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BRAF^{V600E} and BRAF-WT Specific Antitumor Immunity in Papillary Thyroid Cancer

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ABSTRACT

One feature of papillary thyroid cancer (PTC) is the frequently present somatic BRAF^{V600E} mutation. PTCs are also characterized by a lymphocytic infiltration, which may correlate with an improved clinical outcome. The objective of the study was the characterization of BRAF^{V600E} specific anti-immunity in PTC patients and correlation analyses with the clinical outcome. Fourteen HLA A2 positive PTC patients were included into the study of whom tumor tissue samples were also available. Of those, 8 PTC patients revealed a somatic BRAF^{V600E} mutation. All PTC patients were also MHC class II typed. Tetramer analyses for detection of MHC class I and MHC class II-restricted, BRAF^{V600E} epitope-specific T cells using unstimulated and peptide-stimulated T cells were performed; correlation analyses between MHC phenotypes, T cell immunity, and the clinical course were performed. In regard to unstimulated T cells, a significantly higher amount of BRAF^{V600E} epitope specific T cells was detected compared to a control tetramer. Importantly, after overnight peptide stimulation a significantly higher number of BRAF^{V600E} positive and BRAF WT epitope-specific T cells could be seen. In regard to the clinical course, however, no significant differences were seen, neither in the context of the initial tumor size, nor in the context of lymph node metastases or peripheral metastastic spread. In conclusion, we clearly demonstrated a BRAF-specific tumor immunity in PTC-patients which is, however, independent of a BRAF^{V600E} status of the PTC patients.

Introduction

Papillary thyroid cancer (PTC) is the most common malignant tumor of the thyroid [1]. The etiology of PTC seems to be multifactorial including genetic predisposition, environmental triggers [2], and simultaneously appearing autoimmune thyroiditis [3]. PTC is characterized by a rather slow tumor growth, a lymphatic spread with rare distant metastases, and in most cases an excellent prognosis with a 10-year survival rate of more than 90 % [4]. Interestingly, it is the only malignancy of the thyroid showing an abundant

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lymphocytic infiltration into the tumor site [5]. Around 30 % of PTC patients additionally suffer from autoimmune thyroiditis (chronic lymphocytic thyroiditis), the most common autoimmune disease of the thyroid. Most recently, we have demonstrated a thyroglobulin (Tg) and thyroperoxidase (TPO) epitope specific antitumor immunity in PTC patients [6].

About 29–83 % of PTC patients reveal a somatic $BRAF^{V600E}$ mutation [7–11]. BRAF is a member of the mitogen-activated protein cascade, which is responsible for the transmission of the mitogen signal to the nucleus. This leads to cell growth, differentiation and cell survival. The $BRAF^{V600E}$ mutation seen in PTC leads to a permanent activation of the BRAF kinase, which results in tumorigenesis and tumor growth. The somatic $BRAF^{V600E}$ mutation is frequently found in several other tumor types, such as melanoma, colon cancer, and ovarian cancer [12]. In malignant melanoma, for instance, a BRAF-specific kinase inhibitor (vermurafenib) is used for the treatment of $BRAF^{V600E}$ positive tumors [13–15]. Most recently it has also been shown that $BRAF^{V600E}$ induces the recruitment of regulatory T cells (Tregs) to tumor sites [16]. Moreover, it has been demonstrated that melanoma cases, which are resistant to BRAF inhibitors, are still sensitive towards $BRAF^{V600E}$ positive T cells [17]. The most direct evidence of a $BRAF^{V600E}$ dependent cytotoxic immunity has been shown by Veatch et al. [18]. The authors reported on an adoptive cell transfer therapy using tumor infiltrating lymphocytes (TIL) in a patient suffering from a metastasized malignant melanoma. Analysis of the specificity of TILs identified CD4 + T cells specific for $BRAF^{V600E}$, restricted by a common class II MHC molecule. After adoptive cell therapy, the patient reached a durable remission. Even 24 months after therapy, CD4 + $BRAF^{V600E}$ specific T cells were still present in the peripheral blood of the patient.

Based on these results, the aim of our present study was to investigate the role of BRAF-specific T cells in the antitumor immunity in PTC. To do so, BRAF-specific T cells were measured in the peripheral blood of PTC patients with and without in vitro stimulation. Here, we could show a BRAF-specific anti-tumor immunity, which was however independent from the underlying BRAF mutation status.

Patients and Methods

Patients and healthy subjects

Out of our patient cohort of n = 150 PTC patients, which were formerly described [19], n = 14 HLA A2 positive PTC patients were identified who additionally underwent surgery at the University Hospital Duesseldorf and their formalin-fixed paraffin-embedded (FFPE) tissue specimens were available (▶ **Table 1**). All 14 patients (n = 8 females) agreed for another blood drawing for MHC class II typing. Additionally, n = 6 healthy subjects also contributed to the study (n = 3 females). The study has been approved by the local ethical committee (2018–264-KFogU).

Microscopic identification of the tumor tissue in paraffin embedded samples

To identify $BRAF^{V600E}$ positive PTC patients, FFPE tumor tissue was used for mutation analysis. Tumor regions were identified and annotated on Hematoxylin and Eosin (H & E) stained tissue sections. Approximately 4 × 10 µm serial tissue sections were dried for 15 minutes at 100°C and paraffin removal was performed in xylene two times for 10 minutes each. Afterwards, sections were washed twice in 100 % ethanol for 5 minutes and dried for 5 minutes at room temperature (RT) or until complete removal of ethanol. Genomic DNA was then isolated from the micro dissected tissue sections, containing tumor region of interest.

Isolation and purification of genomic DNA

In order to extract DNA, further FFPE tumor tissue slides were used. Micro-dissection of tumor tissue was carried out with a 10 µl pipet tip (with aerosol barrier) to avoid any contamination. Scratched tumor material was than sampled in a 1.5 ml safe lock tube (Eppendorf, Germany) for paraffin removal. DNA was isolated using the EZ1 DNA Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions. Tissue pieces were submerged and incubated at 56°C in 190 or 380 µl G2 buffer (190 µl for < 1 cm^2, 380 µl for > 1 cm^2) with proteinase K (10 µl for < 1 cm^2, 20 µl for > 1 cm^2), overnight or until complete tissue lysis. During incubation, samples were kept on a thermal heating block under constant mixing. Insoluble material was removed by centrifugation in a cooling micro centrifuge at full speed for one minute. Homogenized sample solution was than thoroughly transferred to EZ1 reagent cartridges (Qiagen, Germany). Reagent cartridges contain magnetic particles for precise separation and purification of genomic DNA. DNA isolation was automatically performed by the EZ1-Biorobot (Qiagen, Germany). Up to 2 µl of isolated DNA (1 µl: > 40 ng/µl DNA; 2 µl: < 40 ng/µl DNA) was used for amplification of the BRAF gene in a PCR reaction as followed: Taq-DNA-Polymerase Kit with buffer (Qiagen, Germany), dNTPs (Sigma, St. Louis, USA), and Human Genomic DNA positive control (Promega, Madison, USA). For template amplification of BRAF (exon 15, codon 600), forward-primer: TGCTTGCTCTGATAGGAAAATG, and BRAF reverse-primer: AGCCTCAATTCTTACCATCCA were used, resulting in an amplification product of 191 bps. Cycling was performed with an initial denaturation at 94°C for four minutes. Denaturation at 94°C for 30 seconds, primer annealing at 55°C for 45 seconds, and extension at 72°C for 45 seconds were repeated for 40 cycles. Amplification products were controlled on an agarose gel (2 %, Biozym, Germany) and purified with the PCR purification Kit (Qiagen, Germany). Purified DNA was dissolved in 30 µl pure water and stored at –20°C with a sample concentration of 34.5 ± 25.4 ng/µl.

Analyses for $BRAF^{V600E}$ mutations

Sequencing of the BRAF amplification products was done with 5 µl to an adjusted concentration of 9 ng/µl for each sample by the Sanger method using cycle sequencing on an ABI 3130XL Genetic Analyzer (Applied Biosystems/Thermo Scientific, USA) in cooperation with the BMFZ Genomic and Transcriptomic Laboratory (Heinrich-Heine-University Duesseldorf, Germany). Analysis of the sequence data was performed using the Sequencing Analysis Software 5.3.1 (Applied Biosystems/Thermo Scientific, USA) by the BMFZ. We identified $BRAF^{V600E}$ mutation in comparison to wild type sequences (▶ **Fig. 1**).

Tetramer analyses

Peripheral blood mononuclear cells (PBMCs) were isolated by centrifuging heparinized whole blood samples in BD Vacutainer Cell Preparation Tubes (BD Bioscience, San Jose, CA, USA). For FACS

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▶ **Table 1** PTC patients characteristics.

Gender	Age at blood sampling (years)	Age at initial diagnosis (years)	TNM	$BRAF^{V600E}$	HLA-DQ8
f	71	58	T1 N0 M0	neg	neg
f	63	24	T1 N0 M0	neg	neg
m	39	32	T1 N1 M0	neg	neg
m	52	41	T3 N1 M0	neg	neg
f	62	58	T1 N0 M0	neg	neg
f	59	48	T1 N0 M0	neg	neg
m	60	48	T1 N1 M0	neg	neg
m	50	44	T1 N0 M0	pos	pos *
f	50	36	T3 N1 M0	pos	neg *
f	32	27	T1 N1 M0	pos	neg
f	47	41	T3 N1 M0	pos	neg *
f	72	65	T1 N1 M0	pos	pos *
m	58	42	T2 N1 M0	pos	neg *
m	40	26	T2 N1 M0	pos	neg *

All patients were HLA-A2 positive.; * Patients analyzed for HLA-DQ8-restricted BRAF (WT and mutated) positive T cells.

analysis cells were resuspended either in PBS buffer (Gibco, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) or in complete medium [RPMI 1640 GlutaMAX (GIBCO, Saranac, NY, USA), 10 % FCS (GIBCO), 1 % penicillin/streptomycin (GIBCO)] containing recombinant human IL-2 (50 U/ml), IL-7 (20 U/ml), and IL-15 (10 ng/ml). Tetramer staining was performed as follows: 6×10^4 cells and 5 µl tetramer were added to each FACS tube and incubated at 37°C for 15 minutes (MHC-I tetramers) or at room temperature for 2 hours (MHC-II tetramers). MHC class I tetramers were purchased from tetramer shop (Kongens Lyngby, Denmark) while MHC class II tetramers were obtained from the Tetramer Core Laboratory of the Benaroya Research Institute at Virginia Mason, USA, with kind support of Bill Kwok.

Tetramers were chosen based on previous publications: Somasundaram and colleagues described an HLA-A2 restricted response of cytotoxic T lymphocytes to mutated BRAF peptides in melanoma patients [20]. Nevertheless, our sequences differed in one amino acid position due to a higher binding score (predicted by SYFPEITHI). SYFPEITHI (www.syfpeithi.de) is a database of more than 7000 peptide sequences known to bind MHC class I molecules. It calculates the corresponding binding scores to estimate the immunogenic potential of the epitopes (reviewed in ref. [21]). All MHC class I tetramers chosen had binding scores of 13. The following PE-conjugated MHC class I tetramers and corresponding epitopes were used: BRAF wild-type (WT) 1 (amino acid position (AA) 596–604): GLATVKSRW; BRAF WT2 (AA 596–605): GLATVKSRWS; $BRAF^{V600E}$ 1 (AA 596–604): GLATEKSRW; $BRAF^{V600E}$ 2 (AA 596–605): GLATEKSRWS.

Additionally, Veatch and colleagues described tumor-infiltrating $BRAF^{V600E}$-specific CD4 + T cells that correlated with complete clinical response in melanoma patients [18]. In analogy to this publication, the following PE-conjugated MHC class II tetramers and corresponding epitopes were used: BRAF MHC-II WT (AA 593–607): GDFGLATVKSRWSGS; and BRAF MHC-II V600E (AA 596–604): GDFGLATEKSRWSGS.

For FACS analysis, cells were additionally stained with anti-CD4 APC or anti-CD8 FITC, respectively. Despite of the tetramers, all other FACS antibodies were purchased from BD and incubated as described above. Epitope-specific cytotoxic T cells are constantly displayed as a fraction of all PBMC's in percent.

Stimulation experiments

To investigate whether the PTC patient's T cells get activated by BRAF peptides (WT and V600E), stimulation experiments were performed. Therefore, cancer patients derived PBMC's were cultured overnight at a density of 1×10^6 cells/ml in complete medium [RPMI 1640 GlutaMAX (GIBCO, Saranac, NY, USA), 10 % FCS (GIBCO), 1 % penicillin/streptomycin (GIBCO)] containing recombinant human IL-2 (50 U/ml), IL-7 (20 U/ml), and IL-15 (10 ng/ml) and BRAF WT and V600E epitopes, respectively (each: 10 µg/ml). For control, PBMC's were also incubated in complete medium together with the control peptide HIV. All cytokines were purchased from R & D Systems (Minneapolis, USA) and peptides from Proimmune (Oxford, UK). The next day, a FACS staining was performed using fluorescence-labelled antibodies as described for tetramer analysis. Additionally, Ki67 FITC was added to determine the proliferating cells.

HLA typing of papillary thyroid cancer patients

Genomic DNA was extracted from heparin blood samples using the DNAQiamp 96 DNA Blood kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

For genotyping of HLA genes HLA-A, -B, -C, -DRB1, -DRB3, -DRB4, -DQB1, -DPB1, an amplicon-based approach using the Illumina next generation sequencing technology was used. Primers were designed to target exons 2, 3, and 4 for class I genes and HLA-

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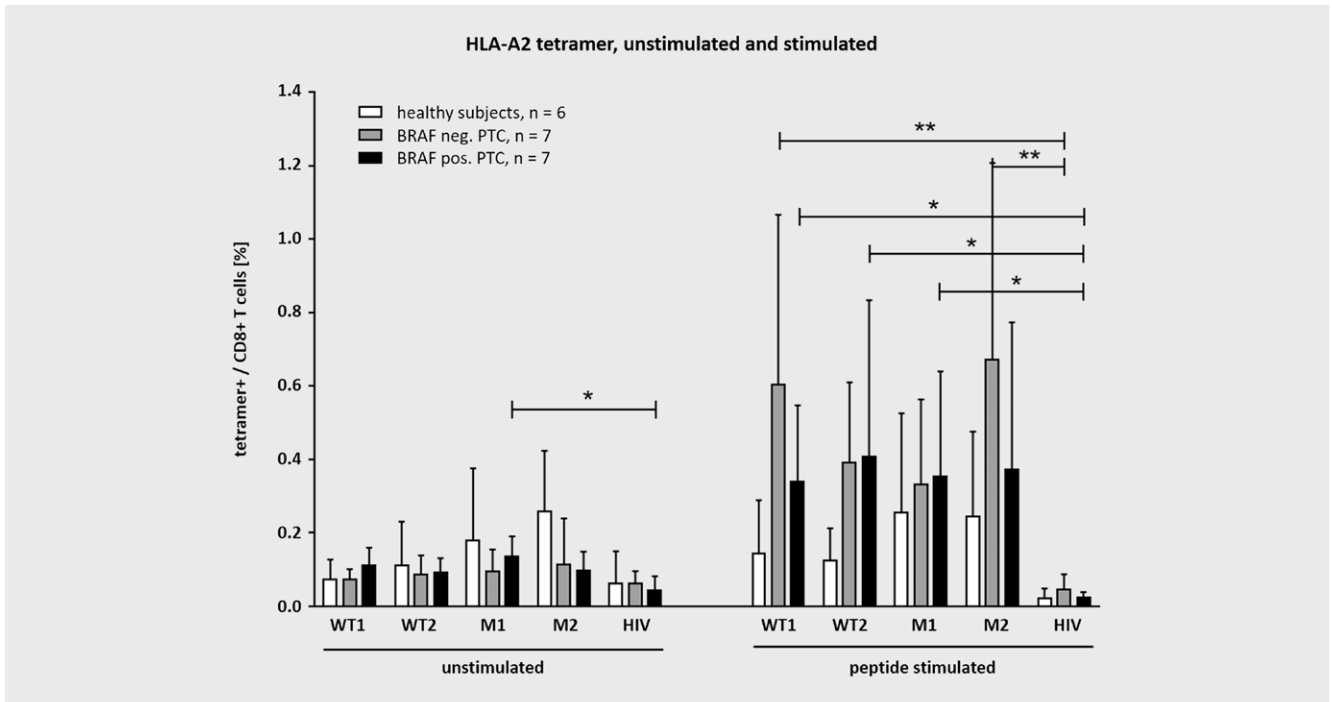

▶ **Fig. 1** Quantification of HLA class I restricted $BRAF^{V600E}$ and BRAF-WT specific T cells in PTC patients using tetramer analyses: $BRAF^{V600E}$ and BRAF-WT specific T cells were measured in PTC patients in unstimulated (left) and peptide-stimulated T cells (right). For one $BRAF^{V600E}$ epitope (M1), a significant increase of tumor-specific T cells could already been detected in unstimulated T cells (p = 0.0169). After stimulation with $BRAF^{V600E}$ and BRAF-WT peptides, however, a large increase of tumor-specific T cells could be seen for all epitopes tested ([mean ± std. deviation of $BRAF^{V600E}$ positive PTC patients: 0.02 ± 0.02 (HIV) vs. 0.34 ± 0.21 (WT1, p = 0.0171); vs. 0.41 ± 0.43 (WT2, p = 0.0242); vs. 0.35 ± 0.29 (M1, p = 0.0242) $BRAF^{V600E}$ negative PTC patients: 0.05 ± 0.04 (HIV) vs. 0.60 ± 0.46 (WT1); vs. 0.67 ± 0.54 (M2)]. Control peptide (HIV) did not reveal an increase of epitope-specific T cells.

DPB1, exons 2 and 3 for HLA-DRB1 and –DQB1 and exon 2 for HLA-DRB3 and -DRB4. The validation of the method was performed according to standard D.4.1.5 of the American Society for Histocompatibility and Immunogenetics (ASHI). The method was approved by ASHI for clinical use [22].

Briefly, the entire set of fragments was amplified in six multiplex PCR reactions and purified step using paramagnetic beads. A second-round PCR served to add sample-specific barcodes and Illumina compatible adapter sequences. The samples were pooled, underwent a second purification step were quantified using the QuantiFluor dsDNA system (Promega, Walldorf, Germany). Seven pM of the NGS library was applied to the MiSeq instrument (Illumina Inc.) for a paired-end 2 × 280 cycles run using a standard v3 cartridge according to the manufacturer's instructions. As an internal quality run control, a spike-in of 15 % PhiX was used. After de-multiplexing of the samples by the MiSeq Reporter software (Illumina Inc.) the analysis of the read sequences was performed by a Visual Basic-based in-house software (NGSAnalyser, Institute of Transplantation Diagnostics and Cell Therapeutics (ITZ), University Hospital of Düsseldorf, Düsseldorf, Germany) approach taking into account quality control values and high coverage to automate data analysis. In order to distinguish between sequencing artefacts such as crossover products and closely related alleles, we developed algorithms.

Statistical analyses

Prism software (PRISM 6, GraphPad Software, Inc., La Jolla, CA, USA) was used for calculation of statistical significances: For data showing a Gaussian distribution, the ANOVA-test and Dunnett's multiple comparison test were performed. For not normally distributed data, the Kruskal–Wallis test and Dunn's multiple comparison test were used. To investigate the distribution of a characteristic HLA molecule, contingency tables were analyed by Chi-square test (in case of more than 5 patients in each group) or Fisher's exact test. p-Values < 0.05 were considered as significant.

Results

Prevalence of $BRAF^{V600E}$ mutation and HLA-type in PTC patients

FFPE tissue specimens were available from 14 HLA A2 positive patients. Of those n = 7 were $BRAF^{V600E}$ positive whereas n = 7 were $BRAF^{V600E}$ negative ($BRAF^{V600E}$ positive: n = 4 females; $BRAF^{V600E}$ negative: n = 4 females). While performing the study, these patients were also tested for the MHC class II haplotype. Here, n = 2 patients were BRAFV600E positive, HLA A2 positive as well as positive for HLA-DQ8. n = 5 patients were BRAFV600E positive, HLA A2 positive and HLA-DQ8 neg. All n = 7 BRAFV600E negative patients were HLA A2 positive and HLA-DQ8 negative.

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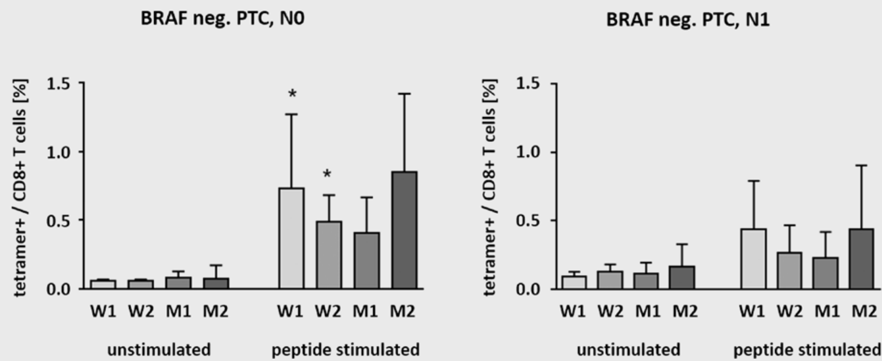

▶ **Fig. 2** Connection between the number of HLA class I restricted, BRAF^{V600E} and BRAF-WT specific T cells and the patient's tumor stadium: An increase of tumor epitope-specific T cells could be seen in all PTC patients after peptide stimulation irrespective of the initial tumor stadium. In regard to the tumor size or the metastatic spread, however, no significant differences could be seen [**a**: BRAF negative PTC patients: n = 4 (N0) and n = 3 (N1); **b**: BRAF positive PTC patients: n = 2 (N0) and n = 6 (N1)]. w = Wild-type BRAF-epitope (epitope 1 and 2, respectively); m = Mutated BRAF-epitope (BRAFV600E; epitope 1 and 2, respectively).

Prevalence of MHC class I restricted BRAF^{V600E} and BRAF-WT specific T cells in PTC patients

We first investigated the number of MHC class I restricted BRAF^{V600E} and BRAF-WT specific T cells in PTC patients (▶ **Fig. 1**). For one BRAF^{V600E} epitope (M1), a significantly higher number of tumor-specific T cells could be detected in unstimulated T cells of BRAF^{V600E} positive PTC patients, compared to the number of HIV (control peptide) specific T cells (mean ± std. deviation: 0.13 ± 0.06 vs. 0.04 ± 0.04; p = 0.0169). After stimulation with BRAF^{V600E} and BRAF-WT peptides, however, a significantly higher number of antigen-specific T cells could be detected for BRAF^{V600E} positive patients (WT1, WT2, M1) as well as BRAF^{V600E} negative patients (WT1 and M2) compared to HIV [(mean ± std. deviation of BRAF^{V600E} positive PTC patients: 0.02 ± 0.02 (HIV) vs. 0.34 ± 0.21 (WT1, p = 0.0171); vs. 0.41 ± 0.43 (WT2, p = 0.0242); vs. 0.35 ± 0.29 (M1, p = 0.0242). BRAF^{V600E} negative PTC patients: 0.05 ± 0.04 (HIV) vs. 0.60 ± 0.46 (WT1); vs. 0.67 ± 0.54 (M2)].

Connection analyses between the number of MHC class I restricted BRAF^{V600E} and BRAF-WT specific T cells and the tumor stadium

All data were also analyzed in regard to the TNM status of the patients (▶ **Fig. 2a**). An increase of tumor epitope-specific T cells could be seen in all PTC patients after peptide stimulation irrespective of the initial tumor stage. In some patients, these differences reached significant changes in regard to the lymph node status in BRAF^{V600E} positive and BRAF WT patients, respectively (▶ **Fig. 2b**). In regard to the tumor size or the metastatic spread, however, no significant differences could be seen.

Prevalence of MHC class II restricted BRAF^{V600E} and BRAF-WT specific T cells in PTC patients

All patients included in the study were also MHC class II typed. Six out of 14 PTC patients revealed an HLA-DQ8 phenotype. Of those, 2 patients were BRAF^{V600E} positive whereas 4 patients showed a BRAF WT. In all patients, HLA-DQ8-restricted, BRAF-specific T cells could be detected. There was a further increase after overnight peptide stimulation in all patients. An increased of IFN gamma production could, however, not be detected.

Discussion

Papillary thyroid cancer is characterized by a lymphatic infiltration and frequently by lymph node metastases without distant metastatic spread. Many PTC patients reveal a somatic BRAF^{V600E} mutation [7–11]. The aim of the present study was to investigate the role of BRAF-specific T cells in the antitumor immunity in PTC. To do so, BRAF-specific T cells were measured in the peripheral blood of PTC patients with and without in vitro stimulation. Moreover, FACS analyses using tumor epitope specific tetramers were also performed. We could clearly show a large increase of BRAF^{V600E} and BRAF-WT tumor-epitope specific T cells in PTC patients. However, this did not correlate with the N-stage.

Based on a previously published paper [18], we initially expected to see a pure BRAF^{V600E} specific T cell immunity in BRAF^{V600E} positive PTC patients. Tumor specific T cells could, however, also be detected in patients with a BRAF wildtype. This is not surprisingly, since strong T cell infiltrations are also observed in PTC patients with the BRAF WT. We also correlated our data with the grade of T cell tumor infiltration. Here, no clear picture could be seen, supposedly due to the low number of tumor epitope specific T cells within the peripheral blood. By using MHC class I and MHC class II restricted tetramers, we also investigated the existence of tumor epitope specific T cells within the tumor sites by using immunofluorescence (data not shown). Here, no BRAF-specific T cells could be detected mainly due to the low number of BRAF epitope-specific cells.

A limitation of this study is the low number of patients included which is however mainly due to the fact that only 28 % (n = 42) of all initially included PTC patients (n = 150) underwent surgery at the University Hospital Duesseldorf and FFPE tissue specimens were available. Of these n = 14 patients were BRAF^{V600E} positive (n = 7

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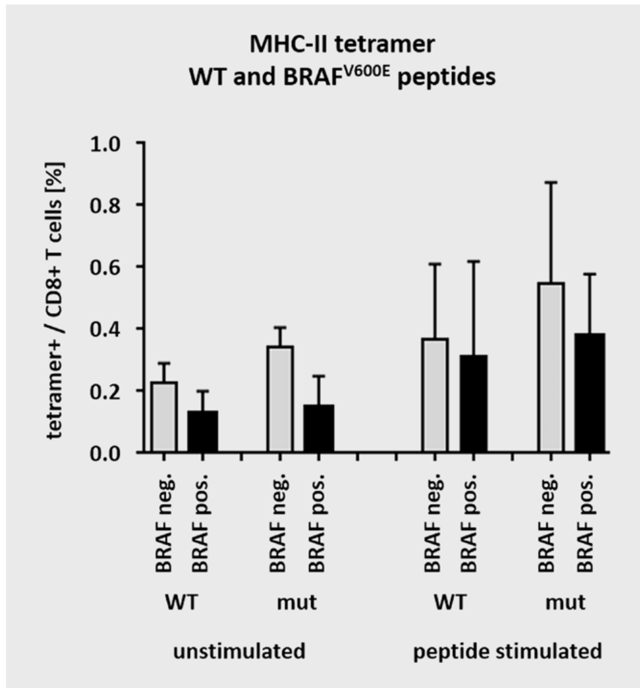

▶ **Fig. 3** Prevalence of HLA class II restricted, BRAF^{V600E} and BRAF-WT specific T cells in PTC patients: All patients included in the study were also HLA class II typed. n = 6 BRAF positive PTC patients were analyzed for the prevalence of DQ8-restricted antigen-specific T cells. Of those, n = 2 were HLA-DQ8 positive and n = 4 were HLA-DQ8 negative. In all patients, HLA-DQ8-restricted, BRAF-specific T cells could be detected. There was a further increase of cell numbers in all patients after overnight peptide stimulation. An increase of IFN gamma production could, however, not be detected (data not shown).

HLA-A2 positive). A higher number of PTC patients fulfilling these criteria is difficult to gain. Another limitation is the fact that all patients were free of disease (initial diagnosis: 3–16 years before, mean 7 years) at the time of the study, possibly affecting the number of tumor epitope-specific T cells. Therefore, the low number of tumor epitope specific T cell detected in our study could also be due to this fact. Our study should therefore be repeated in a prospective design with newly diagnosed PTC patients.

In summary, our study describes for the first time a BRAF-specific tumor immunity in PTC-patients which is, however, independent of a BRAF^{V600E} status of the PTC patients. It can be postulated, that the BRAF-specific tumor immunity, besides further antigen-specific antitumor immunity, might contribute to the excellent prognosis of most PTC patients. This may also have a clinical implication for future BRAF-specific immunotherapy including a potential therapy with (autologous) BRAF-specific T cells.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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