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PD-1 inhibition in patient derived tissue cultures of human gastric and gastroesophageal adenocarcinoma

Marlon Hußtegge[a,b], Ngoc Anh Hoang[b], Jakob Rebstock[a,b], Astrid Monecke[c], Ines Gockel[d], Arved Weimann[e], Guido Schumacher[f], Ingo Bechmann[a], Florian Lordick[b], Sonja Kallendrusch ![ORCID][a]*, and Justus Körfer[b]*

aInstitute of Anatomy, University of Leipzig, Leipzig; bDepartment of Oncology, Gastroenterology, Hepatology, Pulmonology, and Infectious Diseases, University Cancer Center Leipzig, University Hospital Leipzig, Leipzig, Germany; cInstitute of Pathology, University Hospital Leipzig, Leipzig, Germany; dDepartment of Visceral, Transplantation, Thoracic and Vascular Surgery, University Hospital Leipzig, Leipzig, Germany; eDepartment for General and Visceral Surgery, Hospital St. Georg Leipzig, Leipzig, Germany; fDepartment for General and Visceral Surgery, Hospital Braunschweig, Braunschweig, Germany

ABSTRACT

Emerging immunotherapies quest for better patient stratification in cancer treatment decisions. Moderate response rates of PD-1 inhibition in gastric and esophagogastric junction cancers urge for meaningful human model systems that allow for investigating immune responses *ex vivo*. Here, the standardized patient-derived tissue culture (PDTC) model was applied to investigate tumor response to the PD-1 inhibitor Nivolumab and the CD3/CD28 t-lymphocyte activator ImmunoCult™. Resident t-lymphocytes, tumor proliferation and apoptosis, as well as bulk gene expression data were analyzed after 72 h of PD-1 inhibition either as monotherapy or combined with Oxaliplatin or ImmunoCult™. Individual responses to PD-1 inhibition were found *ex vivo* and combination with chemotherapy or t-lymphocyte activation led to enhanced antitumoral effects in PDTCs. T-lymphocyte activation as well as the addition of pre-cultured peripheral blood mononuclear cells improved PDTC for studying t-lymphocyte and tumor cell communication. These data support the potential of PDTC to investigate immunotherapy *ex vivo* in gastric and esophagogastric junction cancer.

Introduction

Gastric cancer (GC) and esophagogastric junction cancer (EGJC) are highly aggressive and heterogeneous tumors.[1,2] Despite ongoing progress in new treatment concepts, GC and EGJC still have a poor prognosis[3] and clinical response to standard chemotherapy regimens is unpredictable.[4] In the last decade, targeted treatment strategies (*e.g.*, Her2 blockade or VEGFR inhibition) and immune checkpoint inhibitors (*e.g.*, PD-1/PD-L1 blockade) have complemented chemotherapy.[5] Immune checkpoint molecules, expressed on immune and cancer cells suppress the immune response and favor tumor progression.[6] In GC and EGJC, anti PD-1 monotherapies display moderate efficiency.[7,8] Combined treatment of immune checkpoint inhibition and chemotherapy are currently investigated in several clinical trials.[9–11] Still, with the rise of novel therapeutic choices, individual patient stratification becomes more important. Predictive biomarkers in GC and EGJC are still limited.[12,13] Microsatellite instability, PD-L1 expression, tumor mutational burden, high amount of tumor infiltrating lymphocytes and specific mRNA expression profiles (*e.g.*, IFNg-related signature) can indicate potential benefit of immune checkpoint inhibition.[14,15] Despite these biomarkers, the overall response to checkpoint inhibition remains limited and individual response is not efficiently predictable.

Basic cell culture and immunocompromised mouse models depict established models for examination of tumor biology and development of novel treatments with limitations in restricted complexity and species differences.[16,17] Therefore, translation from bench to bedside often fails as the majority of drugs selected by preclinical testing are not effective in patients.[18,19] The important role of the tumor microenvironment and immune targets are recognized and urge for more complex approaches to investigate tumor cells in a clinical relevant model system. For enhanced understanding of treatment mechanisms in immune checkpoint blockade and more precise patient stratification, human tumor models, providing enhanced representation of the biological complexity of cancer and the tumor microenvironment, are needed.

Patient-derived tissue cultures (PDTC) were successfully established for several tumor entities and preserve intra- and intertumoral heterogeneity as well as individual tumor microenvironment (TME) for several days *ex vivo*.[20–24] Besides, PDTC provide an unique *ex vivo* system to investigate treatment with chemotherapy, radiation or molecularly targeted therapies in individual patients.[20–22,25–30] Therefore, in this study, we analyzed the preservation and functionality of the resident t-lymphocyte population and further investigated the effect of different immune modulating drugs in PDTC of GC and EGJC *ex vivo*.

Methods

Specimens

Patient-derived tumor specimens were received from clinical departments of the University Hospital Leipzig and two academic hospitals in Braunschweig and Leipzig. In total, material from 15 patients with GC and EGJC was included in this study and used for qualitative and quantitative analyses. Patient's characteristics are shown in Supplemental Table 1. This study was approved by the ethics committee of the Medical Faculty, University of Leipzig. All patients had given their informed written consent.

Preparation of tumor tissue cultures

PDTC were processed immediately after surgical resection and pathological assessment. The preparation was performed as previously described.[28] In brief, tumor specimen were cut into 350 µm slices with a tissue chopper (McIlwain TC752; Campden Instruments, Leicestershire, England). Afterward the size of the slices was standardized by a coring tool (kai Europe, Solingen, Germany) into slices of 3 mm in diameter. Then, tumor slices were cultivated on 6-well-plates on 0.4 µm PFTE membrane inserts (Millipore Corporation, Billerica, MA, USA) under standard conditions (5% CO_2, 37°C). Baseline tissue was fixed with 4% PFA after preparation and cultivated tissue was fixed with 4% PFA after 78–96 h of cultivation.

Experimental setup

Slices were cultured 6–24 h in culture medium, which was composed of RPMI-1640 (Gibco, Life Technologies, Paisly, UK), 10% fetal bovine serum (fetal bovine serum (FBS), Gibco), 1% amphotericin B (Carl Roth, Karlsruhe, Germany), 1% penicillin/streptomycin (Gibco) and 1% L-glutamine (Gibco). Then, tumor slices were treated with Nivolumab (3 µg/ml and 10 µg/ml, Bristol Myers Squibb, New York, NY, USA), ImmunoCult™ (CD3/CD28 t-cell activator, 25 µl/ml, Stemcell Technologies, Vancouver, BC, Canada), Oxaliplatin (20 µmol/l and 100 µmol/l, Sanofi-Aventis, Paris, France), Oxaliplatin (20 µmol/l) plus Nivolumab (3 µg/ml) and ImmunoCult™ (25 µl/ml) plus Nivolumab (3 µg/ml) for 72 h. Media were changed after 6–24 h, after 24 h and at 72 h.

Besides using our standard established culture medium, different compositions of media were tested. We have used RPMI-1640 (Gibco), Hibernate A (Gibco) and X–Vivo (Lonza, Basel, Switzerland) supplemented with 10% FBS (Gibco), 1% amphotericin B (Carl Roth), 1% penicillin/streptomycin (Gibco), 1% L-glutamine (Gibco) and 1% insulin, transferrin, selenium (insulin, transferrin, selenium [ITS]; Gibco) in various combinations (Supplemental Table 2).

Staining procedure

Slices were embedded in paraffin and cut into 5 mm sections. After antigen retrieval and blocking with normal goat serum (Jackson ImmunoResearch, Ely, Cambridgeshire, UK) primary antibodies, diluted in 0.5% bovine serum albumin (Carl Roth), were applied overnight at 4°C. Following antibodies were used:

AE1/3 (BioGenex, Fremont, CA, USA, mouse, 1:100), cPARP (abcam, Cambridge, UK, rabbit, 1:100), Ki67 (DCS, Hamburg, Germany, rabbit, 1:200), CD3 (Bio-Rad laboratories, Hercules, CA, USA, rat, 1:200), CD8a (Cell Signaling Technology, Danvers, MA, USA, mouse, 1:100) and FoxP3 (Invitrogen, mouse, 1:100). Then, sections were washed with 0,3% PBS/Triton and secondary antibodies were added for 1 h at room temperature. Tissue stained with diaminobenzidine (DAB, Sigma Aldrich) and counterstained with hematoxylin. The following secondary antibodies were used: biotinylated goat anti rat (Vector laboratories, Burlingame, CA, USA, 1:100) and biotinylated goat anti-mouse (Sigma Aldrich, 1:100) diluted in 0,3% phosphate buffered saline/TritonX. For staining of FoxP3 0,5% tris buffered saline/Tween. Following secondary antibodies were used for immunofluorescence staining: goat anti rabbit 568 (AlexaFluor, Invitrogen; Eugene, OR, USA, 1:500) and goat anti mouse (AlexaFluor, Invitrogen, Eugene, OR, USA, 1:500). Nuclei were stained with Hoechst 33342 (Sigma-Aldrich, St.Louis, MO, USA, 1:10.000).

Peripheral mononuclear blood cells (PBMC) isolation and cultivation

PBMCs were isolated from EDTA stabilized patient's blood 5 days before surgery took place. PBMCs were isolated with Lymphoprep™ density gradient medium (15 ml; Stemcell Technologies) according to manufacturer's protocol. PBMCs were cultured in a 48 well plate ($2x10^6$ cells per well, 200 µl/well) for 5 days in t-cell expansion medium (ImmunCult™-; Stemcell Technologies) supplemented with 1% penicillin/streptomycin and 1% L-glutamine. Besides, one part of PBMCs were pre-treated with ImmunoCult™ (25 µl/ml) for 3 days. Each cultured slice was treated with 500.000 PBMCs after 2 h of cultivation, applied within a polypropylene ring around the slice for 30 min. Then slices were treated as described above with Nivolumab over 72 h.

mRNA expression analysis with Nanostring nCounter platform

RNA was isolated from formalin-fixed paraffin-embedded (FFPE) tissue sections. A total of 10–15 FFPE section (5 µm) were used for RNA isolation using the FFPE RNA isolation kit (Quiagen N.V., RNeasy FFPE Kit (50), ID: 73504, Venlo, the Netherlands) according to the manufacturer's protocol. For gene expression analysis, NanoString nCounter platform was used. The PanCancer IO Panel 360™ Panel was applied, and analysis was performed according to the manufacturer's protocol.

Analysis

Hematoxylin-eosin (H&E) staining was performed to estimate histomorphology of PDTC. H&E sections were evaluated by using slide scans (Panoramic SCAN and Panoramic Viewer, 3D Histech, Budapest, Hungary). Experiments representing no tumor cells in baseline tissue specimen were excluded from analysis. Further basic parameters like tumor cell fraction, proliferating tumor cell fraction and apoptotic tumor cell fraction were determined by quantification of immunofluorescence stainings (AE1/

3, Ki67 and cPARP). Five pictures per slice (3x5 pictures per condition) were taken manually by using an Olympus BX51 fluorescent microscope (Olympus Deutschland, Hamburg, Germany). In order to analyze these pictures, we have used a semi automatized pixel counting algorithm (created by Sönnichsen et al. 2018).[28] Total positive pixel of Hoechst 33342, AE1/3, cPARP, Ki67 were counted to quantify cell nuclei (Hoechst 33342-positive), tumor cell fraction (Hoechst 33342-, AE1/3-positive), apoptotic tumor cell fraction (Hoechst 33342-, AE1/3-, cPARP-positive) and proliferating tumor cell fraction (Hoechst 33342-, AE1/3-, Ki67-positive). Only pictures with more than 2% tumor cells (Hoechst 3342-, AE1/3-positive) were considered for further quantification of apoptotic and proliferating tumor cell fractions. Due to experimental variance that were similarly described in Sönnichsen et al[28] and Körfer et al[26] we normalized each condition to the control condition (CTR = 1). To differentiate responding vs. non-responding specimens the cutoff was determined to be equal or higher than 2.5 times of the CTR.

Immunohistochemistry sections of immune cell stainings were scanned by a slide scanner and quantified manually by counting positive immune cells (CD3, CD8, and FoxP3) in QuPath software (QuPath, Edinburgh, UK). DAB-positive cells were illustrated in proportion to hematoxylin-positive cells.

Statistical analysis

For statistical analysis of pictures of immunofluorescence/immunohistochemical stainings, mean values for conditions were calculated using mean slice values and standard error of the mean (SEM). For combination of different experiments mean values and SEM were calculated from mean condition values. Gene expression data were normalized to 15 housekeeping genes (ABCF1, DNAJC14, ERCC3, G6PD, GUSB, MRPL19, NRDE2, OAZ1, POLR2A, PSMC4, PUM1, SDHA, SF3A1, STK11IP, TBC1D10B, TBP, TFRC, TLK, TMUB2, UBB) and background thresholding was performed with NanoString nSolver software. IFNg signature[15] (CD27, CD274, CD276, CD8A, CMKLR1, CXCL9, CXCR6, HLA-DQA1, HLA-DRB1, HLA-E, IDO1, LAG3, NKG7, PDCD1LG2, PSMB10, STAT1, TIGIT) and immune suppression score[31] (IMS: CCL8, VCAN, CCL2, CD163, CCL13, COL6A3, BCAT1, ADAM12, AXL, ISG15, SIGLEC1, PDGFRB, IL10, STC1, OLFML2B, TWIST2, FAP, INHBA) were calculated according to publications. For each experiment, relative change of gene expression data in treated conditions (*e.g.*, Nivolumab) related to the negative control was calculated.

GraphPad Prism 8 (GraphPad Software, La Jolla, VA, USA) was used for calculating one-way analysis of variance with Bonferroni post test correction and students t-test. $P < 0.05$ was set significant. Non-significant changes were not illustrated in the figures.

Results

Preservation of tissue resident t-lymphocytes in PDTC of GC and EGJC over four days *ex vivo*

In order to evaluate PDTC in GC and EGJC as a model for exploring immunotherapy *ex vivo*, we have first looked for t-lymphocyte morphology and functionality preservation in PDTC of GC and EGJC by analyzing overall t-lymphocyte populations (CD3+) at the beginning of cultivation and after four days in culture (Figure 1a). Prior to cultivation, we observed a high individual variance in total t-lymphocyte infiltration as it has been already observed in GC and EGJC tumor specimens[32] (supplemental Figure 2). Quantification of CD3 staining was done in 11 cases (Figure 1b). Here, we can observe that the fraction of CD3+ cells remains stable on day 4 of cultivation compared to baseline tissue. Moreover, the subpopulations of cytotoxic t-lymphocytes (CD8+) and regulatory t-lymphocytes (Tregs, FoxP3+) did not show significant change throughout culture period of four days *ex vivo* (Figure 1c and 1d).

Exploration of modified culture media for PDTC

We examined whether histomorphological preservation of PDTC in general and especially of t-lymphocytes after 4 days in culture can be improved by altered culture media composition (media A-H) (supplemental Table 2). Taking four cases into account, no significant difference of apoptotic or proliferating tumor cell fractions was detected between the investigated compositions (supplemental Figure 1b and 1c). Intratumoral heterogeneity, represented by three tissue slices forming one condition demonstrated a high variability of apoptotic-/proliferating tumor cell fractions that was also observed in a previous study of colorectal cancer.[28] Investigating t-lymphocyte population (CD3+) in different media composition conditions, no significant difference in t-lymphocyte preservation was observed (Supplemental Figure 1d). All further functional experiments were subsequently conducted with medium A.

PBMCs can be co-cultivated with PDTC of GC and EGJC after preceding t-lymphocyte stimulation *ex vivo*

PDTC preserve tissue resident t-lymphocytes during culture period as shown above. To examine potential migration of immune cells into PDTC and opportunities of immune modification in PDTC *ex vivo*, co-culture experiment with patient-specific PBMCs are shown as proof of principle (n = 1). PMBCs were isolated and stimulated with CD3/CD28 t-cell activator (25 μl/ml) *ex vivo* before they were applied to tissue cultures. Tissue cultures were analyzed after 4 days of co-cultivation with stimulated PBMCs. A higher infiltration of CD3 + t-lymphocytes was seen in co-cultured tissue with PBMCs. Figure 2 shows apoptotic and proliferating tumor cell fractions of this case, demonstrating altered cPARP and Ki67 expression together of tumor cells.

Nivolumab treatment *ex vivo* results in heterogeneous antitumoral effects

In order to investigate functionality of resident t-lymphocytes in PDTC, we have treated 10 different tumor specimens with Nivolumab *ex vivo*. Two clinical relevant concentrations of Nivolumab were applied and we did not see significant changes in apoptotic tumor cell fractions between both concentrations (supplemental Figure 4b, n = 3). Furthermore, the IgG vehicle
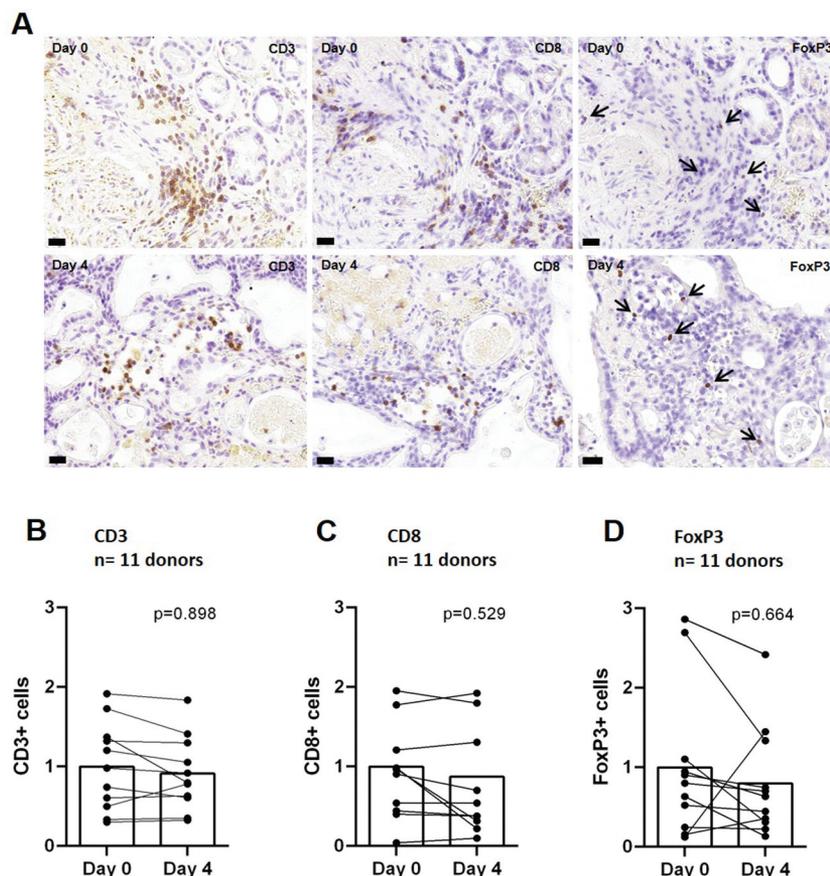
Figure 1. Preservation of t-lymphocytes in PDTC of GC and EGJC after 4 days (a) Representative images of immunohistochemical staining, showing t-lymphocytes (CD3), cytotoxic t-lymphocytes (CD8) and regulatory t-lymphocytes (FoxP3) at day 0 and after 4 days of cultivation. (b) Quantification of DAB stainings for t-lymphocytes (CD3) and (c, d) subtypes (CD8, FoxP3) at day 0 compared to 4 days *ex vivo*. Matched tissue specimens of 11 specimen donors were analyzed. Mann-Whitney-U test (P ≤ 0,05) showed no significant change of t-lymphocyte amounts. Day 0 normalized to 1; bars = 40 µm; error bars: SEM.

treatment did not lead to differences in basic parameters like apoptosis and proliferation compared to the control (supplemental Figure 5, n = 7). Concluding these observations, further experiments were performed with Nivolumab 3 µg/ml and with no additional control for the IgG vehicle. In 10 cases treated with Nivolumab alone, we determined 7 cases in which the relative change of apoptotic tumor cell fraction did not differ significantly from control conditions and we observed 3 cases in which the relative change of tumor cell fraction increased compared to control (Figure 3e and 3f). Two cases are shown in detail demonstrating that differentiation in responding and non-responding tissue can be observed. Case #M41 showed a non-significant increase of apoptosis rate after treatment with single Nivolumab supplementation compared to the control condition (Δ1,66, Figure 3a and 3c). Furthermore, a higher fraction of t-lymphocytes was measured after Nivolumab treatment compared to control in this case (supplemental Figure 3b). Contrasting these results, we also have observed in case #M38 no increase of apoptosis rate after Nivolumab treatment (Δ0,24, Figure 3b and 3d).

was applied in combination with CD3/CD28 t-cell activator (ImmunoCult^TM) in four cases for 72 h. CD3/CD28 t-cell activator was used for general t-cell stimulation to examine overall t-cell vitality and functionality. The combined supplementation of Nivolumab and the t-cell activator was applied to investigate the potency of immune activation in potential immune suppressed tissue specimens. Tumor apoptosis rate was increased significantly by the combination compared to monotherapy with Nivolumab in all cases (Δ3.77, p≤0.01) or compared to monotherapy with CD3/CD28 t-cell activator (Δ2.68, p≤0.05, Figure 4a). Furthermore, monotherapy with CD3/CD28 t-cell activator showed an increased apoptotic tumor cell fraction compared to control, however due to high variances, the difference was not statistically significant (Δ1.87, Figure 4a). Regarding individual specimens, tissue responding to Nivolumab treatment showed benefits of combinational supplementation (#M41, Δ4.16) as well as non-responding tissue demonstrated a general enhancement of the tumor apoptosis rate after treatment with Nivolumab and the CD3/CD28 t-cell activator (#M38, Δ7.20, Figure 4b-c).

Combined therapy with t-lymphocyte stimulation and Nivolumab enhances response in GC and EGJC

We investigated if tissue not responding to single Nivolumab therapy respond to combinational therapies *ex vivo*. Nivolumab

Response of PDTC to combined therapy with Nivolumab and Oxaliplatin

To evaluate clinical relevant therapies in PDTC of GC and EGJC, Nivolumab treatment was combined with Oxaliplatin.
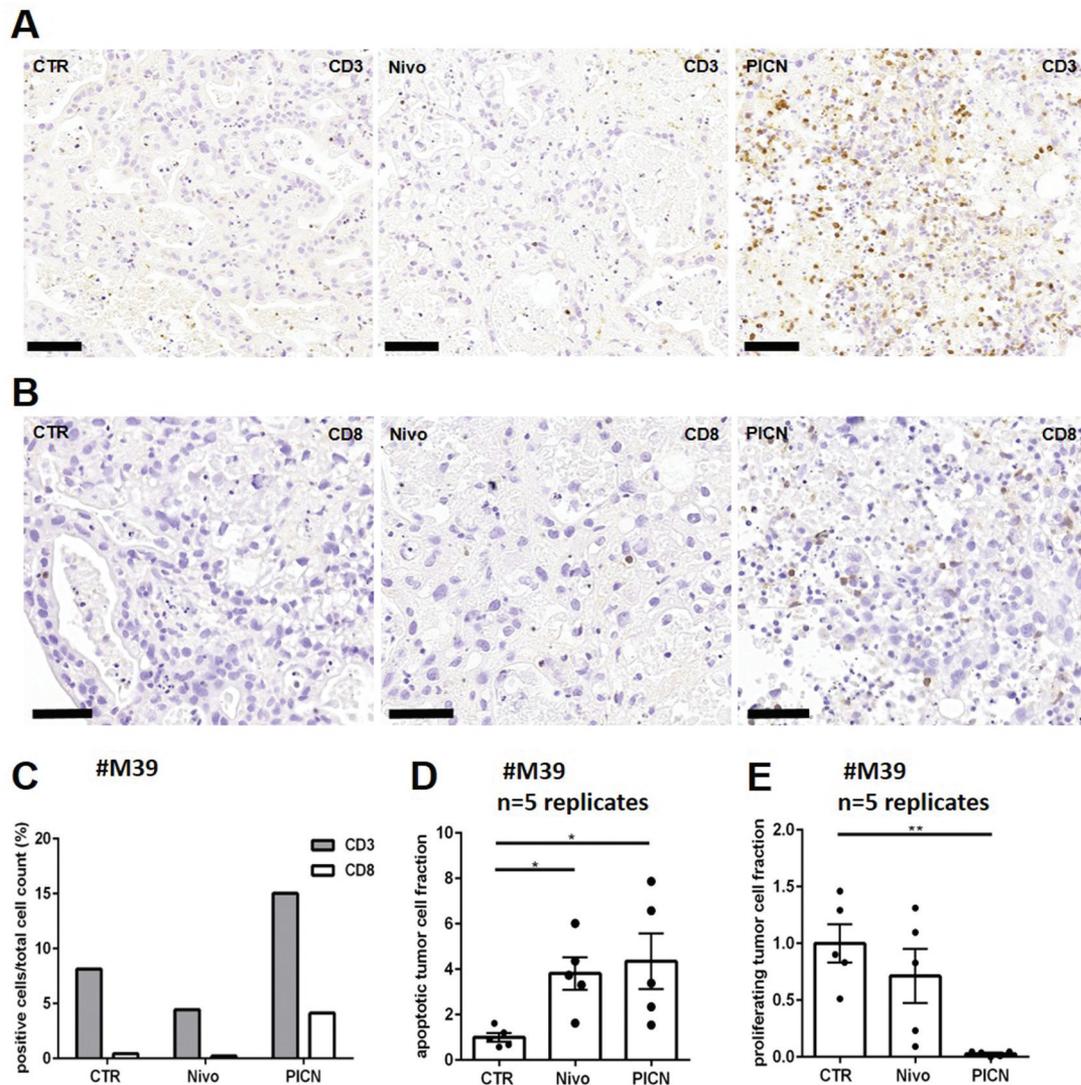
Figure 2. Co-cultivation of peripheral mononuclear blood cells (PBMC) with PDTC Tissue cultures of #M39 were supplemented with either Nivolumab (Nivo) or together with stimulated PBMCs (PICN) and analyzed after 72 h of treatment. (a, b) Representative pictures of DAB stainings with antibodies against CD3 (t-lymphocyte) and CD8 (cytotoxic t-lymphocyte). (c) Quantification of CD3- and CD8 DAB staining. (d, e) Normalized effects of Nivolumab and PBMC co-cultivation on tumor cell apoptosis (cPARP) and tumor cell proliferation (Ki67). bars = 40 µm; CTR, control; error bars: SEM; *p ≤ 0,05, **p ≤ 0,01; Kruskal-Wallis test.

Shown previously, application of chemotherapy in PDTC results in dose-dependent effects.[23,26,28] Here, two different concentrations (20 µmol/l and 100 µmol/l) of Oxaliplatin were investigated. We observed dose-dependent enhancement of the apoptotic tumor cell fraction compared to control conditions (Ox20, Δ1.75; Ox100, Δ5.06, p≤0.01) and between the applied concentrations (Δ3.31, p≤0.05) (supplemental Figure 4a). Additionally, PDTC were treated with Nivolumab combined with Oxaliplatin (20 µmol/l, NOx) in five cases. Here, we observed an non-significant increase of apoptotic tumor cells compared to monotherapy with Nivolumab (Δ2.21) or Oxaliplatin (Δ1.36, Figure 5a). By analyzing individual cases, we observed major differences in tissue response to NOx (Figure 5b-db). Comparing several experiments, non-responding tissue to Nivolumab as well as to Oxaliplatin showed minor benefits of a combinational therapy compared to Nivolumab (#M32, Δ0.71) whereas non-responding tissue to Nivolumab and responding tissue to Oxaliplatin displayed measurably higher amount of apoptotic tumor cell fraction after treatment with NOx compared to monotherapy with Nivolumab (#M38, Δ5.16, Figure 5b-c). Further we observed an enhancement of apoptotic tumor cell fraction in Nivolumab and Oxaliplatin responder after combined treatment (#M41, Δ7.02, Figure 5d).

Increase of t-cell effector gene expression in PDTC after immunomodulatory treatment *ex vivo*

To investigate molecular changes in PDTC, RNA bulk analysis of formalin-fixed tissue slices was performed by the NanoString nCounter platform. Analysis was conducted with six experiments of different patient specimens. Regarding tumor cell apoptosis, we categorized six tissue specimens in responding tissue to Nivolumab (Δ>2.5) and non-responding tissue (Δ<2.5), defined by immunohistochemistry analysis. We evaluated a gene expression set of
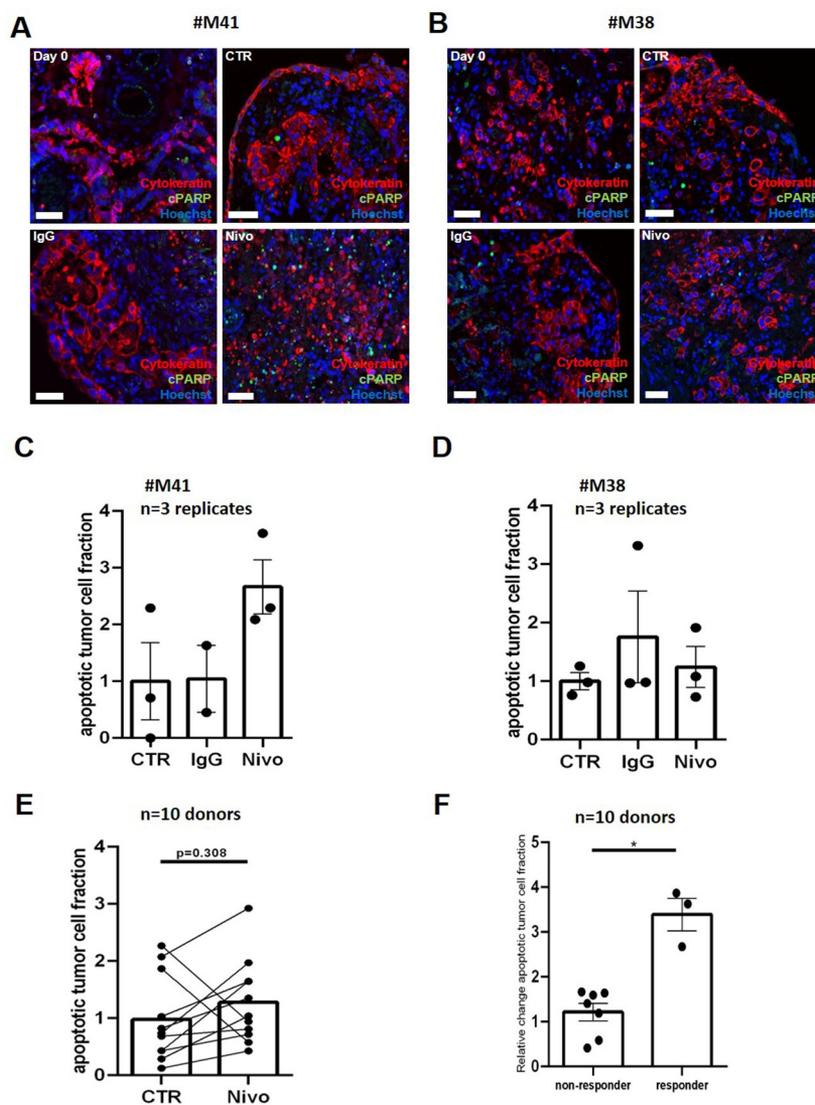
Figure 3. PD-1 inhibition in PDTC of GC and EGJC. (a, b) Representative immunofluorescence images of apoptotic tumor cells at day 0 and 4 days (CTR) *ex vivo*. Nivolumab and its respective IgG were applied for 72 h. (C, D, E) Quantification of the apoptotic tumor cell fraction after treatment normalized to the respective control condition in two single cases (#M38 and #M41) and in 10 different donor specimens respectively. (f) A change of the apoptotic tumor cell fraction greater than Δ2.5 was set as cut off for discrimination of Nivolumab responder and non-responder (*p ≤ 0,05; Mann-Whitney-U test, n = 10 patients). Hoechst, blue; Cytokeratin, red; cPARP, green, control (CTR), Nivolumab (Nivo, 3 μg/ml) and IgG (3 μg/ml); bars = 20 μm; error bars: SEM.
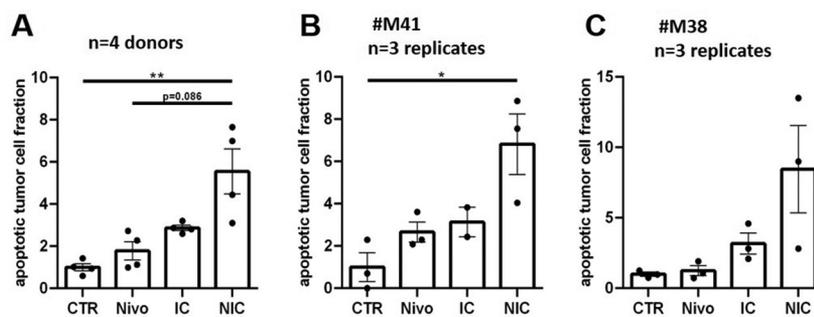

Figure 4. Combined therapy of CD3/CD28-T-cell stimulation and Nivolumab in PDTC of GC and EGJC. (a) Quantification of apoptotic tumor cell fraction after 4 days *ex vivo* in PDTC of four tissue donors (b) Quantification of apoptotic tumor cell fraction of potential Nivolumab responder (#M41) and (c) potential non-responder (#M38) after 4 days *ex vivo*. Control (CTR), Nivolumab (Nivo; 3 μg/ml), t-cell activator (IC, 25 μg/ml) and combined supplementation with Nivolumab and t-cell activator (NIC); error bars: SEM; *p ≤ 0,05, **p ≤ 0,01; Kruskal-Wallis test.
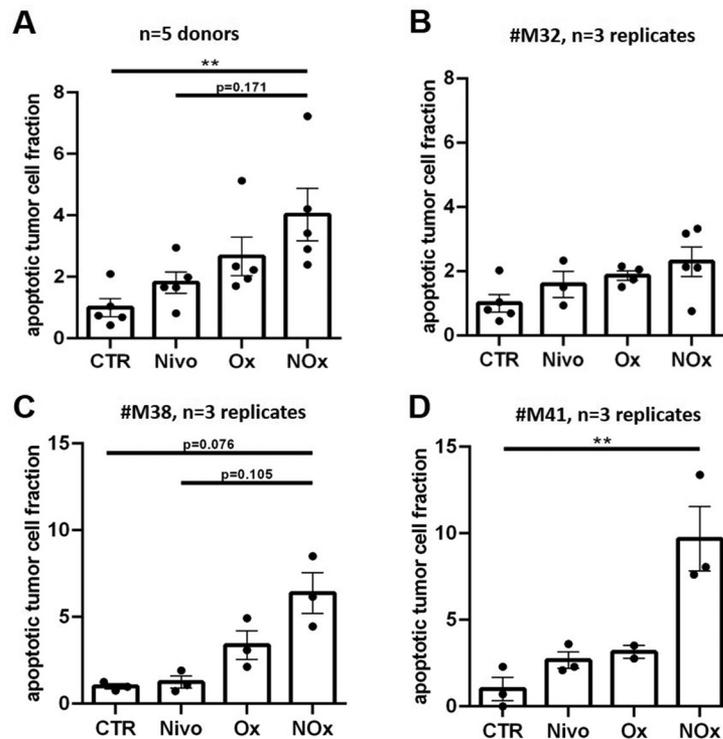
Figure 5. Combined therapy with Nivolumab and chemotherapy in PDTC of GC and EGJC. (a) Quantification of apoptotic tumor cell fraction after 4 days *ex vivo* in in PDTC of five tissue donors. (b-d) Quantification of apoptotic tumor cell fraction in different specific cases (#M32, #M38, #M41) which show various responses to monotherapy and combinational therapy. Control (CTR), Nivolumab (Nivo; 3 µg/ml), Oxaliplatin (Ox; 20 µmol/l) and combined therapy with Nivolumab and Oxaliplatin (NOx); error bars: SEM; **p ≤ 0,01; Kruskal-Wallis test.

five genes (IFNg, GMZA, GMZB, PRF1, FASLG) representing cytotoxic t-cell effector function as a surrogate for determination of Nivolumab response in all PDTC *ex vivo*.[33–36] By comparing the relative change of expression of the t-cell effector gene set, responding tissue show a higher relative change of gene expression compared to non-responding tissue (Δ0.54, p≤0.05; relative change: Nivo vs CTR; Figure 6a). Further, we analyzed two established gene expression scores, the interferon gamma score (IFNg) by Ayers et al.[15] and immune suppression score (IMS) by Cui et al.[31] were related to the t-cell effector gene set established in PDTC. A correlation between relative change of the t-cell effector gene set expression and the IFNg score were observed. Especially, one responder (#M41) shows in both scores high values compared to all other PDTCs (Figure 6b). Regarding the immune suppression score, an inverse correlation of mean expression level of IMS compared to relative change of t-cell effector gene set can be demonstrated (Figure 6c).

Discussion

Human model systems to investigate cancer cells in their relevant organotypic environment, displaying main characteristics, *e.g.* relevant immune cells, hypoxic areas and heterogeneous tumor populations, are desperately needed.[37] Here, we hypothesized that PDTC is a promising model to examine immunotherapy in GC and EGJC. This study shows that the resident t-lymphocytes can be manipulated and investigated in PDTC of GC and EGJC.

Heterogeneity and tumor mediated immunosuppression are characteristics for solid cancers leading to poor individual patient survival.[5,38] Interaction of cancer cells with micro-environmental immune, mesenchymal or endothelial cells has major impact on tumor progression, invasion, metastases as well as on drug resistance.[38–41] Especially for immune checkpoint blockade, primary and adapted resistance is observed in the majority of patients.[42] Intratumoral or systemic immunosuppression mediated by Tregs, myeloid cells (e.g., myeloid derived suppressor cells) or neutrophils has been suggested as one reason for minor response to PD-1 blockade.[38] Therefore, the presented tissue model may represent a useful complementary system in translational research.[17,37]

Antitumoral but also immunosuppressive functions are captured by t-lymphocytes within the TME, mediating tumor cell death by secreting effector molecules like granzymes A/B, perforin or interferon gamma.[34] T-lymphocyte activation can be inhibited by immune cells like Tregs secreting immunosuppressive cytokines (IL-10, TGFß) or upregulation of immune checkpoint molecules (*e.g.* PD-1, PDL-1, LAG-3, TIM-3, VISTA) by t-lymphocyte-activation.[43] Inhibition of these checkpoint molecules subsequently enhance antitumoral effects in individual patients activating CD8 + t-lymphocytes.[41] Here, we can show that the number of tumor infiltrating t-lymphocyte subgroups like CD8 + t-lymphocytes and Tregs remain stable over four days of culture compared to baseline tissue. These results and similar observations in other tumor entities show that tissue resident t-lymphocytes and macrophages are preserved and can be analyzed in PDTC of GC and EGJC.[20,22–24]
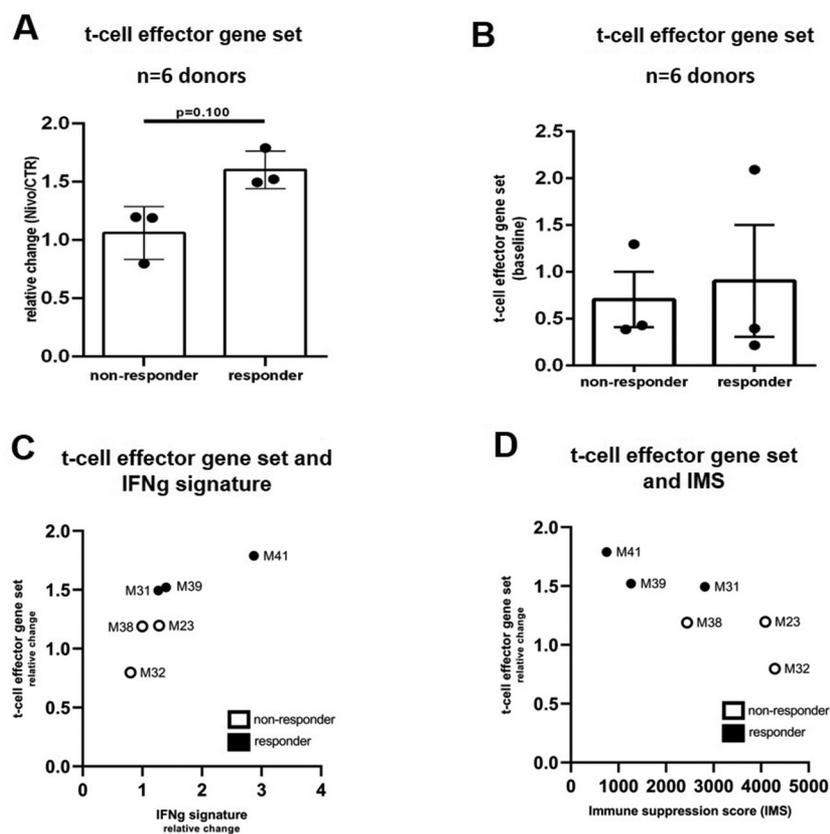
Figure 6. Clinical related gene expression profiles confirm histological tissue analysis of PDTC in GC and EGJC (a) Relative change of the t-cell effector gene expression set determined in PDTC *ex vivo* (relative change of Nivo vs CTR, n = 6). (b) Histologically pre-determined cases are compared by the relative change of the t-cell effector gene set and the relative change of the clinical related Interferon gamma signature. (c) Histologically pre-determined cases are compared by the relative change of the t-cell effector gene set and the mean absolute count of gene expression of immune suppression score (IMS). Error bars: SEM, Mann-Whitney-U test.

PD-1 or PD-L1 blockade is approved in several tumor entities.[44–47] However, monotherapy with PD-1 antibodies have limited clinical benefit and response rates are low in most tumor entities.[42] Predictive markers, providing advice for clinical immune checkpoint therapy indication is however still under intensive investigation.[14] Here, we treated PDTC of GC and EGJC with Nivolumab monotherapy and observed similar low response rates as it has been described in clinical studies.[12,13] Still, tissue specimens responding well to Nivolumab treatment did not necessarily express high PD-L1 levels. By maintaining the organotypic cellular composition, this result might suggest the involvement of further immunological tissue-related cells. Macrophages, B-cells and NK-cells are getting in the focus as they interfere in the PD-1/PD-L1 pathway and are suggested as regulators.[38,48–50]

Combinational approaches with other immune checkpoint inhibitors (*e.g.*, CTLA4 blockade) or chemotherapy seem to be promising due to potential synergistic effects of more than one mechanism of action.[51] Treating PDTC *ex vivo* with combinational approaches of PD-1 inhibition together with Oxaliplatin or a commercial CD3/CD28 t-cell activator led to enhanced tumor cell apoptosis in most patient derived tumor specimens. Whereas the combined effect of the t-cell activator and PD-1 inhibition is t-lymphocyte related, the beneficial effect of the clinical relevant combination of oxaliplatin and PD-1 inhibition is supported by recent literature, despite the concept that immunogenic chemotherapies can lead to adaptive tolerance.[52]

However, recent literature highlight the beneficial effect of combined therapy as found in the present study. It was revealed that therapeutic FOLFOX regimens induced CD8 T-cell activation in clinical specimens and that oxaliplatin can eliminate type 2 tumor-associated macrophages, concomantly restoring t-cell reactivity in immunocompetent mice.[53,54] These results indicate that PDTC can reflect clinical observations and might be a promising tool for further investigations of novel combinational therapy approaches prior to clinical phase 1 trials.

Investigating functionality of resident t-lymphoctyes in PDTC, protein and gene expression analysis were performed. In clinical trials of immunotherapy-specific t-lymphocyte inflammation, gene expression signatures have been established and have been proven of predictive value.[15,31] In future, cancer therapy may become more and more complex, and precision medicine will be part of daily practice. Novel biomarkers can guide oncologist in immunotherapy treatment decisions.[14] Nonetheless, primary and adapted resistance compromise efficacy of immunotherapy or cancer treatment and the underlying mechanism of profitable combinational therapies are still not fully understood.[38,55] Here, we have noticed a heterogeneous inter-individual gene expression after treatment with Nivolumab monotherapy. However, *ex vivo* PDTCs responding to PD-1 inhibition showed a significant higher change of cytotoxic t-cell effector gene expression compared to non-responding PDTCs. We utilized the INFg gene expression profile of Ayers et al, established in clinical specimens from PD-1

clinical trials of 9 cancer entities. This t-lymphocyte inflammation based profile, still under investigation in current PD-1 inhibition trials, could be correlated to the obtained overall effects.[15] Further, the immune suppressor score investigated by Cui et al[31] for their predictive potency in clinical melanoma and gastric cancer specimens was aplied in the current investigation as well as a gene set of t-cell effector molecules that are involved in t-lymphocyte mediated cytotoxicity.[33–36] Though, t-cell effector gene set supported our classification of tissue response by immunohistochemical determination with statistical power. Yet, the t-cell effector gene set is not entitled to predict Nivolumab response in a clinical setting and need to be confirmed therein. While, lower immune suppression scores have been observed in responding patients, it is suggested that the t-cell effector gene set and the immune suppression score might be applied together for more precise patient stratification. Therefore, prospective correlation with clinical data in PDTC of GC and EGJC must be pursued by endoscopic or core needle tumor specimens. In lung cancer, first successful clinical correlations of treatment susceptibilies *ex vivo* and clinical data have been published in PDTC and are ongoing.[29,56]

Still, the system model holds certain limitations as tumor specimens are scarce and have a limited culture period. Though, PDTC can provide preservation of heterogeneous tumors with typical features of immunosuppression or impact of mesenchymal cells.[20,22–24] Functional experiments regarding migration and differentiation of immune cells can be performed in PDTC by co-culturing patient specific PBMCs tissue to enhance the understanding of systemic effectiveness.

In summary, we demonstrate a stable and functional t-lymphocyte population in PDTC of GC and EGJC. We were able to show that individual responses to PD-1 inhibition can be depicted in PDTC and combinational therapy approaches with chemotherapy are able to enhance antitumoral effects.

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Disclosure statement

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ORCID

Sonja Kallendrusch http://orcid.org/0000-0003-2474-8307

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