Impaired CD8+ T-Cell Reactivity against Viral Antigens in Cancer Patients with Solid Tumors

A. Trojan, R. Giger, N. Rist, R. F. Speck

Abstract

Background: Patients with hematological malignancies are at increased risk for various infections. In patients with solid cancer, a variety of immunosuppressive mechanisms affecting T-cell response are described. We hypothesized that patients with advanced solid tumors may exhibit an impaired recognition of viral antigens. To test this, the capability of CD8+ T cells to recognize recall antigens from influenza and vaccinia virus was compared in patients and healthy individuals. Since all patients and most of the healthy individuals had been vaccinated against vaccinia years ago, comparison of the two groups was expected to be especially informative with respect to distinct effector T-cell reactivity.

Materials and Methods: Our test population included 16 healthy individuals and 12 patients with advanced solid cancers who were currently not receiving chemotherapy. We stimulated peripheral blood mononuclear cells (PBMC) *ex vivo* with the well-characterized influenza A matrix 58–66 peptide and the immunogenic and HLA-A*0201 restricted peptide epitope SLSAYIIRV derived from the modified vaccinia virus Ankara (MVA). A specific CD8+ T-cell reactivity was determined by quantitative real-time polymerase chain reaction (qRT-PCR) measuring changes in interferon gamma (IFN-γ) mRNA expression levels.

Results: We found that significantly fewer cancer patients than healthy individuals exhibited specific T-cell recognition of the vaccinia epitope (25% and 69%, respectively). In addition, strength of the T-cell responses against both viral peptides was significantly reduced in cancer patients.

Conclusion: Patients with advanced tumors are less likely to mount a T-cell response against viral epitopes. These findings may have implications for the design of immunotherapeutic interventions against virus-induced diseases, including tumors.

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Introduction

Vaccines and other immunotherapies hold great promise as treatments for viral diseases and cancer, but are premised on the patient's ability to mount an appropriate T-cell response to a specific antigen. A vast amount of viral- and tumor-derived antigens have been identified that are recognized by cytotoxic T lymphocytes (CTL) in a major histocompatibility complex (MHC) class I restricted fashion [1–5]. In contrast, little is known about the frequency and function of antigen-specific CD8+ T cells in selective patient populations, information that might be critical for the development of immunotherapeutic regimens.

In an attempt to determine CTL reactivity in patients and healthy individuals, we characterized the antigen-specific CD8+ T-cell response against epitopes from two viral model antigens, influenza and vaccinia [2, 6]. In adults, repeated exposure to influenza results in stimulation of the virus-specific memory and naive CTL precursor repertoire [1, 7]. In contrast, vaccination with modified vaccinia virus strains decades ago supposedly induced a life-long protective immune response provided by long-lasting and virus-specific CTL memory [8, 9]. HLA-A*0201-restricted CTL responses have been identified for both model viral antigens. For influenza, healthy individuals and patients with advanced cancer show a CTL response against the matrix peptide 58–66 [2, 3, 10, 11]. A recently identified immunogenic epitope derived from modified MVA, allows for direct monitoring of specific CD8+ T-cell responses induced by different vaccinia virus strains [6, 12–14].

Patients with hematological malignancies are at increased risk for various infections [15, 16]. In patients with solid cancer, a variety of immunosuppressive mechanisms

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Table 1
Characteristics of patients and healthy volunteers.

Healthy individuals	Gender	Age (years)	Smallpox vaccination	Patients	Gender	Age (years)	Smallpox vaccination	Cancer	TNM	Stage
HD1	f	40	-	P1	f	44	+	Breast	T4N1M1	IV
HD2	m	29	+	P2	m	33	+	HN	T4N2M1	IV
HD3	m	27	-	P3	m	56	+	Lung	T2N3M1	IV
HD4	f	35	+	P4	f	70	+	Breast	T4N3M1	IV
HD5	f	46	+	P5	f	60	+	Lung	T4N2M1	IV
HD6	f	60	+	P6	m	53	+	HN	T2N1M1	IV
HD7	m	34	+	P7	m	65	+	Colon	T3N2M1	IV
HD8	m	56	+	P8	m	77	+	Pancreas	T3N1M1	IV
HD9	m	38	+	P9	m	59	+	RCC	T4NxM1	IV
HD10	m	30	+	P10	m	57	+	Pancreas	T2N1M1	IV
HD11	f	36	+	P11	f	57	+	Colon	T2N1M1	IV
HD12	f	38	-	P12	m	71	+	LS	T4NxM1	IV
HD13	f	38	+							
HD14	f	51	+							
HD15	f	53	+							
HD16	m	34	+							

TNM staging of all patients with metastatic cancer (stage IV) at the time of evaluation was performed according to the updated 6th edition of the American Joint Committee on Cancer 2002 Cancer Staging Manual. Individuals who had received vaccinia vaccination (at least) once in their lifetime: +, those without vaccination: -; RCC: the patient with renal cell cancer; HN: head and neck cancer; LS: liposarcoma; f: female; m: male.

affecting T-cell response systemically and in the tumor milieu are described [17, 18]. Therefore, we hypothesized that effector T cells from peripheral blood of cancer patients are less likely to mount an efficient response against the two viral recall antigens than T cells from healthy donors. Upon stimulation with an antigenic peptide epitope, CD8+ T cells generate the cytokine Interferon gamma (IFN-γ) [17, 18]. Here, we quantified this response in healthy individuals and in cancer patients by measuring IFN-γ mRNA expression following stimulation with antigenic peptides derived from influenza and vaccinia virus.

Materials and Methods

Patients

Inclusion criteria included the presence of HLA-A*0201 MHC class I genotype, as determined by sequence-specific primer PCR (Protrans GmbH, Ketsch, Germany). Blood samples were obtained after informed consent. 16 healthy individuals, all health-care workers aged 27–60 years (mean 40 years), and 12 cancer patients (age 33–77 years, mean 58 years) were recruited. All patients were suffering from locally advanced and metastatic disease; in particular, two patients each suffered from solid cancers of the lung, colon, pancreas, breast, and oropharynx, one patient had renal cell cancer, and another had liposarcoma. None of the cancer patients had received systemic chemotherapy or immunotherapy within 4 weeks prior to examination of T cell reactivity. Detailed patient characteristics are shown in table 1. According to the detailed and reliable medical history, 13 healthy volunteers and all 12 patients had received the smallpox vaccination once in their lifetime (Table 1). The time since the last smallpox vaccination was 7–38 years (mean 31 years, median 33 years). The donors were not tested for antibodies to influenza A since almost all individuals are seropositive due to previous exposure. Current influenza vaccines consist of surface antigens such as hemagglutinin and neuraminidase; thus, they do not induce T-cell reactivity against the conserved matrix peptide from influenza A virus. Vaccination status of the individuals tested was therefore not relevant.

Stimulation of Cytotoxic T Lymphocytes

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by Ficoll-Hypaque density gradient centrifugation (Pharmacia Biotech, Uppsala, Sweden). For *ex vivo* stimulation the vaccinia virus-derived epitope SLSAYIIRV which is present in different vaccine strains was used [6, 14]. T-cell reactivity against influenza A virus was determined using the matrix peptide 58–66 GILGFVFTL [2]. Reactivity to this antigen is demonstrated in most individuals representing the individual memory T cell reactivation with respect to annual exposure [4, 10, 19–23]. All peptides were generated with standard solid phase chemistry on a multiple peptide synthesizer and analyzed by mass spectrometry [90% purity shown by high-pressure liquid chromatograhy (HPLC)], as described [23].

Autologous PBMC displaying vaccinia- and influenza-derived candidate peptides and a non-immunogenic control peptide (also not binding to HLA-A2) were used to expand specific CTL precursors from patients and healthy individuals as previously described [23–25]. Briefly, ~2×10^6 PBMCs were pulsed with the appropriate peptide (5 μg/ml) and incubated for 1 h at 37 °C in X-VIVO 10 medium (BioWhitaker, Gagny, France) supplemented with 3 μg/ml β2-microglobulin, recombinant IL-7 (10 ng/ml) and IL-2 (10 U/ml) (R&D, Wiesbaden, Germany). One restimulation was performed after 1 week (day 8) with the appropriate peptide at the same concentration without adding cytokines. To obtain the highest IFN-γ mRNA yield, 2×10^6 PBMCs

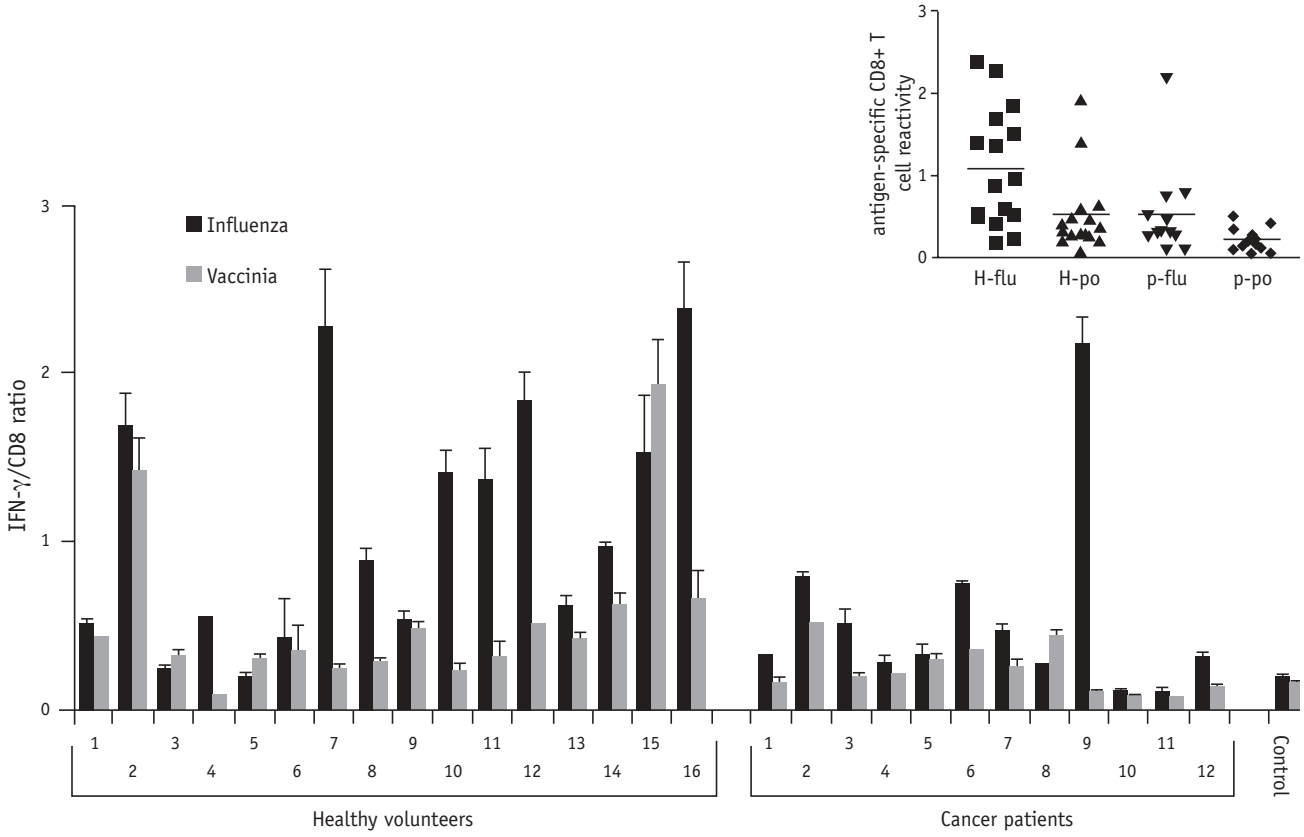

Figure 1. Assessment of immune reactivity for the influenza A peptide$_{58-66}$ and the smallpox peptide with the TaqMan qRT-PCR in 16 healthy donors (HD) and 12 patients (P). Reactivity is displayed as the mean IFN-γ/CD8 ratio ± SD of healthy donors against the influenza matrix peptide (H-flu) and the vaccinia peptide (H-po). Results are demonstrated for the patient (p-flu; p-po) group (according to individuals and conditions a scatter graph is inserted). For characteristics of all individuals and clinical data of the cancer patients please refer to table 1 and the Materials and Methods section. IFN-γ/CD8 ratios were assessed as well after exposure to the irrelevant control peptide from a subset of ten individuals; mean values were calculated for all negative controls as indicated.

were harvested 2 h after the last peptide-triggered restimulation [3, 4] and used immediately for RNA extraction and cDNA synthesis [20, 23].

Real-Time Quantitative PCR

RNA (1 µg) was reverse-transcribed using oligo-p(dT)$_{15}$ priming and avian myeloblastosis virus (AMV) reverse transcriptase (1st Strand cDNA Synthesis Kit for RT-PCR [AMV], [Roche Molecular Biochemicals – RMB; GmbH, Mannheim, Germany] at 42 °C for 1 h. Gene expression was measured with the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). PCR amplifications were carried out in triplicate. Primer sequences for IFN-γ and CD8 were as follows: IFN-γ (forward) 5'-AGCTCTGCATCGTTTTGGGTT-3', IFN-γ (reverse) 5'-GTTCCATTATCCGCTACATCTGAA-3'; CD8 (forward) 5'-CCCTGAGCAACTCCATCATGT-3', CD8 (reverse) 5'-GTGGGCTTCGCTGGC-3'. Corresponding TaqMan probes were used as described [3, 20]. DNA standards were generated by PCR amplification of gene products, purification, and quantification by spectrophotometry (absorbance at 260 nm); standard curves were generated for both IFN-γ and CD8 and allowed for extrapolation of the corresponding copy numbers. Since stimulation with HLA class I-presented epitope defines CD8+ T cells as the only relevant population, mRNA copy numbers were corrected for CD8 mRNA copies from the same sample creating a ratio that reflects the status of CD8+ T cell activation [3, 4, 20, 23]. This method of mRNA cytokine analysis after *ex vivo* exposure to viral or tumor peptides represents a simple and sensitive alternative to other methods that test epitope-induced T-lymphocyte reactivation for identifying immune dominant epitopes and investigation of clinically relevant T-cell responses [26]. Negative control IFN-γ/CD8 ratios (baseline ratios) were assessed for the irrelevant peptide AHTKDGFNF previously shown not to bind to HLA-A2 and not to be immunogenic [5, 23]. To determine the baseline ratios, T-cell reactivity against this irrelevant peptide was analyzed; the mean results of ten individual donors tested served as baseline. A positive response was defined as one greater than three standard deviations above background. All assays were performed in triplicate. Quantitative PCR results are displayed as CD8-normalized IFN-γ levels (IFN-γ/CD8 ratio ± SD).

Statistics

The Kruskal-Wallis test was used for non-parametric comparisons of multiple groups. The Mann-Whitney test was used for

comparisons between groups, the Wilcoxon signed rank test for comparisons within the groups. The χ^2 test was used to compare the frequency of T cell reactivity between groups.

Results

Quantitative PCR Assessment of Peptide-Specific Reactivity

We defined reactivity as the IFN-γ/CD8 ratio of *ex vivo* expanded peptide-specific precursors. Reactivity was monitored as changes in IFN-γ gene expression as determined by real-time qPCR [5, 20, 23, 26]. Significant reactivity against the vaccinia virus peptide was demonstrated in 69% of the vaccinated healthy individuals (9/13), but in only 25% of the patients (P2, P6, and P8). Frequency of T-cell reactivity significantly differed between healthy individuals and patients (χ^2 $p < 0.027$). Similar results for CD8+ T cell response were obtained in a cohort of 306 healthy vaccine recipients 20–75 years after vaccination, substantiating our findings [27]. The strength of T cell reactivity against the vaccinia peptide in the entire cohort of cancer patients was significantly lower than was observed in healthy individuals (cancer patients: mean ± SEM was 0.23 ± 0.04, median 0.19; and healthy donors: mean ± SEM was 0.53 ± 0.12, median 0.36, respectively; Wilcoxon signed rank test $p < 0.05$). Two healthy donors had received a booster vaccination against smallpox 9 years (HD 6) and 7 years (HD 11) prior to the study. Surprisingly, they did not reveal any reactivity against the vaccinia peptide as compared to the irrelevant control peptide [8].

A significant increase in IFN-γ/CD8 ratio upon stimulation with the influenza matrix peptide was demonstrated in 13 of 16 healthy donors (81%) and seven of 12 patients (58%), respectively (Figure 1) (frequency of T-cell reactivity $p = 0.1841$). Except for patient 9 (P9), who had previously undergone resection of a primary metastatic renal cell cancer, the average increase of IFN-γ /CD8 ratio was significantly higher in healthy individuals as compared to cancer patients (healthy donors: the mean ± SEM was 1.08 ± 0.2, median 0.92 and cancer patients: mean 0.53 ± 0.16, median 0.315, respectively; Mann-Whitney test $p < 0.008$). Ratios for IFN-γ/CD8 copies were 4.5 and 2.2 times, respectively, above baseline indicating that *ex vivo* kinetics of the virus-specific effector T-cell repertoire and the corresponding functional state are comparable to data obtained from tetramer-guided sorting and *in vitro* studies in humans [26–30]. We also found an increased reactivity (indicated as IFN-γ/CD8 ratio) against the influenza peptide in comparison to that against the vaccinia peptide in both healthy individuals and cancer patients ($p = 0.0082$ and $p = 0.0093$, respectively).

Discussion

The importance of a strong and specific CD8+ T cell response has become increasingly evident for the control of viral infection and tumor [1, 31, 32]. Alterations in cell-mediated immune function are associated with frequent bacterial and viral infections; in cancer patients, however, lack of efficient T cell responses against viral pathogens is mainly reported in patients with hematological malignancies [15, 16, 31, 33, 34]. Here we examined the reactivity against viral epitopes of influenza and vaccinia in patients with advanced solid tumors.

We found that the frequency of immune response to the vaccinia epitope was significantly lower in cancer patients than in healthy individuals (25% vs 69%, respectively). Also, strength of the specific T-cell response was significantly impaired in cancer patients when compared to the group of healthy individuals. These results suggest that advanced solid cancer may negatively influence specific CTL responses. However, frequency for recognition of the influenza virus epitope was similar in the group of healthy individuals and cancer patients. It is therefore conceivable that cancer patients maintain their ability to react to recall antigens like influenza A matrix to which they might be exposed repeatedly during the annual influenza season. Strength of the T cell reactivity against the influenza A epitope again was significantly lower in cancer patients, indicating that the recognition of both the vaccinia and the influenza antigen may be impaired by immunosuppressive conditions with respect to tumor burden and progression of disease, a reduced T cell repertoire or disrupted time frame for T cell recovery [15, 16, 35]. The increase in the CD8 normalized T cell reactivity may appear moderate but is significant and in line with other representative studies [26–30].

Of note, in all individuals tested the strength of T cell reactivity against the antigenic influenza peptide was significantly higher than against the vaccinia antigen; a superior T cell reactivity that may be due to boosting and expansion of a virus-specific memory and naive CTL precursor repertoire [31, 36].

Unexpectedly, the two healthy volunteers who had received a smallpox booster 7 and 9 years previously did not exhibit an enhanced T cell reactivity against this immunogenic peptide. Whether this observation may be due to a potential disproportionate loss of specific CD8+ T cells early after boosting remains unclear [27, 36].

Results of this pilot study require validation in prospective clinical trials also involving a greater number of individuals and complementary techniques such as tetramer staining, ELISPOT and chromium release assays [26, 37]. It should also be stressed that we have not evaluated other shared modes of protective immunity such as CD4+ T cell response and serum antibody levels [31, 36].

In conclusion, frequency and strength of the CD8+ T cell reactivity to the vaccinia epitope seemed to be impaired in cancer patients. Nonetheless, it appears that cancer patients maintain the ability to mount a specific T cell reactivity against viral epitopes. Whether quantification of T cell reactivity against antigens will serve as surrogate marker for monitoring in therapeutic and experimental vaccination settings needs to be explored.

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