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Immune responses against shared antigens are common in esophago-gastric cancer and can be enhanced using CD40-activated B cells

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ABSTRACT

Background Specific immune response is a hallmark of cancer immunotherapy and shared tumor-associated antigens (TAAs) are important targets. Recent advances using combined cellular therapy against multiple TAAs renewed the interest in this class of antigens. Our study aims to determine the role of TAAs in esophago-gastric adenocarcinoma (EGA).

Methods RNA expression was assessed by NanoString in tumor samples of 41 treatment-naïve EGA patients. Endogenous T cell and antibody responses against the 10 most relevant TAAs were determined by FluoroSpot and protein-bound bead assays. Digital image analysis was used to evaluate the correlation of TAAs and T-cell abundance. T-cell receptor sequencing, in vitro expansion with autologous CD40-activated B cells (CD40Bs) and in vitro cytotoxicity assays were applied to determine specific expansion, clonality and cytotoxic activity of expanded T cells.

Results 68.3% of patients expressed ≥5 TAAs simultaneously with coregulated clusters, which were similar to data from The Cancer Genome Atlas (n=505). Endogenous cellular or humoral responses against ≥1 TAA were detectable in 75.0% and 53.7% of patients, respectively. We found a correlation of T-cell abundance and the expression of TAAs and genes related to antigen presentation. TAA-specific T-cell responses were polyclonal, could be induced or enhanced using autologous CD40Bs and were cytotoxic in vitro. Despite the frequent expression of TAAs co-occurrence with immune responses was rare.

Conclusions We identified the most relevant TAAs in EGA for monitoring of clinical trials and as therapeutic targets. Antigen-escape rather than missing immune response should be considered as mechanism underlying immunotherapy resistance of EGA.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Tumor-associated antigens (TAAs) represent important targets for immunotherapy and have been described in multiple cancer types. Endogenous cellular and/or humoral responses to TAAs contribute to antitumor immune response and efficacy of immunotherapies. Although TAAs are often used for translational analyses of clinical trials, expression patterns and endogenous responses are poorly described for most cancer types.

WHAT THIS STUDY ADDS

⇒ TAAs are frequently expressed in esophago-gastric adenocarcinoma (EGA) with coexpression in distinct clusters. Endogenous responses to TAAs are common, but co-occurrence of expression with cellular and/or humoral immune response against the same TAA is rare. Parallel expansion of specific T cells targeting multiple TAAs is feasible using CD40Bs and these polyclonal TAA-specific T cells show cytotoxic activity in vitro.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Results of this study affect immune monitoring of clinical trials in EGA. Our data suggest evaluation of combined targeting of multiple TAAs and therapeutic enhancement of TAA-expression or antigen presentation.

INTRODUCTION

Esophago-gastric adenocarcinoma (EGA) is among the most common causes of cancer-related deaths worldwide with a rising incidence in western countries.[1] Combinations of surgical resection, radiotherapy, and chemotherapy led to some progress over the last years, but the overall prognosis remains poor. Patients treated with curative intent have a very high risk of disease recurrence, especially in cases with nodal involvement.[2 3] Immunotherapy represents a novel treatment option for a variety of cancer types and targeting the programmed cell death-1

(PD-1) has recently proven efficacy in EGA. Anti-PD-1 therapy (nivolumab, Checkmate 649; pembrolizumab, Keynote 059, Keynote 590) is effective in the metastatic setting and nivolumab also prolongs disease-free survival as adjuvant treatment following neoadjuvant chemoradiotherapy and surgical resection (Checkmate 577).[4–7] However, objective response rates are low and mechanisms underlying susceptibility to anti-PD-1 therapy are poorly understood in EGA and other types of cancer.[8] For example, almost 80% of patients with metastatic EGA did not respond to pembrolizumab therapy.[9] Enhancement of pre-existing or de novo induction of antitumor T-cell responses is assumed as crucial mechanism of anti-PD1 therapy and other immunotherapeutic approaches.[10] Mutation-associated neoantigens (MANAs) or tumor-associated antigens (TAAs) are the major targets of tumor-specific immune responses.[11 12] In contrast to the highly individual MANAs, TAAs are often shared between patients and types of cancer. Due to their broad applicability even across different types of cancer, TAAs are classical targets for various immunotherapeutic approaches such as vaccination,[13] adoptive T-cell transfer,[14] or CAR T cells.[15] However, targeting a single TAA often showed limited efficacy and lead to selection of clones not expressing the respective TAA.[16] Other mechanisms of immune escape such as defective antigen presentation or silencing of target genes are additional obstacles.[17–19] Recent data and emerging therapeutic strategies such as mRNA-vaccination renewed the interest in TAAs.[12] Especially, attempts using combined targeting of multiple TAAs have shown promising results in lymphoma[20] and myeloma[21] and appear synergistic with immune checkpoint inhibition.[22] Moreover, TAA-specific immune responses are important parameters for immune monitoring of clinical trials.[23]

This study aims to elucidate endogenous TAA-directed immune responses in previously untreated EGA as important prerequisite for immune monitoring and design of immunotherapeutic trials. We identified the most relevant TAAs in EGA, describe prevalence of TAA-specific cellular and humoral responses and demonstrate the antitumor efficacy of TAA-directed T-cell responses in vitro.

MATERIALS AND METHODS

Patients and samples

Forty-one treatment-naïve patients with EGA were enrolled in this study. Peripheral blood mononuclear cells (PBMCs), matched serum (n=41) and lymphocytes of a tumor-draining lymph node (TDLN, n=1) were analyzed. Serum of 24 kidney donors served as healthy controls. Patient-matched formalin-fixed paraffin-embedded (FFPE) tumor and healthy tissue sections (n=41) were used for digital pathology (details in online supplemental methods). Detailed patient characteristics are summarized in online supplemental table 1. PBMCs were isolated and tissue specimens were processed using a standardized protocol (online supplemental methods) and stored in liquid nitrogen until analysis.

FluoroSpot

PBMCs were cocultured with/without peptide pools of TAAs (1 µg of each peptide/mL, peptides and elephants, Germany, details in online supplemental table 2) on precoated FluoroSpot plates (Mabtech, Sweden) for 20 hours. Patients who showed ≥10 specific spots per 2×10^5 PBMCs were counted as responder for the respective peptide. Spot detection was performed on an AID iSpot Spectrum Reader (AID, Germany). Assay details in online supplemental methods.

RNA isolation and NanoString

FFPE tumor blocks of 41 treatment-naïve EGA patients and patient-matched blocks of normal tissue from the aboral resection front were selected for RNA isolation. RNA was isolated using the Maxwell RSC RNA FFPE Kit according to the manufacturer's instructions (Promega, USA). NanoString was performed using 250 ng RNA (NanoString, USA) (see online supplemental methods). For detailed information on the customized code-set, see online supplemental table 3.

Generation of antigen-presenting B cells and T-cell expansion

Antigen-presenting B cells (CD40Bs) were generated from PBMCs using the human CD40-Ligand Multimer Kit (Miltenyi, Germany) with a standardized protocol (online supplemental methods). T cells were isolated from PBMCs using a EasySep Human CD3 Positive Selection Kit II (StemCell, USA). For expansion, irradiated CD40Bs and T cells were mixed in a 1:1 ratio and cocultured in the presence of peptide pools of TAAs (peptide&elephants, Germany) for 10 days (online supplemental methods).

Cytotoxicity assay with OE19 cells

To determine antigen-specific cytotoxicity, PBMCs with/without T cells previously expanded with autologous TAA-pulsed CD40 activated B cells were cocultured with the EGA cell line OE19. After 1- and 3 days of coculture, cells were harvested and used for flow cytometry (online supplemental methods).

HLA genotyping and prediction of binding affinity

HLA genotyping was performed with Illumina's NGS method (online supplemental methods). The amino acid sequences of 10 frequently expressed TAAs in EGA (online supplemental table 2) were used in combination with HLA-genotyping results to identify the patient-specific repertoire of high-affinity binding HLA I-restricted 9-10mers using the IEDB analysis resource NetMHCpan (V.4.1) tool (IEDB.org).[24] The complete list of predicted peptides was filtered based on the percentile rank (PR). Peptides with a PR ≤1 were considered high-affinity binders.

T-cell receptor repertoire

DNA was isolated from 1 to 2×10^5 T cells before and after T-cell expansion with individual TAA-peptide pools using the DNA Gentra Puregene Cell and Tissue Kit (Qiagen, Netherlands). Amplicon TRB-VJ next-generation sequencing of baseline and expanded T cells was performed as described previously in the EuroClonality-NGS assay[25] (see online supplemental methods for details).

Detection of tumor-specific antibodies

A protein-bound bead assay was used to detect antibodies against TAA in patient sera (n=41). Proteins of TAAs were coupled to magnetic carboxylated polystyrene microparticles (MagPlex Microspheres, Luminex, USA). For coupling validation, commercially available anti-TAA antibodies were used in titration experiments (proteins and antibodies in online supplemental table 4). Assay details in online supplemental methods. Median fluorescence intensity was determined on a Cytoflex LX flow cytometer (Beckman Coulter, USA).

Statistical analyses, visualizations, TCGA data, and software

Graphs and statistical tests were created using GraphPad Prism V.9.0.2 (GraphPad, USA). Figures were generated with Inkscape V.1.0.1. Group sizes, statistical significance levels, definition of error bars and applied tests are included in the respective figure legends. For detailed software, see online supplemental methods. RNA expression data from the TCGA database were downloaded from cBioPortal.[26 27]

RESULTS

TAAs are frequently coexpressed in EGA

We performed RNA expression analysis using NanoString to characterize the expression patterns of 26 TAAs in EGA, which were highly heterogeneous (figure 1A). The 26 TAAs were selected based on previous description in cancer using the cancer testis antigen database (http://www.cta.lncc.br), previous publications and preferably also clinical trials or expression in EGA tumor samples using The Cancer Genome Atlas (TCGA)-based GEPIA2 platform (http://gepia2.cancer-pku.cn). Selective expression in the tumor microenvironment is another important feature of TAAs. The average difference between TAA-expression in tumor and normal tissue was positively correlated with RNA expression in tumor samples (figure 1B). We defined tumor-specific expression based on absolute expression in the tumor and the patient-specific difference between normal and tumor. All analyzed TAAs were specifically expressed in at least one patient. While some TAAs showed homogeneous negativity or positivity across most patients (eg, IGF2BP3 and SPA17, respectively), expression was highly variable for others (figure 1C). Of 41, 40 (97.6%) patients expressed at least one of the included TAAs (figure 1D). 68.3% (28/41) of the patients in our study showed coexpression of ≥5 TAAs (figure 1D), which is particularly relevant as a recent study in lymphoma demonstrated durable clinical benefit using cellular therapy for combined targeting of multiple TAAs.[20] We performed hierarchical clustering and Pearson correlation to estimate coregulation of TAAs. This analysis identified distinct clusters with similar expression of for example CSAG1, MAGEA12 and NY-ESO-1 or PBK, TTK and Survivin (figure 1E). Stratification of our data according to tumor stage demonstrated that some TAAs were enriched in patients with advanced tumor stages (Union Internationale Contre le Cancer (UICC) high, eg, CTCFL or CT45A1) and others were predominantly expressed at early stages (UICC low, eg, GAGE1 or NY-ESO-1, figure 1F). Overall expression patterns and coregulated clusters were similar, when we compared our results with data from the TCGA database (n=505, online supplemental figure 1A,B).

EGA patients show frequent endogenous cellular immune response against TAAs

We performed FluoroSpot assays, which allow the simultaneous detection of interferon gamma (IFN-γ) and interleukin 2 (IL-2) on coculture with peptide pools of TAAs (figure 2A). We included peptide pools of 10 selected TAAs which showed high expression in our cohort (CEP55, MAGEA3/6, PBK, CT83, IGF2BP3, TTK), or were previously described in EGA (Survivin, PRAME, NY-ESO-1) (figure 1A). The analyses revealed that 75.0% of patients (15/20) showed an endogenous cellular response (IFN-γ or IL-2) to at least one of the included TAAs (figure 2B). Cellular responses in ≥25% of patients were detected for PBK, MAGEA3, IGF2BP3, CT83, NY-ESO-1 and Survivin with the highest fraction of patients responding to Survivin (65.0%) and NY-ESO-1 (52.6%) (figure 2C). These responses were rare in healthy donors, which were analyzed for endogenous T cell responses to the same set of shared antigens ($p<0.0001$, online supplemental figure 2A). IFN-γ responses were more common than IL-2 responses, with few patients showing simultaneous IFN-γ and IL-2 release (eg, Survivin) (figure 2C). In 9 out of 15 responders, we detected endogenous immune responses to 1–4 TAAs and 6 patients showed a simultaneous response against≥5 TAAs (figure 2D). The magnitude of responses varied between the different TAAs and patients. The number of specific spots was highest in Survivin (mean of 35.7 spots, figure 2E). IFN-γ responses were not only more frequent in general, but also showed more specific spots per 2×10^5 PBMCs compared with IL-2 (figure 2C,E).

The observed variation in immunogenicity of the different TAAs may be related to the number of immunogenic peptides predicted to bind to MHC-I. To determine the number of high-affinity peptides arising from the 10 TAAs, we calculated in-situ the percentile rank (PR) of each possible 9-10mer that binds the patient's unique HLA genotype. In our cohort of EGA patients, the highest number of high-affinity peptides (PR≤1) was calculated for PRAME and TTK (326.7±58.4 and

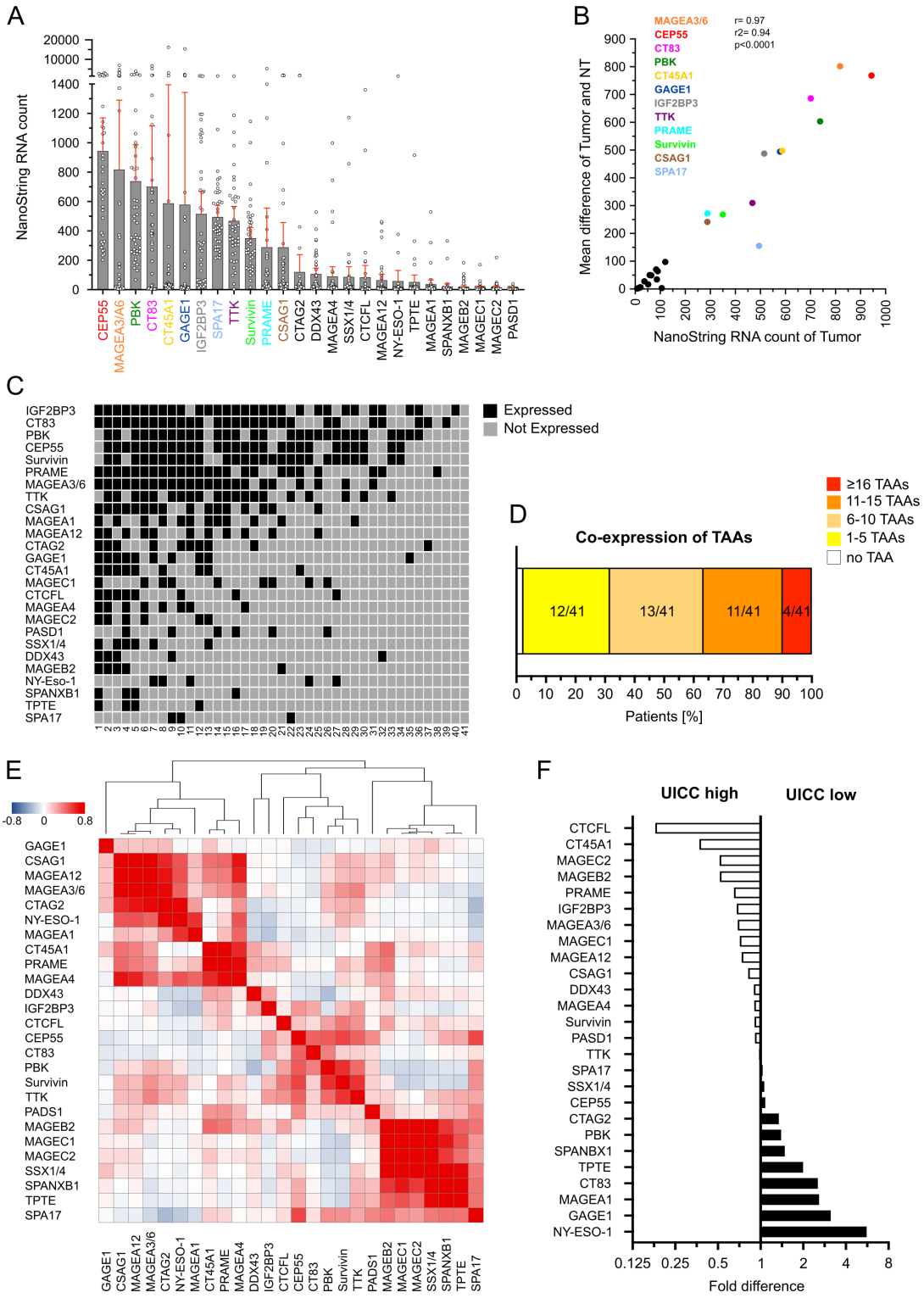

Figure 1 Tumor-associated antigens (TAAs) are frequently coexpressed in esophago-gastric adenocarcinoma. (A) NanoString based RNA expression analysis of 26 TAAs in 41 treatment-naïve esophago-gastric adenocarcinoma (EGA) tumors. (B) Correlation of the absolute RNA count of the tumor and the mean difference of tumor and normal tissue (n=41) for the indicated TAAs. (C) A tumor sample with a TAA-expression higher than the mean expression of the respective TAA in NT (n=41) and a threefold change compared with patient-matched normal tissue was considered positive. (D) Coexpression of 26 TAAs in EGA patients (n=41). (E) Similarity matrix with correlation of TAA-expression in EGA tumor samples. The expression of 26 TAAs in tumors from 41 EGA patients was assessed by NanoString. Hierarchical clustering of TAAs was performed by one minus Pearson correlation with average linkage. (F) Tumors were categorized in early (UICC I+II, eighth edition) and advanced (UICC III+UICC IV) stage tumors. The fold-difference of the mean expression in UICC high and UICC low tumors was calculated. Tow-tailed, Pearson correlation coefficient was calculated (B). When appropriate, mean±95% CI is indicated. UICC, Union internationale contre le cancer

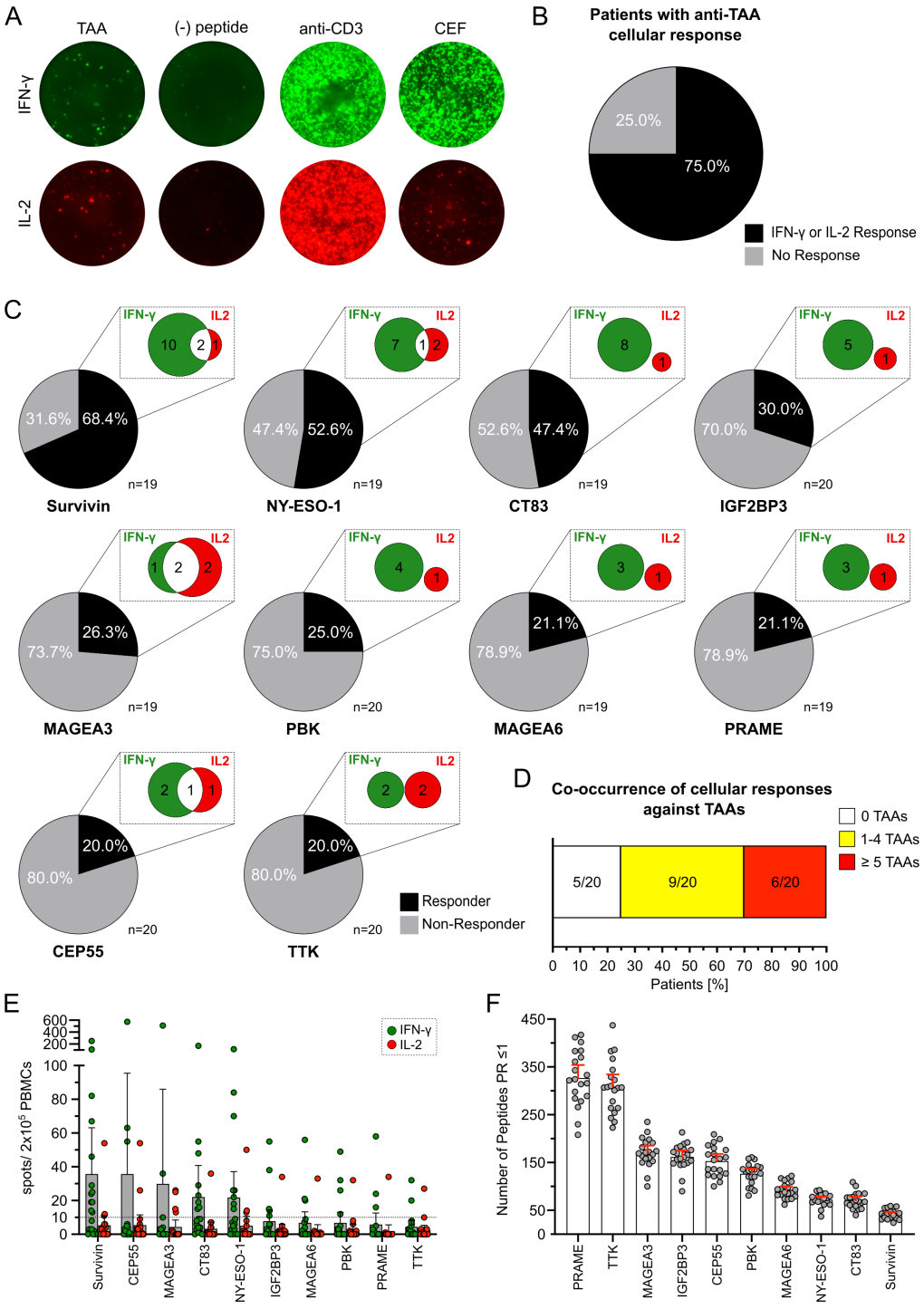

Figure 2 Esophago-gastric adenocarcinoma (EGA) patients show frequent endogenous cellular immune response against tumor-associated antigens. T-cell responses against tumor-associated antigens (TAAs) were assessed by FluoroSpot analysis. (A) Representative plots of a FluoroSpot assay showing an interferon gamma (IFN-γ) for one and an Interleukin 2 (IL-2) response for another EGA patient. plots of 2×10^5 PBMCs per well after 20 hours of coculture with a tumor-associated antigen (TAA, here Survivin), without peptide, anti-CD3 (technical positive control) and CEF (cytomegalovirus, Epstein-Barr virus and influenza, biological positive control) are shown. (B) Percentage of EGA patients (n=20) with either IFN-γ or IL-2 response (black) or no response (gray) against at least 1/10 of the tested TAAs (TAAs shown in C). (C) Percentage of EGA patients with either IFN-γ or IL-2 response (black) or no response (gray) against the indicated TAA. Overlay plots show the overlap of IFN-γ (green) and IL-2 (red) response. (D) Percentages and proportion of EGA patients (n=20) without specific FluoroSpot response to one of the investigated 10 TAAs (white), simultaneous response to 1–4 TAAs (yellow) or≥5 TAAs (red). (E) Number of specific spots after coculture with TAA peptide pools. The absolute number of specific spots for IFN-γ and IL-2 and the indicated TAAs are shown. (F) The amino acid sequence of each TAA was used to predict the number of high-affinity peptides (percentile rank, PR≤1) binding to the HLA genotype of each patient (n=41). When appropriate, mean±95% CI is shown. PBMCs, peripheral blood mononuclear cells.

308.5±55.4, respectively). Interestingly, for these TAAs we detected only rare cellular responses (21.1% and 20.0%, respectively), while the TAAs with the smallest number of high-affinity peptides (CT83, 70.9±16.8 and survivin, 42.0±10.0) showed high frequencies of cellular immune responses (47.4% and 68.4%, respectively) (figure 2C,F). We normalized the number of high-affinity peptides to control for the variable protein size (online supplemental table 2), which changed the ranking of TAAs. With this normalization, CT83 ranked second with higher numbers of predicted peptides per 100aa (online supplemental figure 2B). Central tolerance mediated by expression of a TAA on medullary thymic epithelial cells (mTECs) may also contribute to differences in immunogenicity. Expression of most TAAs included in this study on human mTECs is low, as determined by reanalysis of published data by Brennecke *et al* (online supplemental figure 2C).[28]

Tumor-specific antibodies are frequent, but co-occurrence with expression of the target gene and/or cellular immune response is rare

We performed a protein-bound bead assay to detect TAA-specific antibodies. 53.7% (22/41) of the analyzed sera of EGA patients contained antibodies against at least one of the 10 TAAs (1–4 TAAs in 21 samples and ≥5 TAAs in one sample) (figure 3A–C). In serum samples of EGA patients, antibodies against all TAAs except for TTK were detected. Antibodies against NY-ESO-1 (8/41) and CEP55 (7/41) were most frequent, while antibodies binding to PRAME, CT83 and Survivin where only detectable in 2/41 patients. The percentage of TAA-specific antibodies was similar in serum samples from healthy controls, but strong responses (fold change ≥10) were more frequent in EGA patients 17.1% (7/41) vs 4.2% (1/24) (figure 3C). When comparing the three different aspects of TAAs included in our analyses, expression of TAAs was most abundant, followed by T-cell responses and humoral responses (figure 3D). Expression by more than half of the EGA patients was found for all TAAs except for NY-ESO-1 (12.2%) and TTK (48.8%), while strong cellular responses against Survivin (68.4%) and NY-ESO-1 (52.6%) and the highest percentage of humoral responses was detected for NY-ESO-1 (19.5%) and CEP55 (17.1%) (figure 3D). We did not observe clear correlations between immune response and expression of the different TAAs. For example, CEP55 showed strong expression but few cellular responses, while frequent cellular responses were detected for NY-ESO-1 with only weak gene expression (figures 1A and 2C). Integration of gene expression, cellular and humoral immune response in 19 patients with complete datasets revealed co-occurrence of expression, humoral and cellular immune response was only observed in 15.8% (3/19) of patients. The overlap of expression with cellular and/or humoral immune response was 20.0% (38/190) of the possible combinations (figure 3E). Survivin showed the highest overlap of expression and cellular response (31.6%), while there was no overlap for NY-ESO-1 (online supplemental figure 3A,B).

T-cell abundance in the tumor microenvironment is correlated to higher expression of TAAs and genes of the antigen-presenting machinery

A strong immune infiltrate is associated with superior survival and response to immunotherapy in a variety of cancer types.[29 30] We categorized the included tumor samples of EGA patients in immune-score (IS) high, intermediate and low using digital quantification of CD3 and CD8 T cells in the tumor microenvironment (figure 4A).[31 32] These data were used to evaluate the impact of TAAs on T-cell abundance. In addition to expression of the target gene, appropriate processing and presentation of immunogenic peptides is needed for an effective T-cell response. Hence, we also included RNA expression analysis of 25 genes of the antigen-presenting machinery (APM) (figure 4B). In general, antigen presentation was increased in the tumor microenvironment, as 14/25 (56.0%) genes associated with various steps of the APM were increased in tumors compared with normal tissue (online supplemental figure 4A). However, patient-specific analyses also revealed impaired expression of one or multiple APM genes (figure 4B). Genes of the HLA molecule (β–2M, HLA-A, HLA-B and HLA-C), the immuno-proteasome (LMP2, LMP7, LMP10) and genes involved in MHC loading (Tapasin, TAP1, TAP2) showed higher expression in patients with IS-high tumors (figure 4C and online supplemental figure 4B). Expression of APM-genes but also expression of TAAs was correlated to immune-cell abundance as 52.0% (13/25) of APM genes and 23.1% (6/26) of the TAAs were upregulated in IS-high patients, while non showed upregulation in IS-low patients (figure 4D+E). In total, 23.1% (6/26) of the analyzed TAAs showed higher expression in IS-high patients (PBK, IGF2BP3, TTK, Survivin, CTAG2 and PRAME, figure 4C,E).

Autologous CD40-activated B cells (CD40Bs) can be used to enhance or induce TAA-specific T cells, which are cytotoxic in vitro

To assess feasibility of induction or enhancement of specific T-cell responses to the 10 TAAs, we generated TAA-pulsed autologous CD40-activated B cells (CD40Bs) as antigen-presenting cells from PBMCs of EGA patients (EGA, n=3). These cells were coincubated with autologous T cells and each of the 10 peptide pools. T-cell receptor (TCR, CDR3ß) sequencing before and after expansion demonstrated clonal expansion of specific TCRs as indicated by a lower number of unique clonotypes in expanded T cells (before 476.3±51.6 vs expanded 305.5±100.1, p=0.0274, figure 5A). Analysis of the distribution of the ranked clones showed less diversity in the expanded samples with the most expanded clones (top 20) accounting for 52.2% of the repertoire (figure 5B). Comparison of the repertoire of clones within each of the analyzed TAAs revealed that expansion using CD40Bs

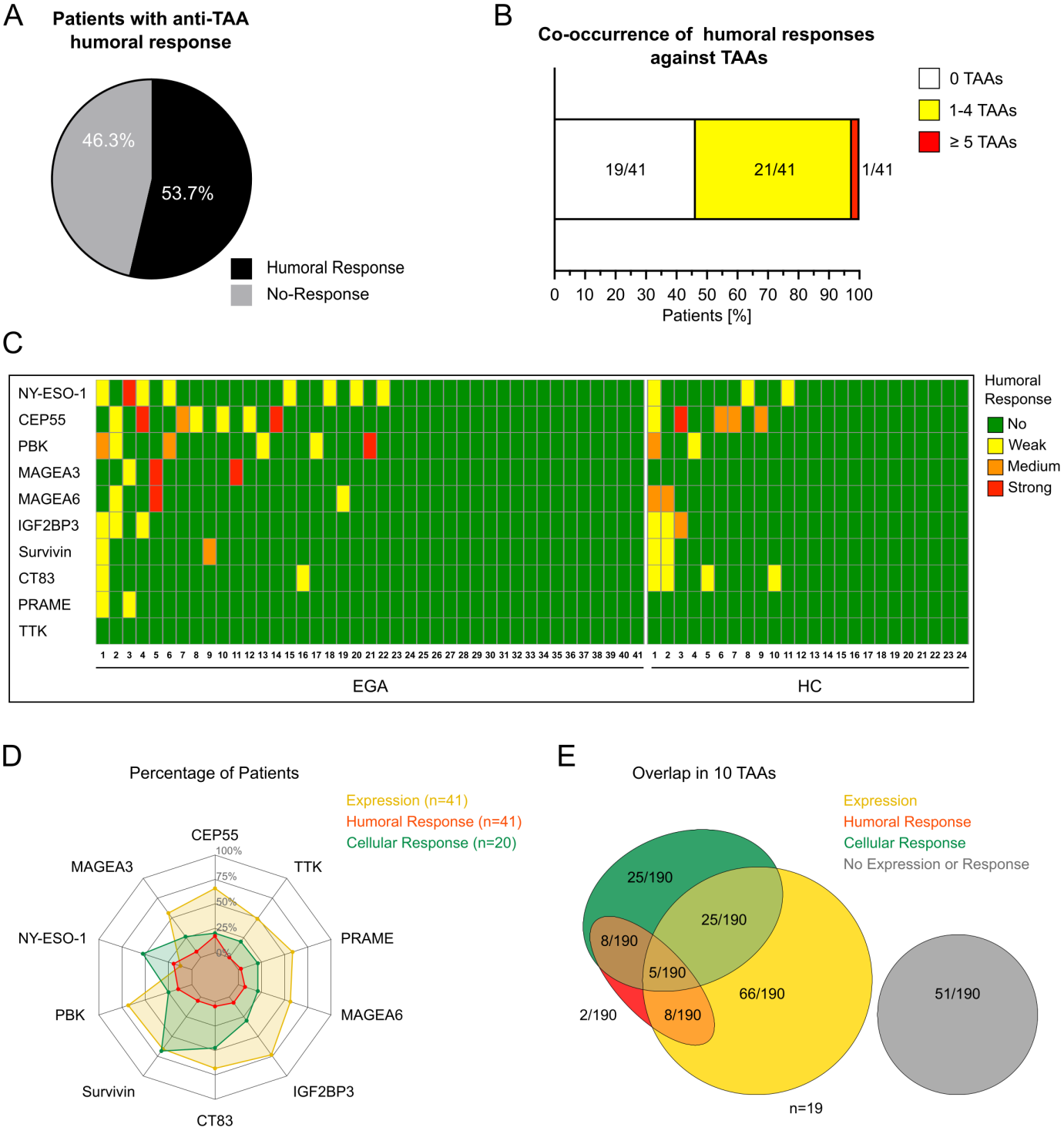

Figure 3 Tumor-specific antibodies are common, but co-occurrence with expression and/or cellular immune response is rare. Bead-bound proteins of tumor-associated antigens (TAAs) were used to detected antibodies against TAAs in patient sera (n=41). (A) Percentage of patients with antibody responses against at least one of the included TAAs (details of proteins and antibodies in online supplemental table 4). Bead-assay was performed in technical triplicates. The median fluorescence intensity (MFI) of sample-matched beads coupled with human serum albumin (HSA) was subtracted as unspecific background. (B) Percentages and proportions of esophago-gastric adenocarcinoma (EGA) patients (n=41) without specific antibody response to one of the investigated 10 TAAs (white), simultaneous response to 1–4 TAAs (yellow) or ≥5 TAAs (red). (C) Fold change was calculated by subtracting the twofold SD of HSA of healthy controls (HCs, n=24) and dividing by the MFI of the respective TAA in HCs. Samples with a fold change ≥2 were considered positive. Samples were categorized in weak (fold change≥2 and ≤5, yellow), medium (>5 and ≤10, orange), strong (>10, red) or no humoral response (<2, green). (D) Radarchart shows the percentage of patients with expression (yellow), humoral response (red) or cellular response (green). (E) The co-occurrence of expression, humoral response, and cellular responses to 10 TAAs (list of TAAs in C) was analyzed in patients with matched samples (n=19).

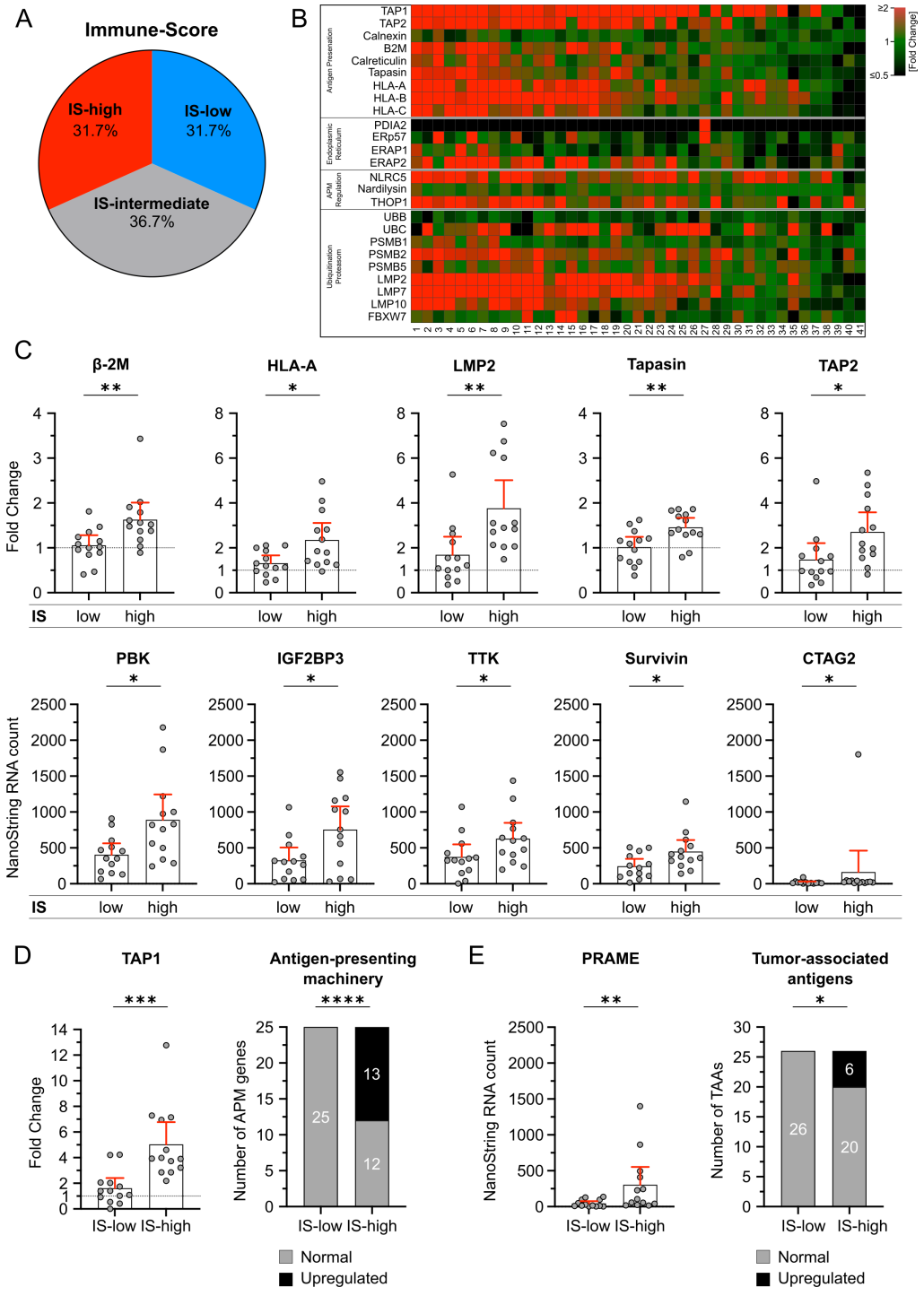

Figure 4 T cell abundance in the tumor microenvironment is correlated to higher expression of tumor-associated antigens (TAAs) and genes of the antigen-presenting machinery. (A) CD3 and CD8 T-cell abundance in the tumor and invasive margin was used to generate an Immune-Score (IS) for each patient with esophago-gastric adenocarcinoma (EGA, n=41). Percentage of patients with high (IS-high, n=13), low (IS-low, n=13) or intermediate (IS-intermediate, n=15) Immune-Score is shown. (B) RNA expression of 25 genes associated with antigen presentation or processing was determined by NanoString. Fold change of each gene was calculated with patient-matched healthy tissue. Heatmap shows the fold change of the indicated gene for each patient individually. (C) Patient-specific fold change of the indicated genes associated with antigen presentation or absolute RNA expression of the indicated tumor-associated antigens in the tumor is shown for IS-low and IS-high patients. (D) Representative fold change of TAP1 in IS-low and IS-high patients is shown. Number of normally expressed (gray) or upregulated (black) genes associated with antigen presentation or processing in IS-low and IS-high patients. (E) Representative expression of PRAME in the tumor of IS-low and IS-high patients is shown. Number of normally expressed (gray) or upregulated (black) genes of TAAs in IS-low and IS-high patients. Significant differences calculated by nonparametric, unpaired and two-tailed Mann-Whitney test (C–E) or two-sided Fisher's exact test (D and E) are indicated by asterisks. *p≤0.05, **p≤0.01, ***p≥0.001, ****p≤0.0001. When appropriate, mean±95% CI is indicated.

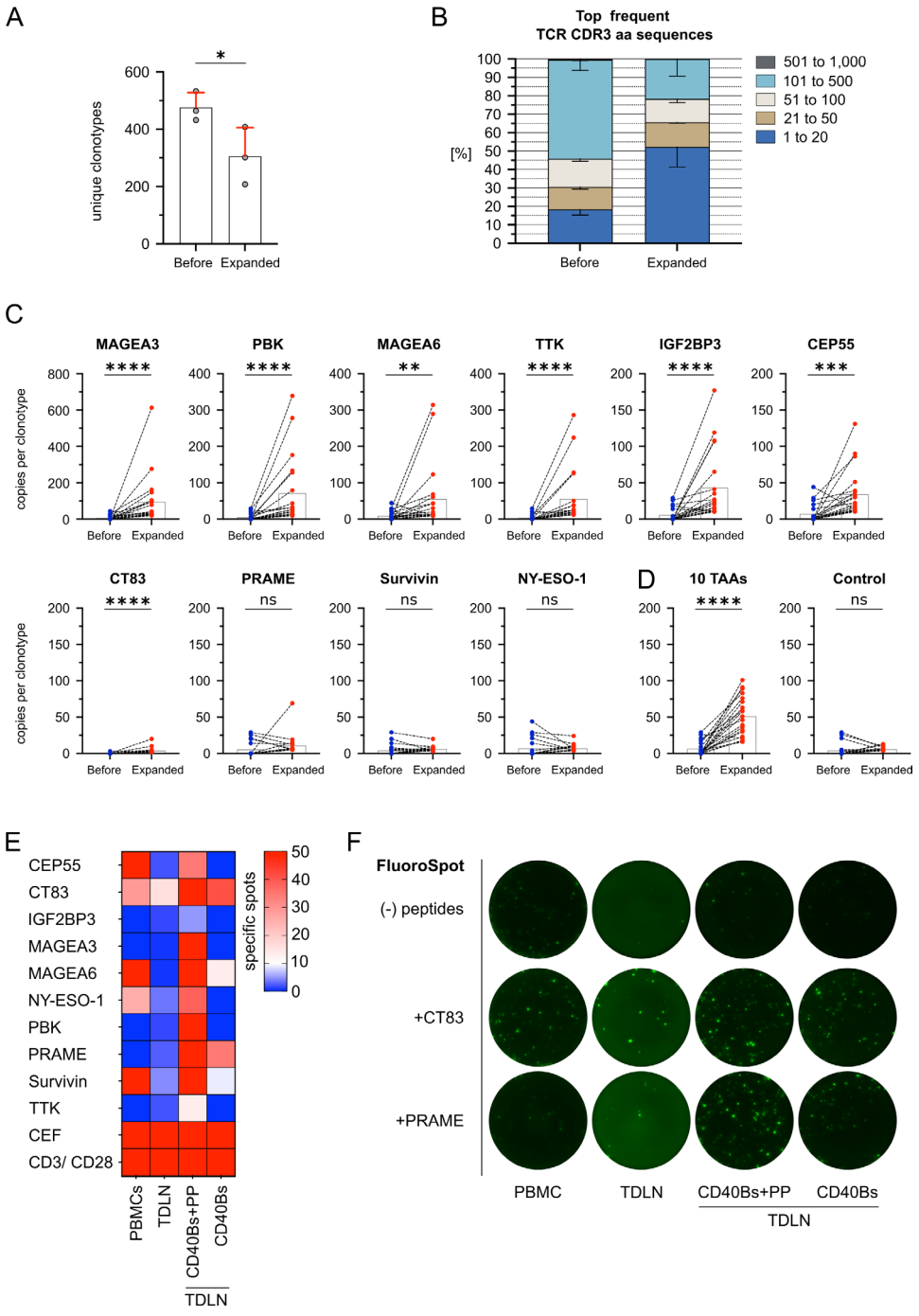

Figure 5 Autologous CD40-activated B cells (CD40Bs) can be used to enhance or induce TAA-specific T cells. T cells were expanded using autologous CD40-activated B cells (CD40Bs) and peptide pools of tumor-associated antigens (TAAs). DNA before and after expansion was isolated for T-cell receptor sequencing. (A) Unique clonotypes before and after expansion with TAA-pulsed CD40Bs. (B) Mean percentages of the top frequent TCR CDR3 aa sequences in PBMCs of three esophago-gastric adenocarcinoma (EGA) patients (n=3) before and after expansion with TAA-pulsed CD40Bs. (C) Representative plots showing copies per clonotype before and after expansion with the indicated peptide pools and autologous CD40Bs for one EGA patient. (D) Copies per clonotype before and after expansion with 10 TAAs simultaneously or with CD40Bs without peptide pools (Control). (E) TAA-specific interferon gamma (IFN-γ) secretion was assessed by FluoroSpot assay. Triplicates of peripheral blood mononuclear cells (PBMCs) and lymphocytes of a tumor-draining lymph node (TDLN) from an EGA patient were cocultured with peptide pools of the indicated TAAs. A peptide pool of Cytomegalovirus, Epstein-Barr virus, and influenza virus (CEF) was used as biological positive control, while antibodies against CD3 and CD28 were used as technical positive control. Median number of spots from triplicates without peptide was subtracted as background. Number of specific spots after coculture with the individual TAAs in a FluoroSpot assay are shown as heatmap. (F) Representative FluoroSpot plots after coculture without peptide ((-) peptides) and peptide pools for CT83 or PRAME. Significant differences calculated by parametric, paired, two-tailed t-test (A), or nonparametric, paired, two-tailed Wilcoxon test (C+D), are indicated by asterisks. *p≤0.05, **p≤0.01, ***p≥0.001, ****p≤0.0001. When appropriate, mean±SD.

in combination with TAAs induced highly clonal T-cell populations (online supplemental figure 5A). Analysis of the copy numbers per clonotype of the 20 most expanded clones confirmed the observed polyclonality. The increase in copy numbers per clonotype compared with the baseline T-cell repertoire was significant for 7, 9 and 10 of the 10 TAA peptide pools in the three analyzed patients (representative results for one patient in figure 5C). Of note, expansion in a pooled approach containing all 10 TAA peptide pools together also resulted in specific expansion of TCRs with a high clonal overlap to the individual expansions. In contrast, coculture with CD40Bs without peptides (Control) showed no specific TCR expansion (figure 5D). To confirm their antigen specificity, we performed FluoroSpot analysis of expanded T cells. Since the number of PBMCs was limited, we used T cells isolated from a TDLN and all 10 TAAs pooled together for this additional T-cell expansion experiment. The FluoroSpot analyses was performed with the individual peptide pools. We found an endogenous T-cell response to 5/10 (50.0%) TAAs (CEP55, CT83, MAGEA6, NY-ESO-1 and survivin) in PBMCs, while lymphocytes isolated from a TDLN of the same patient showed only an endogenous response against CT83 (figure 5E). After coculture of TDLN-derived T cells with autologous TAA-pulsed CD40Bs, TAA-specific T-cell responses against all TAAs except for IGF2BP3 and TTK were detectable in FluoroSpot analysis (figure 5E,F).

To demonstrate cytotoxic activity of T cells expanded with TAA-pulsed autologous CD40Bs, we used the gastric adenocarcinoma cell line (OE19) as target. HLA genotyping of the OE19 cell line revealed homozygosity for HLA-A and HLA-C and a loss of HLA-B. Thus, an HLA-A*02:01-positive healthy donor was selected to reduce allogenic effects. As we found coexpression of multiple TAAs per patient and targeting multiple TAAs showed promising results in lymphoma[20] and myeloma,[21] we expanded T cells with CD40Bs and a peptide pool of CT83, MAGEA3, MAGEA6, NY-ESO-1 and survivin. To validate specific expansion, we performed FluoroSpot analysis before and after expansion with and without peptides (figure 6A). NY-ESO-1 showed the strongest endogenous response (83±13 spots) and the strongest response after expansion (251±81 spots). T cells expanded with CD40Bs but without peptides, showed no difference in FluoroSpot analysis compared with the endogenous response in PBMCs (figure 6B). In coculture experiments with OE19, T cells expanded with TAA-pulsed CD40Bs showed increased activation ($CD69^+$ %Tcells) at day 1 and day 3 (19.1%±1.9 and 19.6%±2.1, respectively), which was lower or absent when unexpanded PBMCs or T cells expanded with CD40Bs but without peptides where used (figure 6C). Results were similar for the secretion of IFN-γ on coculture with OE19 cells (figure 6C). In addition to specific activation, T cells expanded with peptide pools of multiple TAAs and autologous CD40Bs exhibit antigen-specific tumor killing in vitro. This was reflected by increased expression of the tumor necrosis factor receptor 1 (TNF-R1) on tumor cells and increase of late apoptotic (Annexin V^+PI^+) OE19 cells after 3 days of coculture with TAA-expanded T cells (figure 6D).

DISCUSSION

Specific response to tumor-specific antigens (MANAs or TAAs) is a hallmark of cancer immunotherapy. As they are often shared between different individuals, TAAs (especially cancer testis antigens) are important targets for various immunotherapy approaches. Our study thoroughly characterizes 10 of the most important TAAs in EGA, integrating gene expression with cellular and humoral TAA-specific immune responses. We highlight that expression of TAAs as well as TAA-specific cellular and/or humoral immune responses are present in the majority of EGA patients, but these three different aspects rarely overlap in one individual.

We identified a set of 10 TAAs, which were expressed in tumor samples of a large fraction of our cohort with a strong difference compared with matched normal mucosa. Expression levels and interestingly also the observed patterns of coexpression were similar in data from 505 patients from the TCGA database. In a comprehensive analysis of cancer cell lines from different origins (prostate, breast, non-small cell lung cancer (NSCLC), sarcoma, melanoma and ovarian), Maxfield *et al* also described frequent coexpression of multiple TAAs.[33] Co-occurrence of multiple TAAs has also been described in primary tumor samples for various types of solid cancer such as hepatocellular carcinoma (HCC)[34 35] or NSCLC.[36] Epigenetic mechanisms and key transcription factors underlying the coregulation of these clusters are poorly understood. In colorectal cancer, Kutilin observed that changes in the expression pattern of TAAs (eg, BAGE; SSX2 and PRAME1) was correlated with the activity of specific DNA methyltransferases (DNMT3A and DNMT3B).[37] Regulation of TAA-expression by such epigenetic modifications could represent important mechanisms of antigen-escape which are shared across different types of cancer.[38] The most relevant TAAs seem to differ between cancer types as observed in cross-cancer analyses using a proteomic approach.[39] The observed stage-dependent differences of TAA-expression and an increased expression of TAAs in tumors with high T-cell infiltrates may be correlates of reciprocal shaping between tumor cells and immune response.[40–42] In line with our results, KK-LC-1 (CT83) showed a higher expression in early gastric cancer.[40] ujita *et al* found higher RNA and protein expression of NY-ESO-1 in advanced stages of esophageal squamous cell carcinoma, which also has a different mutational landscape than EGA.[43 44]

Seventy-five per cent of the patients included in our study showed a cellular response against at least one of the included 10 TAAs. Comparably high frequencies of TAA-specific T cells in PBMCs have been described for patients with HCC,[45] melanoma[46 47] or NSCLC.[48] T-cell abundance is often used as a surrogate for immunogenicity and is

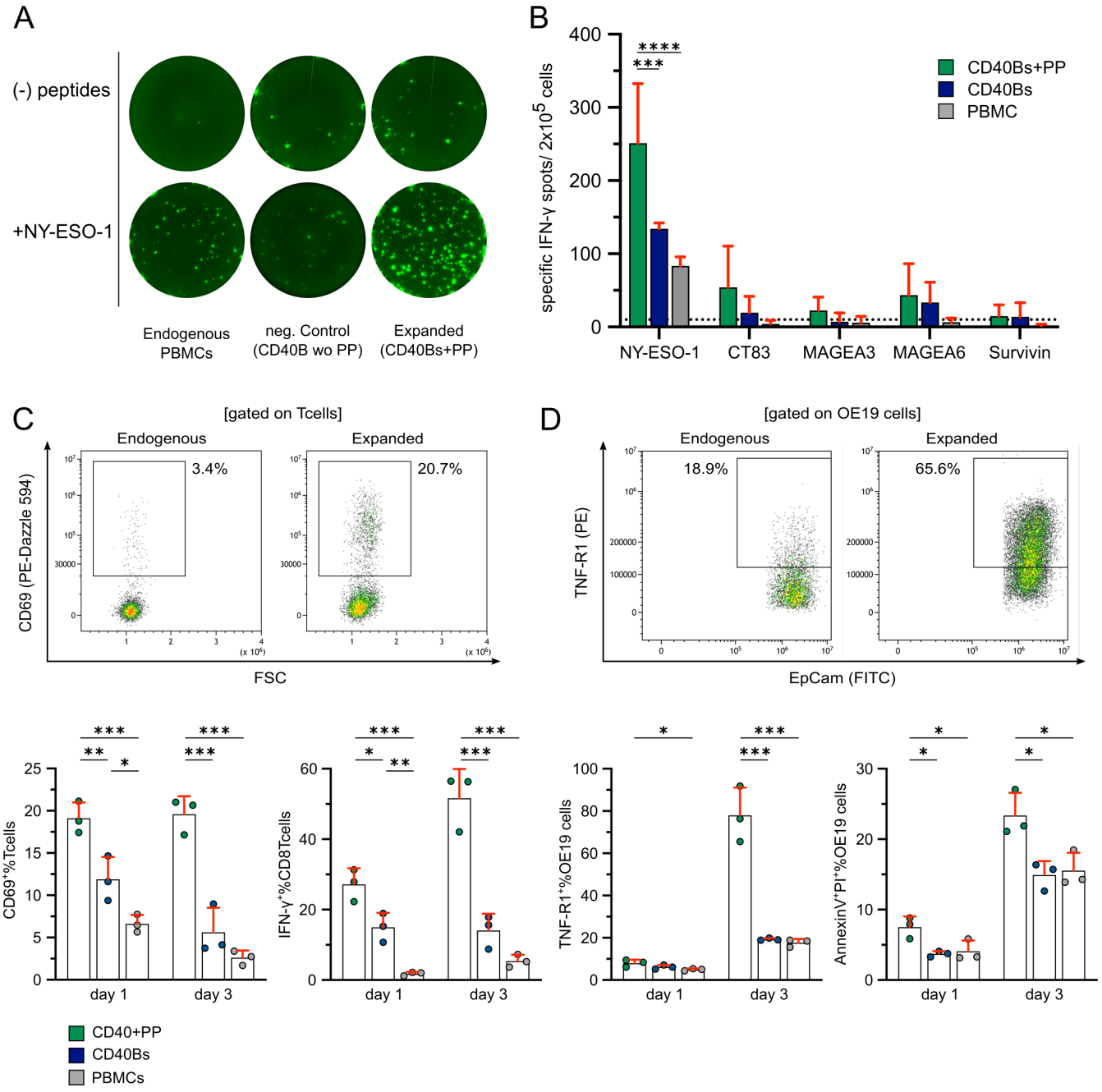

Figure 6 T cells expanded with autologous TAA-pulsed CD40-activated B cells (CD40Bs) show cytotoxic capacities in vitro. T cells of a donor were cocultured with autologous CD40Bs and peptide pools of tumor-associated antigens (TAAs, NY-ESO-1, CT83, MAGEA3, MAGEA6). (A) Representative plots of a FluoroSpot assay. Plots of the endogenous response in peripheral blood mononuclear cells (Endogenous PBMCs), PBMCs expanded with autologous CD40Bs without (CD40Bs wo PP) and with (CD40Bs+PP) peptide pools of TAAs (PP, NY-ESO-1, CT83, MAGEA3, MAGEA6, survivin) are shown for one donor from three independent experiments. (B)A number of specific interferon gamma (IFN-γ) spots per 2×10^5 cells in FluoroSpot analysis are shown for PBMCs before expansion (gray) and after expansion with autologous CD40Bs without (blue) and with (green) the indicated peptide pool. (C) OE19 cells were cocultured with PBMCs (gray) or PBMCs containing T cells expanded without (blue) or with (green) TAA-pulsed autologous CD40Bs. Representative FACS plots showing T-cell activation ($CD69^+$ expression) after 3 days of coculture with PBMCs (Endogenous) or PBMCs containing TAA-expanded T cells (Expanded) are shown. CD69 expression by T cells ($CD69^+$%Tcells) and interferon gamma (IFN-γ) secretion by cytotoxic T cells (IFN-γ^+%CD8Tcells) is shown for day 1 and day 3 of coculture. (D) Representative FACS plots showing OE19 apoptosis (tumor necrosis factor receptor 1, TNF-R1) after 3 days of coculture with PBMCs (Endogenous) or PBMCs containing TAA-expanded T cells. TNF-R1 expression on OE19 cells and percentage of late apoptotic ($AnnexinV^+PI^+$) OE19 cells is shown for day 1 and day 3 of coculture. Results from three independent experiments are shown (B–D). Significant differences calculated by two-way ANOVA (B) or one-way ANOVA test (C and D) for day 1 or day 3 of the coculture followed by Tukey's post hoc test to correct for multiple comparison are indicated by asterisks. *p≤0.05, **p≤0.01, ***p≥0.001, ****p≤0.0001. When appropriate, mean±SD is indicated. ANOVA, analysis of variance.

related to prognosis, risk of recurrence and susceptibility to immunotherapy.[49 50] Expression of 6/26 TAAs was higher in T cell rich tumors, but T-cell abundance appeared to be more related to expression of genes associated with antigen-presentation. A low or absent correlation between T-cell abundance and expression of TAAs was also described in other cancer types.[51–53] Consistent with the frequent coexpression of multiple TAAs in our cohort, combined T-cell responses to more than one TAA were also commonly detected. A similar fraction of patients with combined responses has been described in NSCLC.[48]

68.4% of the patients showed a cellular response against Survivin while responses to other TAAs (eg, CEP55 or TTK) were detected in less than 25% of patients. Previous publications described endogenous T cell responses to Survivin in 63.6%, 50.0% and 23.1% of analyzed patients with melanoma, breast cancer and lung cancer, respectively.[54 55] 52.6% of patients in our study showed endogenous NY-ESO-1 specific T cell responses compared with 31.3% described for melanoma and 26.1% described for NSCLC.[47 48] The different frequencies can be cancer-dependent, but also patient-derived or assay-dependent. Most studies used selected HLA-02:01 specific polypeptides, whereas we applied peptide pools spanning the whole TAA sequence. We combined HLA-genotyping of our cohort with in-silico prediction of HLA-binding affinities to evaluate the role of differential immunogenicity of included TAAs. In addition to an expected correlation between protein size and the absolute number of high-affinity peptides, we also observed protein-related differences. For example, CT83 was among the proteins with the highest number of high-affinity peptides per 100 amino acids after normalizing for the protein length. Posttranscriptional modifications may also contribute to differences regarding immunogenicity of the different TAAs and merit further investigation. These additional studies should also consider expression of a TAA of interest on mTEC, which may lead to central tolerance. Cancer testis antigens appear as the most promising group of TAAs, as they are often not expressed on mTEC.[56 57] For example, p53-specific T cells undergo negative selection because of expression on mTEC whereas NY-ESO-1-specific T cells are often not depleted.[58] Expression of cancer testis antigens on mTEC needs further investigation, but available data from bulk gene expression and single-cell transcriptomic analyses suggest low expression of the TAAs included in our analyses.[28 57] Binding affinity of endogenous TAA-specific T cells can be low and may be improved by application of recombinant high-affinity TCRs identified by in vivo stimulation of antigen-negative animals.[59]

In addition to direct ex vivo analyses of T-cell responses, which were detected in majority of patients (75.0%), we demonstrate expansion of TAA-specific T cells using CD40-activated B cells (CD40Bs). Schultze *et al* introduced in vitro generated CD40Bs as an alternative source of highly efficient antigen presenting cells for the generation of autologous antigen-specific T cells for adoptive immunotherapy in various settings.[60 61] In this combination of specific expansion and TCR sequencing, we mostly observed expansion of multiple CDR3 sequences suggesting polyclonal T-cell responses. Importantly, tumor-specific T cells expanded with CD40Bs also induced tumor-specific cytotoxicity in vitro.

Presence of specific antibodies often improves immune responses against infection or cancer.[62 63] Despite controversies regarding the intracellular (cytosolic) localization of most TAAs, antibody responses to a variety of TAAs have been described.[64] For example, antibodies binding NY-ESO-1, a cytosolic antigen originally identified in esophageal cancer, were found to correlate with NY-ESO-1 expression of tumors and also with response to anti-CTLA-4 treatment in melanoma.[64–66] We found a humoral response against at least one of the 10 included TAAs in 53.7% of patients, but these responses were also present in healthy controls. This may be a result of previous expression of the target in these individuals or cross-reactivity. Co-occurrence of cellular and humoral immune response is poorly described in cancer. In melanoma, combined responses are comparably rare to EGA.[47] In this study, T cell responses were stronger for $CD8^+$ T cells. Cellular responses in IFN-γ EliSpot or FluoroSpot assays can be derived from $CD4^+$ and $CD8^+$ T cells and this should be considered whenever possible. However, combined cellular and humoral immune responses correlate with superior prognosis of melanoma patients treated with immune checkpoint inhibition.[66]

Cellular therapies such as adoptive T-cell transfer or CAR T-cell therapy targeting a single TAA (eg, Melan-A) showed only limited efficacy due to loss or downregulation of target expression.[16 19] Recent multitarget approaches in lymphoma and multiple myeloma showed durable clinical effects and renewed the interest in TAAs.[20 21] For example, combined targeting of five different TAAs (PRAME, SSX2, MAGEA4, SURVIVIN and NY-ESO-1) with antigen-specific autologous T cells was highly effective in lymphoma.[20] We demonstrate feasibility of combined expansion of T cells reactive to multiple TAAs using CD40B cells as a broadly applicable and autologous source of antigen-presenting cells. Hence, combined targeting of multiple TAAs appears promising also in EGA. However, tumor-specific T-cell response is only one crucial aspect, and our study highlights that co-occurrence of expression of the target gene, cellular and humoral immune response is rare. This suggests immune escape rather than a lack of endogenous immune response as mechanism underlying resistance of EGA to immunotherapy. Targeting of epigenetic mechanisms leading to loss of antigenicity of TAAs appears highly promising and may further enhance susceptibility of EGA to immunotherapy.

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