unique mutant and cancer/testes Ag groups approximate this expectation well (2, 31). However, the majority of Ags discovered until now are self proteins with enhanced expression in tumors. Considering self proteins as targets inevitably

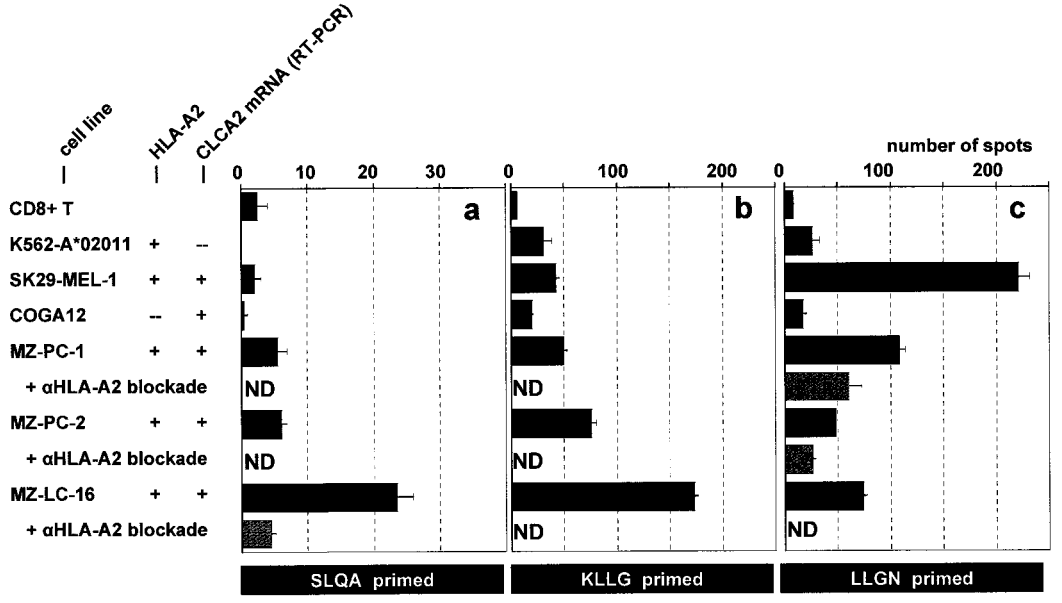

FIGURE 7. In vitro-primed T cells recognize allogeneic tumor cells. Three different T cell lines (10,000/assay condition) that were repeatedly stimulated with the peptides SLQA, KLLG, and LLGN were then tested against a panel of tumor cell lines. Expression of HLA-A2 was determined by FACS analysis and expression of CLCA2 at the mRNA level by RT-PCR. Recognition of some tumor cell by certain T cells was also tested in the presence of the blocking mAb MA2.1 (αHLA-A2; ▨).

raises two major issues: 1) is it possible to provoke an effective immune response against such an Ag, asking whether tolerance or clonal deletion would prevent vaccination; and 2) is it safe to vaccinate against self proteins, raising questions about autoimmunity. With this study, we wanted to find an answer to the first question, and to determine whether that CLCA2 can be considered as a useful anti-tumor immunogen despite being a self-protein.

Using extensive genetic screening methods, we have previously identified a transcript, markedly overexpressed in a number of tumors, that was identical to the CLCA2. CLCA2 was predicted to contain five transmembrane domains with a large amino-terminal extracellular domain, and was found to function as a calcium-regulated outwardly rectifying anion channel. This molecule is also expressed in normal trachea and apparently in mammary gland epithelium (32), and qualifies thus as a "self" Ag. Furthermore, a mouse and a bovine homologue were implicated to play a role in lung metastasis formation (33). Although its relevance for malignant cells is unclear at this time, fast proliferation and altered environment of tumors may be connected to its higher expression in lung squamous cell carcinomas and tumors of the gastrointestinal tract (U. König and W. Sommergruber, manuscript in preparation).

The present study reports that the *CLCA2* gene encodes antigenic epitopes that can be recognized by $CD8^+$ T cells. From a pool of peptides that were selected by computer algorithms a small subset that bound to HLA-A2 was selected. Although binding affinity of peptides generally correlates well with their immunogenicity (30), melanoma-derived tumor Ag peptides often display

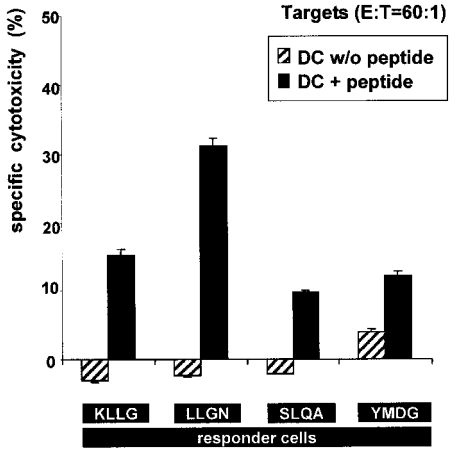

FIGURE 8. Cytotoxic activity of $CD8^+$ T cells generated by in vitro priming. Cells from donor 5 were primed with the peptides KLLG, LLGN, SLQA, and the tyrosinase-derived peptide YMDG. Target cells were native autologous DCs (▨) or the same autologous DCs pulsed with the respective peptides (■). Lytic activity was evaluated in a dissociation-enhanced lanthanide fluorescent immunoassay EuTDA cytotoxicity assay. Background lytic activity was <20% and was subtracted from the results.

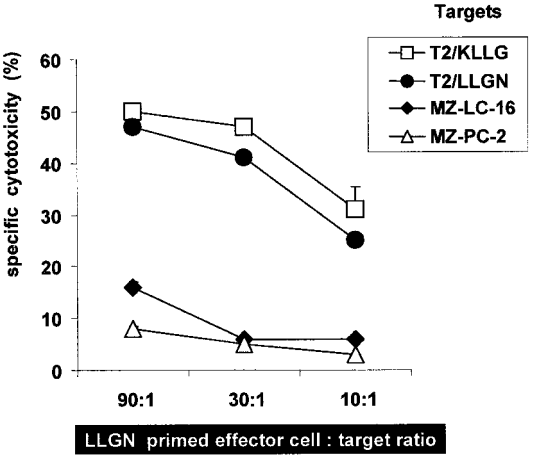

FIGURE 9. Peptide-pulsed T2 cells, MZ-LC-16 and MZ-PC-2, were labeled and used as targets in a dissociation-enhanced lanthanide fluorescent immunoassay EuTDA cytotoxicity assay. Effector cells were generated by in vitro priming with the peptide LLGN from donor 4.

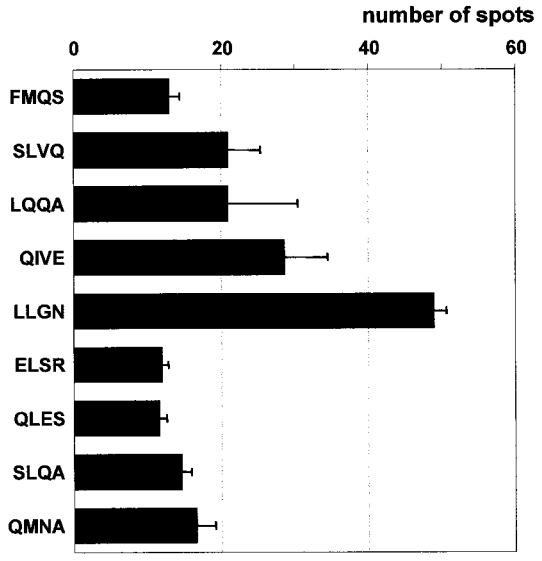

FIGURE 10. T cells primed with MZ-PC-2 cells recognize the LLGN epitope. $CD8^+$ T cells were expanded by repeated stimulation with the partially matched allogeneic MZ-PC-2 pancreas carcinoma cells. Effector cells (10,000 cells/well) were tested in ELISPOT assay with T2 cells coated with various CLCA2-derived peptides as APCs. Full sequences of peptides are listed in Table I.

only intermediate affinity (16, 34). We have taken the tyrosinase-derived YMDG peptide as a positive control throughout the experiments, because of its high binding affinity, and also because successful in vitro priming with this peptide has been demonstrated (35). Compared with YMDG, the KLLG and LLGN peptides qualify as high-affinity, and the SLQA epitope as medium-affinity, binders. In vitro stimulation with these peptides repeatedly induced expansion of specific $CD8^+$ T cells. In contrast to the KLLG, LLGN, and SLQA peptides, we were unable to raise specific T cells against the medium-affinity IMFQ epitope.

Peptide- and HLA-specificity of the expanded $CD8^+$ T cells was confirmed by titration and Ab-blocking experiments. This indicates that despite potential tolerance or clonal deletion concerns, the T cell repertoire available in healthy donors would not preclude immunization against CLCA2.

Specificity of T cells was further confirmed in experiments where stimulator cells were cotransfected with plasmids encoding for the CLCA2-derived epitopes in addition to HLA-A2-encoding plasmids. Plasmid-mediated expression of full-length CLCA2 was not successful. This can be explained either by its function as a chloride channel or by the complicated structure of the protein itself that includes multiple hydrophobic domains. Notably, the KLLG/LLGN epitopes are incorporated in such a hydrophobic putative transmembrane region. van der Bruggen et al. (36) have identified a MAGE-3 epitope that was recognized by T cells only when it was provided in a synthetic peptide form, but not as a product of natural processing in tumor cells (37). In sharp contrast to this case, our T cell lines also recognized various $HLA\text{-}A2^+$ and $CLCA2^+$ allogeneic tumor cell lines. This finding is important, because it could indicate that the corresponding epitopes might be processed and displayed on tumor cells. We also found that tumor cell lines are recognized by the different T cell lines with variable efficiency. For example, SK29-MEL-1 and MZ-PC-1 were better recognized by LLGN-specific T cells, while SLQA-primed T cells reacted best with the MZ-LC-16 lung carcinoma, although natural processing has not yet been demonstrated. The variability in the efficiency of recognition of the tumor cell lines is likely due to the different sensitivity of the various T cell lines. Low or intermediate avidity is not uncommon among in vitro primed and expanded T cells and may be a result of the employed IVP protocol (38, 39). Alternatively, variability in epitope processing and display among tumor cells may contribute to the differential recognition of tumor cells. Although HLA-A2 surface expression and CLCA2 expression were similar on the tested tumor cells, it is very likely that from the overlapping KLLG and LLGN epitopes only LLGN is produced. That LLGN, but not KLLG, is the naturally produced variant was also suggested by the fact that cells transfected with the vector that was encoding the common K/LLGN fragment were recognized by the LLGN-primed cells (Fig. 6), but only marginally provoked reactivity from KLLG-primed cells (data not shown). Differential reactivity was also seen with SK29-MEL-1 cells (Fig. 6): they were recognized by LLGN-, but not by KLLG-, primed T cells. However, after synthetic KLLG peptide was exogenously loaded onto the SK29-MEL-1 line, the cells turned successively into a potent target in a dose-dependent manner for KLLG-primed cells (data not shown).

The preeminent role of the CLCA2-derived peptide LLGN was also supported by results from a different approach: T cells from cocultures with the pancreatic carcinoma cell line MZ-PC-2 were found to specifically recognize this peptide. These data indicate that peptides derived from the CLCA2 protein are displayed in a form that can be recognized by T cells and thus CLCA2-expressing tumor cells can be targets of Ag-specific immunotherapy.

Precursors against CLCA2-derived epitopes could not be detected in ex vivo peripheral blood $CD8^+$ T cells isolated from healthy $HLA\text{-}A*0201^+$ donors. Specific T cell precursors against other tumor Ags, e.g. MAGE-3 and tyrosinase, are also generally rare, and only for MELAN-A were specific T cells readily found (40, 41). Furthermore, absence of tumor Ag-specific T cells in the peripheral blood, even if the Ag is a self-protein, does not necessarily reflect the complete absence of such cells. Indeed, in close spatial association with tumors among tumor-infiltrating lymphocytes, but also in lymph nodes, it has repeatedly been possible to identify T cells specific for self tumor Ags (6, 42).

Taken together, our data indicate that a T cell response can be evoked against the CLCA2 protein. Although our analysis did not include all possible HLA-A*0201-restricted peptides, nor was it extended to other histocompatibility alleles, one can assume that additional epitopes restricted by HLA class I or class II may be contained in CLCA2.

Identification of novel functional tumor Ags is a prerequisite for effective anti-tumor immunotherapies. As CLCA2 was found to be expressed on a broad variety of tumors, including gastrointestinal and lung cancers, results from this study may contribute to future vaccination protocols.

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