Naturally Occurring T-Cell Response against Mutated p21 Ras Oncoprotein in Pancreatic Cancer

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Abstract Mutated p21 ras proteins (muRas) are present in ~90% of pancreatic adenocarcinomas and express mutants which can function as cancer-specific antigens. To evaluate the frequency and magnitude of the natural T-cell response against muRas in 19 HLA-A2-positive patients with muRas-positive pancreatic carcinomas, antigen-experienced T lymphocytes in fresh peripheral blood mononuclear cells were shown by IFN-γ enzyme-linked immunospot using muRas peptides (5-21) that encompass both HLA class I (HLA-A2) – and class II – restricted (HLA-DRB1) epitopes. Six of 19 patients (32%) were found to have a specific T-cell response against individual mutation-specific ras_{5-21} but not against other ras mutations or wild-type ras. In contrast, none of 19 healthy subjects had T cells specifically secreting IFN-γ ($P = 0.004$). The T-cell response consisted of both $CD8^+$ and $CD4^+$ T cells but was dominated by CD8 T cells in three of four patients. $MuRas_{5-14}$ and $muRas_{6-14}$ were shown to specifically induce $CD8^+$ T-cell mediated cytotoxicity against HLA-A2-positive, muRas-bearing pancreatic carcinoma cells. The T-cell response was not correlated with prognostic or clinical variables such as tumor-node-metastasis status, stage, or survival. In conclusion, a natural T-cell response against muRas proteins that could be exploited for immunostimulatory therapeutic approaches has been shown in a significant proportion of patients with pancreatic cancer.

The *ras* proto-oncogenes (H-*ras*, K-*ras*, and N-*ras*) encode 21-kDa proteins with intrinsic GTPase activity that are involved in cell proliferation and differentiation. Point mutations in the *ras* proto-oncogenes have been identified in a wide range of human solid tumors, in particular carcinomas of the pancreas, colon, lung, and thyroid as well as myeloid malignancies. The majority of point mutations of the *ras* gene reside at codons 12, 13, and 61 (1). These mutations result in the production of aberrant proteins, which are structurally and functionally distinct from normal endogenous ras and, due to their strict tumor specificity, can function as targets for cellular and humoral immune responses in cancer patients (1–5) and healthy donors (6–8).

In pancreatic adenocarcinomas where *ras* point mutations occur in 80% to 90% of cases, it is the K-*ras* gene at codon 12 that is found frequently mutated. Replacement of the normal glycine (Gly) residue by an aspartatic acid (Asp), valine (Val), cysteine (Cys), or arginine (Arg) residue accounts for ~98% of all *ras* mutations in pancreatic carcinomas, rendering pancreatic cancer an ideal model for the investigations of T-cell responses against *ras* mutations at position 12. Previous studies have shown that $CD4^+$ T-cell immunity to position 12 mutated Ras (muRas) can be detected in the majority of patients with pancreatic cancer (3, 5). The proof of this T-cell response and the early presence of muRas in the tumoral process have prompted the implementation of clinical trials aimed at inducing CTL responses against defined muRas antigens (9–11). In these studies, immunizations with peptides of 13 to 17 amino acids in length were able to induce $CD4^+$ and $CD8^+$ T lymphocytes specific for muRas. The latter were shown to be cytotoxic against autologous tumor cells or tumor cell lines (12–15).

However, the demonstration of these muRas-specific T cells required the expansion of the T cells *in vitro* by repeated stimulation with antigen and interleukin 2, which may result in quantitative and qualitative changes (5, 12–15), and the observed responses were not quantified (3). However, the recent development of accurate methods for assessment of T-cell responses, such as enzyme-linked immunospot (ELISPOT) or IFN-γ fluorescence-activated cell sorting (FACS) analysis, allows for a more sensitive and more specific investigation of the naturally occurring T-cell response against tumor antigens (16, 17). The use of these methods should contribute to a better understanding of the occurrence and function of both $CD4^+$ and $CD8^+$ T-cell responses against muRas. Moreover, with respect to the development and application of therapeutic vaccine strategies using muRas as the target antigen, a method for immunomonitoring is warranted, which allows for the assessment of quantitative changes in muRas-specific T cells.

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Against this background, the objectives of this study were (*a*) to quantify and analyze the naturally occurring $CD4^+/CD8^+$ T-cell response against muRas in HLA-A2 pancreatic carcinoma patients and healthy donors directly *ex vivo*; (*b*) to compare the magnitude and quality of the observed $CD8^+$ T-cell response against muRas peptides with the T-cell response against selected virus peptides; and (*c*) to establish a method adapted for immunomonitoring of muRas cancer vaccines.

Materials and Methods

Peripheral blood mononuclear cells and tissue from patients and healthy donors. This study was approved by the local ethical review board ("Ethikkommission der Ärztekammer des Saarlandes") and done according to the Declaration of Helsinki. After informed consent, tumor tissue and peripheral blood mononuclear cells were obtained from 19 patients with histologically proven adenocarcinoma of the pancreas. Peripheral blood mononuclear cells were isolated by density gradient centrifugation using Ficoll-Paque Plus (Pharmacia Biotech, Freiburg, Germany). Pancreatico-duodenoectomy (Whipple operation) or pancreatectomy was done at the University of Saarland Medical School (Homburg, Germany) between January 1997 and November 2000. All patients were typed HLA-A2 positive and their tumors were shown by PCR to bear one of the three most common K-*ras* mutations at amino acid position 12 (valine, aspartatic acid, or arginine replacing glycine). Tumor stage and grading were determined according to the International Union against Cancer (UICC) classification (18). Peripheral blood mononuclear cell samples were also collected from HLA-A2-positive healthy donors who served as controls.

Human leukocyte antigen typing. HLA-A2 expression was shown using the antibody BB7.2 (immunoglobulin G2b; kindly provided by Dr. S. Stevanovic, Institute of Cell Biology, Tübingen, Germany). For indirect staining, dichlorotriazinylaminofluorescein-conjugated goat anti-mouse immunoglobulin G $F(ab)_2$ antibody (Dianova, Hamburg, Germany) was employed and samples were measured by FACScan (Becton Dickinson, Heidelberg, Germany).

DNA extraction. For K-*ras* mutation analysis DNA was extracted from formalin-fixed, paraffin-embedded tissue specimens using the QIAMP Tissue Kit (Qiagen, Hilden, Germany). Sections of 5×20 μm were transferred into a sterile 1.5-mL tube. Samples were taken from areas involved by the neoplasm as shown by staining of adjacent sections. Paraffin was removed by extracting twice with 1-mL xylene and twice with 1-mL absolute ethanol, followed by drying at 37°C for 10 minutes. The samples were digested with proteinase K at a concentration of 1.8 mg/mL at 55°C for 3 to 16 hours and loaded on a spin column afterwards. DNA was then absorbed by short centrifugation onto the QUIAMP silica membrane, washed twice, eluted with water, and stored at 4°C in Tris-EDTA buffer (19).

PCR and sequencing. Special precautions according to the standard operation procedures of the laboratory (available on request) were taken to avoid contamination, including separate handling of PCR reagents and products in different rooms and pipetting PCR reactions under a laminar air flow hood with filtered tips. Appropriate controls [PaTu cell line (mutation GGT to GTT at codon 12), Jurkat cell line (wild-type control at codon 12), and H_2O for PCR contamination] were included in all reactions. Ten microliters of the proteinase-digested DNA from pancreatic carcinoma biopsies served as a template for PCR. PCR, enrichment PCR, and evaluation of their results were done as previously described (20, 21) based on the method of Kahn et al. (22) with minor modifications (20).

Synthetic peptides. The peptides were commercially synthesized (Neosystem, Strasbourg, France). The degree of purity was >95% by reverse-phase high-performance liquid chromatography and their identity was confirmed by mass spectrometric analysis. The peptide sequences derived from muRas are shown in Table 1. To evaluate the immune response against viruses, the following peptides were used: $pp65_{495\text{-}503}$ (NLVPMVATV) derived from cytomegalovirus (23), influenza matrix protein ($IMP_{58\text{-}66}$; GILGFVFTL) derived from the influenza virus (24), $Pol_{476\text{-}484}$ (ILKEPVHGV) derived from the human immunodeficiency virus (25), and $LMP2_{426\text{-}434}$ (CLGGLLTMV; ref. 26) or $BMLF\text{-}1_{280\text{-}288}$ (GLCTLVAML; ref. 27) derived from Epstein-Barr virus.

IFN-γ ELISPOT. IFN-γ ELISPOT tests were done in 96-well multiscreen filtration plates (MAHAN 4550, Millipore, Bedford, MA) as described earlier (28). The number of spots was counted by a computer-assisted video image analyzer (Biosys, Karben, Germany). To determine the frequency of antigen-specific precursor-CTL in peripheral blood mononuclear cells of patients, a total of 2.4×10^6 peripheral blood mononuclear cells per patient were plated into 12 wells (i.e., 2×10^5 peripheral blood mononuclear cells per well) for each peptide of interest. Peripheral blood mononuclear cells simultaneously functioned as responder and stimulator cells in the respective well. The peptide of interest was added at 10 μg/mL of RPMI-AB supplemented with 100 units/mL interleukin 2. Frequencies of antigen-specific IFN-γ-secreting cells were calculated based on the number of spots per well after subtraction of the background (mean value from 12 wells). Background was obtained by culturing the responder cells with unloaded stimulator cells.

Isolation of $CD4^+$ and $CD8^+$ T lymphocytes. To obtain T-cell subpopulations for ELISPOT assays, peripheral blood mononuclear cells were treated with CD4 and CD8 antibody–coated magnetic microbeads (Dynal, Oslo, Norway), respectively, according to the recommendations of the manufacturer. Negative selection resulted in $CD4^+$ or $CD8^+$ cell suspensions with a purity of >95% as determined by FACS analysis. Negatively selected peripheral blood mononuclear cells (2×10^5) were plated per well and a total of 2.4×10^6 negatively selected peripheral blood mononuclear cells per patient were plated into 12 wells for each peptide of interest.

Intracellular FACS analysis. Antigen stimulations with muRas, wild-type ras, or viral peptides were done in peripheral blood mononuclear cells essentially as described for cytomegalovirus-specific T cells (29). Briefly, 2×10^6 nonirradiated peripheral blood mononuclear cells were stimulated with 4 μg of the relevant peptide in the presence of 1 μg/mL anti-CD28 and 1 μg/mL anti-CD49d (clones L293 and L25.3; Becton Dickinson) and were immunostained with anti-CD4 or anti-CD8 (clones SK3 or SK1, peridinin chlorophyll protein; Becton Dickinson), anti-CD69 (clone L78, phycoerythrin; Becton Dickinson), and IFN-γ (clone 6402.3, fluorescin isothiocyanate; Becton Dickinson; ref. 29). At least 10,000 $CD4^+$ or $CD8^+$ lymphocytes were analyzed by FACScan (Becton Dickinson) and the percentage of specific T cells was calculated by subtraction of the frequency obtained by the respective control stimulations.

In vitro *generation of CTL.* $CD4^+$-depleted peripheral blood mononuclear cells were plated at 2.5×10^6 per well in 24-well plates (Nunc, Wiesbaden, Germany) and were stimulated by autologous,

Table 1. Ras peptide sequences to detect T-cell responses against muRas epitopes

Type of Ras	Length (mer)	Sequence*
Ras wild-type (Gly12) 5-21	17	KLVVVGA**G**GVGKSALTI
Mutated Ras (Val12) 5-21	17	KLVVVGA**V**GVGKSALTI
Mutated Ras (Val12) 5-14	10	KLVVVGA**V**GV
Mutated Ras (Val12) 6-14	9	LVVVGA**V**GV
Mutated Ras (Asp12) 5-21	17	KLVVVGA**D**GVGKSALTI
Mutated Ras (Asp12) 6-14	9	LVVVGA**D**GV
Mutated Ras (Arg12) 5-21	17	KLVVVGA**R**GVGKSALTI
Mutated Ras (Arg12) 6-14	9	LVVVGA**R**GV

*Bold-faced letters indicate mutated amino acids as compared with the wild-type sequence.

irradiated (30 Gy), muRas$_{5-14}$-Val12 or muRas$_{6-14}$-Val12 peptide–loaded peripheral blood mononuclear cells (stimulator cells; 2.5×10^6) every week in the presence of 20 units/mL interleukin 2. Stimulator cells were incubated for 2 hours at 37°C in serum-free medium (X-VIVO 15, BioWhittaker Europe, Verviers, Belgium) with 10 μg/mL muRas$_{5-14}$-Val12 or muRas$_{6-14}$-Val12 peptide. After 4 weeks, resulting T-cell cultures were tested for their cytotoxic activity against different targets in varying effector/target ratios (1:5, 1:20, and 1:80): T2 cells (174 × CEM.T2 hybridoma, HLA-A2$^+$, TAP1 and TAP2 deficient) not loaded or loaded (10 μg/mL) with muRas$_{5-14}$-Val12 or muRas$_{6-14}$-Val12 peptide (T2 muRas$_{5-14}$ or T2 muRas$_{6-14}$) or control peptide (T2 HIV-pol). T2 cells without adding effector cells were used as another negative control. Reactivity of CTL lines was also tested against muRas-Val12–bearing pancreatic or colon cancer cell lines (Patu or SW480) positive or negative for HLA-A2 (S8988; kindly provided by B. Simon, Philipps University, Marburg, Germany). To block this reactivity, anti-HLA-A2 monoclonal antibody BB7.2 (kindly provided by S. Stevanovic, Institute of Cell Biology, Department of Immunology, Tuebingen, Germany) was added.

Cytotoxicity assay. As described earlier (28), T-cell cultures were tested for their specific cytotoxicity in a DNA fragmentation assay (30) with [^{3}H]thymidine-labeled T2 cells or tumor cell lines as target cells. Each experiment was carried out at least in triplicate. Cytotoxicity was calculated as % specific killing = 100 − [(spontaneous retained DNA − experimental retained DNA) / spontaneous retained DNA] × 100. Spontaneous release was always <20% of the maximum release. For some target cells (tumor cell lines), standard chromium release assay was done alternatively (30). For this assay, 5×10^5 target cells were labeled with [^{51}Cr]-sodium chromate for 1.5 hours at 37°C and washed thrice. [^{51}Cr]-sodium chromate–labeled target cells (2×10^4) were then added to varying numbers of effector cells in V-bottomed microwells. After incubation for 4 hours at 37°C, plates were centrifugated. Fifty microliters of supernatant per well were harvested and added to 200 μL scintillation solution OptiPhase "Supermix" in PET-G-plastic 96-well plates (Perkin-Elmer Wallac, Freiburg, Germany). After shaking the plates for 5 minutes, they were read with a scintillation gamma counter (1450 MicroBeta Trilux, Perkin-Elmer Wallac). Cytotoxicity was calculated as % specific killing = (experimental release − spontaneous lysis / maximum release − spontaneous release) × 100. Spontaneous release was always <10% of the maximum release.

Statistical analysis. SE values are presented as error bars in graphs. The comparison between precursor T-cell frequencies in patients and healthy donors was drawn by the Mann-Whitney *U* test. Cross tables were tested by χ^2 or Fisher's exact test where appropriate. Concerning the latter tests, a cutoff of six spots per well was applied to define a positive T-cell response in ELISPOT assays. Kaplan-Meier survival curves were calculated and the observed differences in survival were compared by log-rank test. $P < 0.05$ was considered significant.

Results

Diversity of p21 ras mutations in pancreatic adenocarcinoma. A prerequisite for an effective immune response against mutated p21 ras proteins in a cancer patient is the presence of the respective ras mutation in the tumor cells. Therefore, we identified 35 patients with K-*ras* codon 12 mutations in their primary tumor for further analysis of their immune response against muRas. Eighteen of 35 muRas-positive pancreatic adenocarcinomas showed amino acid substitution replacing glycine with valine (51%). Thirteen showed aspartic acid in amino acid position 12 (37%), three arginine in position 12 (9%), and one alanine in position 12 (3%). The determination of the HLA-A2 status, which is present in ~50% of the Caucasian population, was usually done before the analysis of ras mutations. Nineteen of 32 patients with muRas-positive pancreatic adenocarcinoma were shown to carry the HLA-A2 phenotype and were subjected to analysis of T-cell response against HLA-A2-restricted peptides. In the remaining 13 cases, the HLA-A2 analysis was done retrospectively and was negative. No association was observed between the presence of the ras mutation in the tumor sample and the HLA-A2 phenotype of the individual.

Study population. The clinical characteristics of the 19 patients whose T-cell response was measured are given in Table 2. The median age was 61 years and the ratio of women to men was 1:1.37. Nine of 19 patients had stage III or VI of pancreatic cancer, 8 of 19 had stage II (=pT3N0M0), and only 2 had stage I disease according to the UICC classification (18). The primary tumor was resectable in 13 of 19 cases.

Naturally occurring T-cell response against muRas$_{5-21}$ peptides. To determine the naturally occurring T-cell response against mutated p21 ras oncoprotein, peripheral blood mononuclear cells were directly analyzed using IFN-γ ELISPOT assay without prior *in vitro* stimulation. To detect circulating antigen-specific T cells, we induced IFN-γ secretion by 17-mer muRas peptides containing the ras mutation present in the respective primary tumor of the patient. These 17-mer muRas$_{5-21}$ peptides comprised binding motifs for both MHC class I as well as MHC class II molecules (see Table 1) and were chosen based on peptide epitope prediction software SYFPEITHI (31). Following this approach, we found 6 of 19 patients (32%) mounting a specific T-cell response against muRas$_{5-21}$ peptides, with muRas-Val12 in four cases and muRas-Asp12 or muRas-Arg12 in one case, respectively. These patients showed 24 to 88

Table 2. Patient characteristics and T-cell response against muRas$_{5-21}$

Characteristic	Number of patients per group	Number of patients with T-cell responses (%)
	19	6 (32)
Age (y)		
>60	11	5 (45)
≤60	8	1 (13)
Gender		
Male	11	5 (45)
Female	8	1 (13)
pT status		
pT_2	2	0 (0)
pT_{3-4}	17	6 (67)
pN status		
pN_0	11*	4* (36)
pN_1	6*	1* (17)
Stage		
I/II	10	3 (30)
III/IV	9	3 (33)
Resectability		
Resectable	13	4 (31)
Irresectable	6	2 (33)
Anatomic site		
Caput	13	2 (15)†
Corpus	6	4 (67)

*n = 17 cases for pN.
†P = 0.046, caput versus corpus (Fisher's exact test).

spots per 2×10^5 peripheral blood mononuclear cells in ELISPOT assay whereas the remaining patients showed 0 to 5 spots per 2×10^5 peripheral blood mononuclear cells or well (Fig. 1). In contrast, in 19 healthy subjects, no more than four spots per well were detected, indicating a lack of a ras-specific immunity in these individuals (Fig. 1). The difference in the number of IFN-γ-secreting T cells between healthy donors and patients with pancreatic cancer was statistically significant (P = 0.004, Mann-Whitney test). This was also true when a cutoff of six spots per well was used to define a positive T-cell response (P = 0.02, Fisher's exact test). No T-cell response specific for wild-type $ras_{5\text{-}21}$ was detected in any patient (zero to five spots per 2×10^5 peripheral blood mononuclear cells; data not shown). Moreover, an assessment of IL-4 producing cells by ELISPOT did not reveal muRas-specific, IL-4-positive T lymphocytes in 8 healthy donors and 13 pancreatic cancer patients, including 3 with a muRas-specific, IFN-γ-positive T-cell response (data not shown).

Dissection of CD4 and CD8 T-cell responses against $muRas_{5\text{-}21}$ peptides. In patients with a $muRas_{5\text{-}21}$-specific T-cell response (n = 6), we analyzed the fraction of $CD4^+$ and $CD8^+$ T-cell subpopulations by negative selection of peripheral blood mononuclear cells. This showed that the observed T-cell response included both $CD8^+$ and $CD4^+$ T cells; muRas-specific $CD4^+$ cells were found in a range of 12 to 182 per 1×10^5 $CD4^+$ cells and muRas-specific $CD8^+$ cells were found in a range of 29 to 106 per 1×10^5 CD8 cells (Fig. 2). In four of six patients, the T-cell response was dominated by $CD8^+$ cells (Fig. 2). As negative control, we isolated peripheral blood mononuclear cells of patients without a response to $muRas_{5\text{-}21}$. Isolated $CD4^+$ or $CD8^+$ T cells of these patients did not result in positive ELISPOT assays (n = 3). The results of a representative patient are shown in Fig. 2 (patient 7).

Cross-reactivity of the immune response against $muRas_{5\text{-}21}$. In patients with response to $muRas_{5\text{-}21}$, we analyzed the T-cell response against the ras mutation present in the primary tumor as well as the response against other frequent codon 12 mutations (muRas Val12, Asp12, and Arg12). In six of six patients, the detected $CD8^+$ and $CD4^+$ T lymphocytes were specific for the mutation expressed by the primary tumor and the T-cell response against this mutation did not cross-react with other ras mutations or wild-type ras (data not shown).

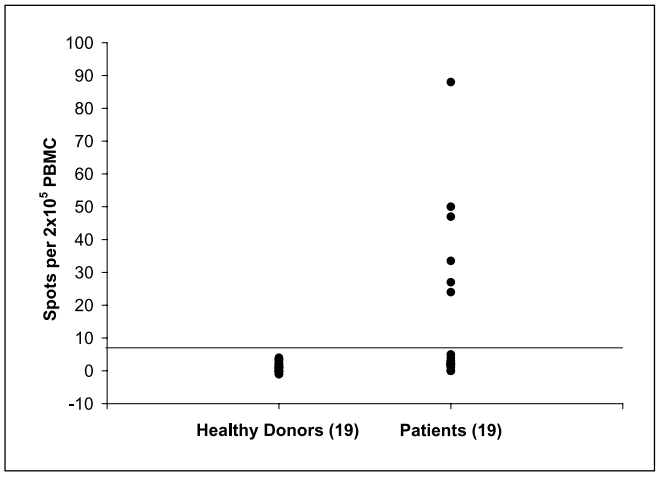

Fig. 1. Frequency of $muRas_{5\text{-}21}$-specific T cells in patients with pancreatic adenocarcinoma and healthy donors. Peripheral blood mononuclear cells of 19 patients with pancreatic cancer and 19 healthy donors were tested against $muRas_{5\text{-}21}$ peptide. Given is the number of spots per well corresponding to the number of IFN-γ-secreting T cells per 200,000 peripheral blood mononuclear cells. Points, median number of 12 tested wells.

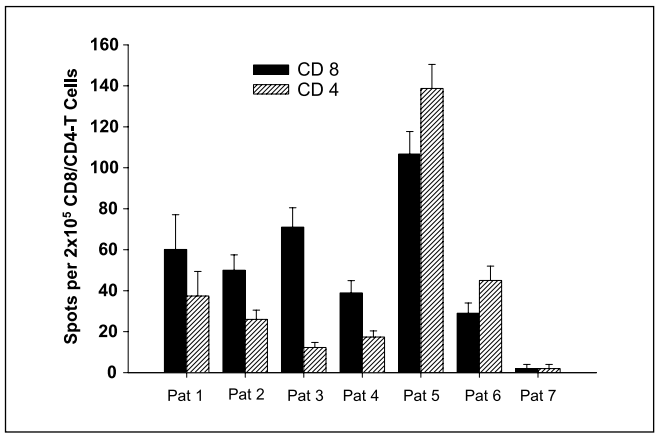

Fig. 2. Proportion of $CD4^+/CD8^+$-muRas 5-21–specific T cells in patients with pancreatic adenocarcinoma. The ratio of $CD4^+/CD8^+$ T-cell subpopulations in response against $muRas_{5\text{-}21}$ was measured in patients with pancreatic cancer. $CD4^+$ and $CD8^+$ T lymphocytes were negatively selected by magnetic beads and subjected to IFN-γ ELISPOT assay. Peripheral blood mononuclear cells were stimulated with peptides corresponding to the ras codon 12 mutation of their primary tumor (Val12, Asp12, and Arg12). Given is the number of spots per well (median of 12 wells) corresponding to the number of IFN-γ secreting T cells per 100,000 $CD4^+$ or $CD8^+$ T lymphocytes. Bars, SD.

$CD8^+$ T-cell response against $muRas_{5\text{-}14}$ and $muRas_{6\text{-}14}$. To determine which epitope elicits the $CD8^+$ T-cell response against muRas-Val12, we used the HLA-A2 class I–restricted peptides $muRas_{5\text{-}14}$-Val12 and $muRas_{6\text{-}14}$-Val12 for stimulation in ELISPOT assays. These epitopes had been predicted to display a high binding capacity to HLA-A*0201 by SYFPEITHI. In all patients who showed a T-cell response to $muRas_{5\text{-}21}$-Val12 (n = 4), we also observed a strong $CD8^+$ T-cell response against $muRas_{6\text{-}14}$ (10-23 spots per 2×10^5 peripheral blood mononuclear cells). Similarly, $CD8^+$ T-cell responses were detected against $muRas_{5\text{-}14}$-Val12 in three of four patients (17-23 spots per 2×10^5 peripheral blood mononuclear cells). It is noteworthy that the magnitude of the T-cell response against the examined muRas-Val12 peptides was different: the 17-mer peptide elicited IFN-γ secretion in a larger proportion of T cells in comparison with the shorter 9- and 10-mer peptides (Fig. 3), suggesting a combined stimulation of IFN-γ secretion of $CD4^+$ and $CD8^+$ T cells by the 17-mer, with the short peptides exclusively activating $CD8^+$ T cells.

In a proportion of patients with pancreatic carcinoma, we analyzed the frequency of a muRas-specific $CD8^+$ T-cell response against nonamer peptides. $MuRas_{6\text{-}14}$-specific $CD8^+$ cells were found in 6 of 13 patients (data not shown). Similarly, $muRas_{5\text{-}14}$ specific $CD8^+$ cells were found in four of nine patients. In contrast, $muRas_{6\text{-}14}$- or $muRas_{5\text{-}14}$-specific T-cell responses were not observed in healthy donors (P = 0.015 and P = 0.026, respectively, Fisher's exact test). In one patient, muRas-specific T cells could only be detected with short peptides (9- and 10-mer) but not with the 17-mer peptide (data not shown).

The $CD8^+$ T-cell response against $muRas_{6\text{-}14}$ or $muRas_{5\text{-}14}$ was not only investigated by ELISPOT but also in parallel by IFN-γ FACS analysis. In all patients (n = 6) with a $CD8^+$ response as shown by ELISPOT, we could confirm the presence of IFN-γ-secreting $CD8^+$ T cells, with frequencies ranging

between 0.05% and 0.1% of all $CD8^+$ T cells (test value minus negative control). A representative example of a $CD8^+$ T-cell response against $muRas_{6-12}$ is shown in Fig. 4.

Function of $muRas_{5-14}$- and $muRas_{6-14}$-specific $CD8^+$ T cells. To evaluate the function of $muRas_{6-14}$- and $muRas_{5-14}$-specific $CD8^+$ T cells, we tested the cytotoxic activity of these T cells after weekly stimulation with muRas-loaded peripheral blood mononuclear cells over a period of 4 weeks. $CD8^+$ T cells stimulated in this way killed $muRas_{6-14}$- and $muRas_{5-14}$-loaded T2 cells but not empty T2 cells or T2 cells loaded with control peptide (HIV-pol; Fig. 5A and B). They also showed cytotoxic activity against HLA-A2-positive, muRas-Val12 – bearing pancreatic or colon cancer cell lines (PaTu and SW480) but not against a HLA-A2-negative, muRas-Val12 – bearing pancreatic carcinoma cell line (S8988; Fig. 6A and B). The cytotoxic activity could be blocked by anti – HLA class I antibodies (Fig. 6A and B). These results indicate that muRas-specific CTLs can be generated from naturally occurring $muRas_{6-14}$- and $muRas_{5-14}$-specific T cells. In addition, they confirm the natural processing of $muRas_{6-14}$ and $muRas_{5-14}$ in pancreatic cancer cells.

Magnitude of $muRas_{6-14}$-specific CD8 T cells in comparison with virus-specific CD8 T-cell response. In addition to the $CD8^+$ response against muRas-Val12 peptide epitopes, we examined the magnitude of the $CD8^+$ response against HLA-A2-restricted viral peptide epitopes (IMP, EBV antigens LMP2 and BMLF1) by ELISPOT: the response against viral peptides revealed a heterogeneous picture with higher, similar or lower frequency of virus-specific $CD8^+$ T cells depending on the respective viral peptide in comparison with the frequency of $muRas_{6-14}$-specific $CD8^+$ T cells (Fig. 3B). These results were confirmed by IFN-γ FACS analysis (data not shown).

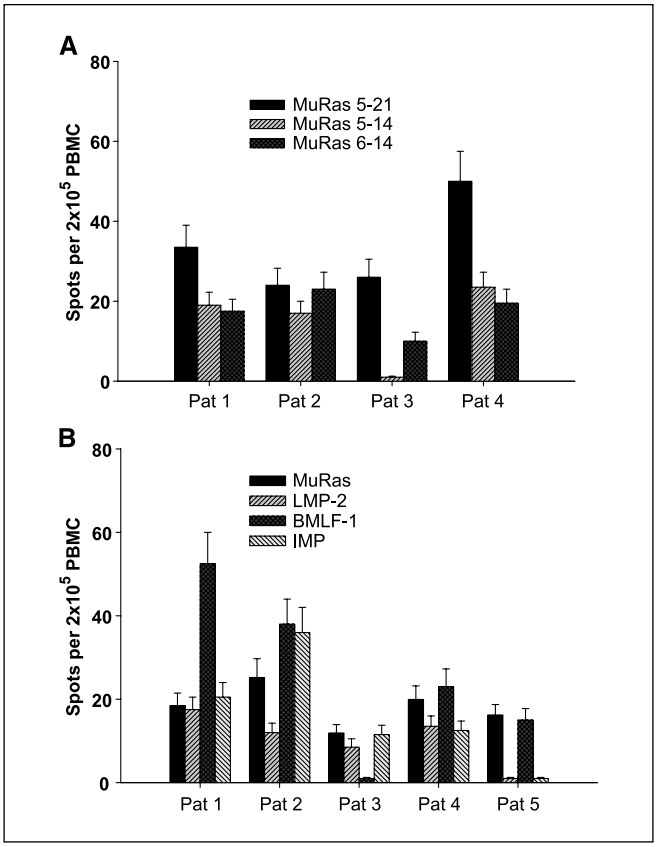

Fig. 3. *A,* comparison of T-cell response against muRas-Val12 peptides of different lengths. Peripheral blood mononuclear cells (*PBMC*) of pancreatic carcinoma patients ($n = 4$) were stimulated with nonamer 6-14, decamer 5-14, and heptadecamer 5-21 peptides derived from muRas. *B,* comparison of muRas-specific with virus-specific CD8 T-cell response. Peripheral blood mononuclear cells of pancreatic carcinoma patients ($n = 5$) were stimulated with nonamer peptides derived from mutated Ras ($muRas_{6-14}$) and different virus proteins: IMP (58-66) from influenza-virus and LMP-2 (426-434) or BMLF-1 (280-88) from Epstein-Barr virus. Columns, number of spots per well (median of 12 wells) corresponding to the number of IFN-γ-secreting T cells per 200,000 peripheral blood mononuclear cells. Bars, SD.

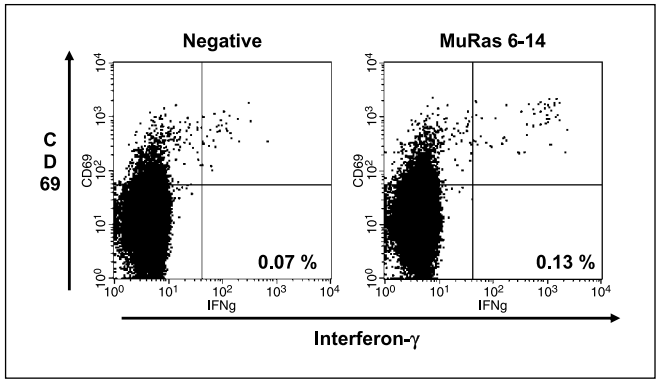

Fig. 4. Frequency of CD8 T-cell response against muRas-Val12 peptide 6-14. Representative example of CD8 T-cell response against muRas 6-12 in a patient with pancreatic cancer as measured by FACS analysis. Peripheral blood mononuclear cells (200,000) were incubated with nonamer 6-14 or without peptide (negative control). Bottom right quadrant, numbers indicate percentage of stimulated CD8 T cells. Frequency of muRas-specific cells is calculated as test value minus negative control (0.13 - 0.07% = 0.06%). Please note the stronger mean fluorescence intensity of IFN-γ in muRas peptide – stimulated T cells in comparison to negative control. *X* axis, IFN-γ. *Y* axis, CD69.

Naturally occurring T-cell response against viral peptides. A comparison of the $CD8^+$ response against HLA-A2-restricted viral peptide epitopes (IMP, EBV antigens LMP2 and BMLF1 and cytomegalovirus-antigen pp65) did not reveal differences in the occurrence of virus-specific T cells between patients with pancreatic carcinoma and healthy donors ($n = 14$; data not shown).

T-cell response against muRas and correlation with clinical and prognostic variables. T-cell responses against muRas were not correlated with clinical or prognostic variables such as tumor-node-metastasis status, stage, grading, or resectability of the primary tumor (Table 2). There was also no association with age, gender, or the concentration of CA 19-9 in their serum. Patients were followed for survival with a median observation time of 4.8 years. There was no statistically significant difference of the survival curves between muRas responders and non-responders. It is noteworthy, however, that differences between groups might not have been discovered because of the limited number of patients. Surprisingly, a T-cell response against muRas was more frequently found if the primary tumor was localized in the cauda or corpus of the pancreas and not in the caput (Table 2, last row; $P = 0.046$, Fisher's exact test).

Discussion

In this study, we provide evidence for a naturally occurring $CD8^+$ and $CD4^+$ T-cell response against muRas proteins in the circulating T lymphocyte pool of a considerable proportion of HLA-A2-positive pancreatic cancer patients (32%). In contrast to previous studies in muRas-bearing cancer patients, we analyzed the T-cell response and quantified its magnitude without prior

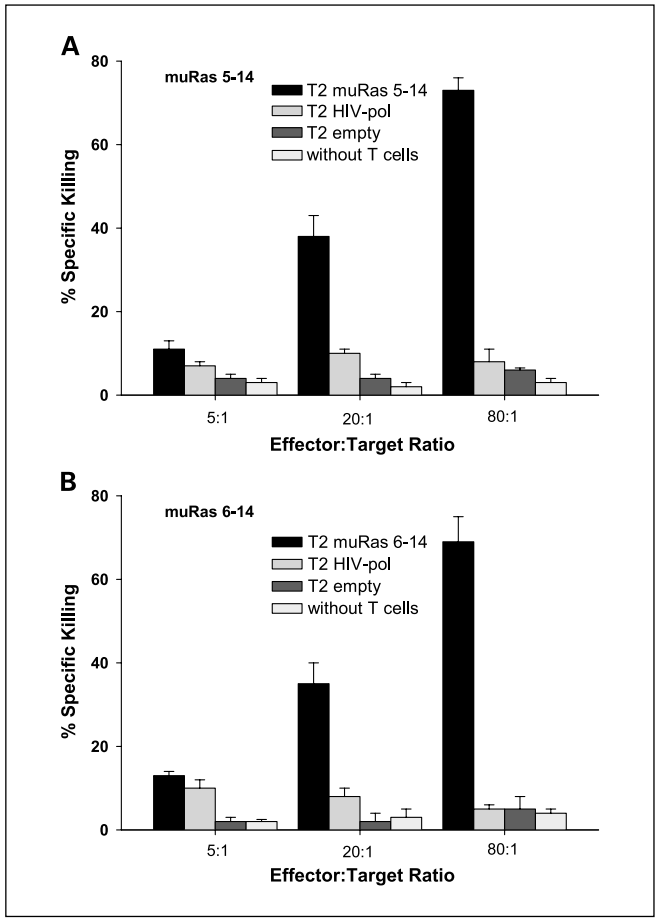

Fig. 5. Cytotoxicity of muRas-Val12 peptide 5-14 (*A*) or 6-14 (*B*) stimulated CD8 T cells against peptide loaded targets. $MuRas_{5-14}$- and $muRas_{6-14}$-specific $CD8^+$ cells kill $muRas_{6-14}$- and $muRas_{5-14}$-loaded T2 cells (*black columns*) but not empty T2 cells (*dark gray columns*) or T2 cells loaded with control peptide (HIV-pol; *left light gray columns*). Columns, percent specific killing representative for T-cell bulk cultures of three (*A*) or two patients (*B*) with pancreatic cancer; bars, SD. T2 cells without adding effector cells were used as another negative control (*right light gray columns*).

in vitro stimulation. The observed T cells can be considered as naturally occurring because the IFN-γ ELISPOT which was used for their assessment detects only antigen-experienced T cells. The investigation of T-cell subgroups revealed that the T-cell response against muRas involved both $CD8^+$ and $CD4^+$ T lymphocytes. Interestingly, in the majority of patients, the $CD8^+$ T-cell response was predominant in comparison with the $CD4^+$ T-cell response. This has not been shown in previous studies in which muRas-specific cytotoxic $CD8^+$ T lymphocytes were only detected after several rounds of *in vitro* stimulation, even in patients vaccinated with muRas 13- to 17-mer peptides (12, 15, 32). This may be explained by the low sensitivity of the assays to detect a $CD8^+$ T-cell response (e.g., limited dilution assay) used in those studies (9, 11) or simply because $CD8^+$ T-cell response was missed due to the fact that the relevant MHC class I–restricted peptides were unknown (3, 5). In contrast, $CD4^+$ T-cell responses have been shown in 44% to 75% of patients with pancreatic cancer (3, 15). Because muRas is an intracellular protein, it is susceptible to the endogenous pathway of antigen processing and loading on MHC class I molecules. Mechanisms related to malignancy such as tumor necrosis might enable access of the sequestered protein to the MHC class II pathway of antigen-presenting cells. Analogous observations have been reported from other cancer-related intracellular proteins such as p53 (33).

Notably, $CD8^+$ as well as $CD4^+$ T-cell responses corresponded to the ras mutation expressed by the primary tumor of a patient and the T-cell response against this mutation did not cross-react with other ras mutations or wild-type ras. To determine the HLA-A2-restricted $CD8^+$ response in more detail, a nonamer and a decamer peptide predicted by peptide prediction software SYFPEITHI (31) to bind to HLA-A2 were tested for their capacity to detect antigen-experienced T-cell responses. The peptides 5-14 and 6-14 derived from muRas-Val12 elicited $CD8^+$ T-cell responses which could be detected by IFN-γ ELISPOT and confirmed by IFN-γ-FACS analysis. We also showed that these peptides are targets of CTLs generated from $muRas_{5-14}$- and $muRas_{6-14}$-loaded antigen-presenting cells and that these CTLs are able to kill HLA-A2-positive, muRas-Val12–bearing pancreatic cancer cells. The latter fact shows that these peptides are naturally processed in pancreatic cancer. Data from another group support the immunogenicity of the $muRas_{5-14}$ peptide.

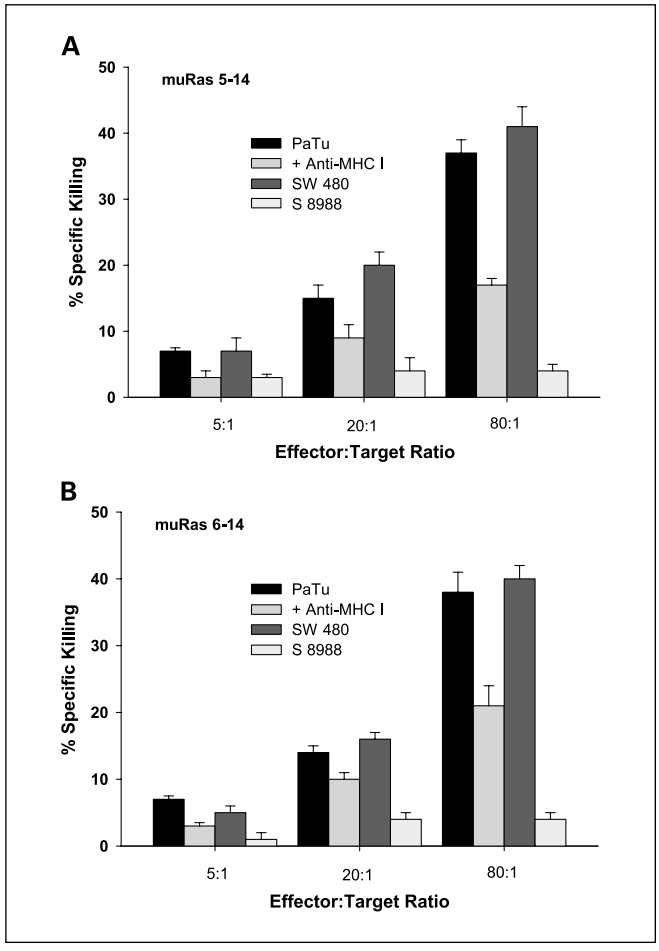

Fig. 6. Cytotoxicity of muRas-Val12 peptide 5-14 (*A*) or 6-14 (*B*) stimulated CD8 T cells against pancreatic carcinoma cell lines. $MuRas_{5-14}$- and $muRas_{6-14}$-specific $CD8^+$ cells killed muRas-Val12–bearing, HLA-A2-positive pancreatic cells (PaTu; *black column*) but not HLA-A2 negative, muRas-Val12–bearing pancreatic cancer cells (S8988; *right light gray columns*). Cytotoxicity could be blocked by an anti–HLA class I antibody (*left light gray columns*). MuRas-Val12–bearing, HLA-A2-positive colon cancer cells are also killed (SW480; *dark gray columns*). Columns, percent specific killing representative for T-cell bulk cultures of three (*A*) or two patients (*B*) with pancreatic cancer; bars, SD.

These investigators established a cytotoxic $CD8^+$ T-cell line against $muRas_{5-14}$-Asp12 in a patient with colon carcinoma (15).

In pancreatic cancer patients, we detected 5 to 12 $CD8^+$ T cells per 100,000 peripheral blood mononuclear cells in an ELISPOT assay using $muRas_{5-14}$ or $muRas_{6-14}$. This frequency is ~1 log beyond the detection limit of IFN-γ ELISPOT (1/100,000 peripheral blood mononuclear cells). In other tumor entities like colorectal carcinoma or melanoma, the frequencies of $CD8^+$ T cells specific for the relevant tumor antigens are in the same range (5, 34–36). For example, Nagorsen et al. (34) showed 3 to 20 Ep-CAM-specific $CD8^+$ T cells and 1 to 8 Her-2/neu-specific $CD8^+$ T cells per 100,000 peripheral blood mononuclear cells in colorectal cancer. However, it must be kept in mind that the ELISPOT assays done in different laboratories are not standardized, making a comparison of the number of the observed spots difficult. Therefore, attempts have been made to standardize this technique and to evaluate the reliability of the T-cell frequency analysis. Consistent frequencies of model viral peptide-specific T cells were observed by ELISPOT assay in different laboratories (36).

In this study, we did not find correlations between a specific immune response against muRas and tumor-node-metastasis status, stage, or serum levels of CA-19-9. This does not support the hypothesis that a high tumor load leads to a strong T-cell response (34) or the opposite hypothesis that a high tumor load is associated with immunosuppression. Although data from selected patients suggest a favorable clinical course in patients with naturally occurring T-cell response in peripheral blood mononuclear cells (37, 38) or intratumorally (12, 39), no study has systematically investigated the prognostic significance of tumor antigen–specific T lymphocytes in peripheral blood mononuclear cells. Because of the limited number of patients with detectable muRas T-cell response, we could also not consider this question in a sufficient way. The observation that pancreatic adenocarcinomas located in the cauda or corpus elicit an immune response more frequently than adenocarcinomas located in the caput is difficult to interpret. Whether this is related to the anatomic characteristics of the pancreatic lymphatics (40) remains open for speculation.

To summarize, we have shown a T-cell response against muRas proteins in a significant proportion of patients with pancreatic carcinoma. The ELISPOT assay using the described muRas derived peptides is a useful tool for immunologic monitoring of muRas cancer vaccine trials because it is more informative than the qualitative tests described thus far. The goal of a vaccination with muRas is to boost the preexistent immune response and/or to overcome their possible functional deficits. To this end, we recently developed a novel cancer vaccine using genetically modified lymphoblastoid cell lines (28) which elicits a strong muRas-specific T-cell response *in vitro*. A clinical pilot study to evaluate this approach is ongoing.

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