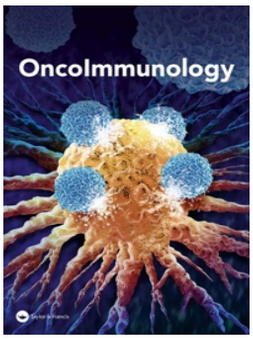

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RESEARCH ARTICLE

Intratumoral regulatory T cells are associated with treatment response to neoadjuvant chemotherapy and prognosis in gastroesophageal adenocarcinoma

Franziska Baenke[a,b,c,1], Antonia Stammberger[d,1], Ulrich Sommer[e], Sascha Brückmann[e], Felix Merboth[a,c], Heike Polster[a,c], David Digomann[a,b,c], Loreen Natusch Bufe[a,b,c], Luise Rupp[d], Daniela E. Aust[e], Jürgen Weitz[a,b,c], Daniel E. Stange[a,b,c], Marc Schmitz[b,c,d], Lena Seifert[a,b,c,f,2] and Adrian M. Seifert[a,b,c,2]

[a]Department of Visceral, Thoracic and Vascular Surgery, Medical Faculty and University Hospital Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany; [b]German Cancer Consortium (DKTK), Partner Site Dresden, and German Cancer Research Center (DKFZ), Heidelberg, Germany; [c]National Center for Tumour Diseases Dresden (NCT/UCC), a partnership between DKFZ, Faculty of Medicine and University Hospital Carl Gustav Carusm, Technische Universität Dresden, and Helmholtz-Zentrum Dresden-Rossendorf (HZDR), Dresden, Germany; [d]Institute of Immunology, Faculty of Medicine Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany; [e]Institute of Pathology and Tumour- and Normal Tissue Bank of the University Cancer Center (UCC), University Hospital Carl Gustav Carus, Medical Faculty, Technische Universität Dresden, Dresden, Germany; [f]Else Kröner Clinician Scientist Professor for Translational Tumor Immunological Research, Technische Universität Dresden, Dresden, Germany

ABSTRACT

Gastroesophageal junction (GEJ) adenocarcinoma is an increasingly common cancer with complex biology and poor prognosis. The treatment strategy for locally advanced tumors involves multimodal treatment with perioperative chemotherapy. However, survival rates remain low, especially for advanced disease. Here, formalin-fixed paraffin-embedded tumor sections from 72 patients with GEJ I and II adenocarcinoma who underwent primary resection or perioperative standard-of-care FLOT treatment were analyzed for their intratumoral T cell composition using multiplex immunohistochemistry. The proportions of T cells and their influence on survival were evaluated using Mann–Whitney *U* and log rank analyses. A comparison of short- and long-term survivors revealed significant differences in the infiltration of regulatory T cells (Tregs). Tumors after neoadjuvant FLOT treatment presented increased proportions of $CD8^+$ T cells with reduced Granzyme B expression, indicating an altered immune response. Overall survival analysis revealed that high infiltration of Tregs was associated with poor survival. Notably, responders to FLOT therapy had a greater T cell frequency and improved survival, whereas nonresponders presented higher levels of Tregs and $CD8^+$ T cells expressing TIM–3. Overall, GEJ cancer patients had increased $CD8^+$ T cells after neoadjuvant chemotherapy with FLOT, and Tregs were associated with treatment response and reduced survival.

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Multiplex immunohistochemistry; GEJ adenocarcinoma; intratumoral T cells; neoadjuvant chemotherapy

Introduction

Gastric and gastroesophageal junction (GEJ) cancers have a poor prognosis, despite recent advancements, including treatment with immune checkpoint blockade.[1-3] The incidence of gastric cancer is particularly high in East Asia, while GEJ cancer is the fastest-growing cancer type in Western populations.[4] GEJ cancers refer to adenocarcinomas located within 5 cm above and below the anatomical junction between the esophagus and stomach.[5] Although GEJ is often grouped with gastric adenocarcinoma (GAC) in cancer registries and clinical trials for targeted therapies, it differs significantly from esophageal

CONTACT Adrian M. Seifert adrian.seifert@ukdd.de Department of Visceral, Thoracic and Vascular Surgery, University Hospital Carl Gustav Carus, Technische Universität Dresden, Fetscherstraße 74, 01307, Dresden, Germany
[1]Co-first authors.
[2]Co-senior authors.
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adenocarcinoma and gastric cancer in terms of transcriptomic characteristics, clinicopathological features, and treatment outcomes.[6-8] Despite advancements in therapeutic strategies, patient outcomes in gastro-esophageal cancer patients have not significantly improved over recent years.[6] Increasing evidence suggests that the immune system might play a critical role in the response to therapy. Factors beyond tumor cell-specific responses, such as the functional state of the tumor immune microenvironment, may significantly influence treatment response and prognosis.[9,10] In the past decade, the development of immune checkpoint inhibitors, advanced molecular profiling, and a deeper understanding of the tumor microenvironment have contributed to the incorporation of immunotherapy into treatment regimens for both localized and advanced esophagogastric cancers, yielding promising outcomes.[11] The phase 2 PANDA and the neoadjuvant ChiCTR1900024428 trials are innovative approaches that combine immunotherapy with chemotherapy or chemoradiotherapy, resulting in significantly improved patient responses and survival rates.[1] The MATTERHORN trial was the first global, randomized phase III trial to show improved event-free survival with an immunotherapy-based regimen in patients with resectable gastric and GEJ cancers.[12] Understanding the complex biology of GEJ adenocarcinomas is essential for developing effective treatment strategies and improving patient prognosis. Multiplex immunohistochemistry (mIHC) analysis can offer a detailed view of the immune landscape within the tumor, which results in uncovering mechanisms of immune evasion and identifying potential targets for immunotherapeutic interventions.[13,14] Currently, there is limited knowledge regarding the expression of immune checkpoints on tumor-infiltrating lymphocytes in response to neoadjuvant therapy (NAT) in GEJ patients. To address this, mIHC was conducted on GEJ tumor tissues after primary resection or neoadjuvant chemotherapy to explore the frequency and phenotype of intratumoral T cell populations.

Material and methods

Patient samples

The cohort for multiplex immunohistochemistry included 72 patients with type I and II GEJ cancers who underwent surgery at the Department of Visceral, Thoracic, and Vascular Surgery at the University Hospital Carl Gustav Carus in Dresden, Germany, between 2018 and 2021. The tumor samples were formalin fixed, paraffin embedded, stained with hematoxylin and eosin (H&E) and evaluated by trained pathologists. The response to the neoadjuvant standard of care treatment was assessed by the tumor regression grade (TRG) following the Becker scoring system.[15] Patient characteristics were collected, including tumor staging according to the TNM classification system, and detailed clinicopathological characteristics are provided in Table 1.

Study approval

The tumor samples used in this study were taken from patients undergoing surgery for GEJ I/II cancer at the Department of Visceral, Thoracic and Vascular Surgery, University Hospital Dresden. All patients provided written informed consent, and the study was approved by the Ethics Committee of Technische Universität Dresden (EK76032013). The study was conducted in accordance with the ethical standards of the Declaration of Helsinki.

Multiplex immunohistochemistry

To characterize the phenotype, frequency, and functional orientation of GEJ I/II-infiltrating T cell subsets, two mIHC panels were established. The first panel comprised markers for cluster of differentiation (CD) 8, granzyme B (GzmB), the marker of proliferation Ki−67, programmed cell death 1 (PD−1), lymphocyte-activation gene 3 (LAG−3), and T cell immunoglobulin and mucin domain-containing protein 3 (TIM−3) to identify $CD8^+$ T cells and assess their functional status within the GEJ I/II tissue sections. The second panel consisted of antibodies against CD3, CD4, T-box transcription factor (Tbet), GATA binding protein 3 (GATA3), RAR-related orphan receptor C (RORγt), and forkhead box protein 3 (FOXP3) to distinguish between infiltrating T helper cell subsets. Patient samples were formalin fixed and paraffin embedded

Table 1. Patient characteristics.

	Overall	*n* (%)
Age		
Median (range)	65	(41–85)
Sex		
Female	7	(10%)
Male	65	(90%)
Tumor location		
GEJ I	33	(46%)
GEJ II	39	(54%)
Neoadjuvant treatment (NAT)		
No	26	(36%)
Yes	46	(64%)
Tumor regression grade		
1	13	(18%)
2	14	(19%)
3	19	(26%)
T stage		
0	1	(1%)
1	20	(28%)
2	11	(15%)
3	35	(49%)
4	5	(7%)
N stage		
0	32	(44%)
1	15	(21%)
2	13	(18%)
3	12	(17%)
M stage		
0	61	(85%)
1	11	(15%)
UICC stage		
0	1	(1%)
I	15	(21%)
II	8	(11%)
III	28	(39%)
IV	20	(28%)

(FFPE), cut into 2.5 µm thick sections and placed on adhesive immunohistochemistry slides. The staining of the FFPE tissues was performed using the Ventana DISCOVERY Ultra system (RRID:SCR_021254; Ventana Medical Systems, Basel, Switzerland), which employs tyramide signal amplification-based OPAL technology (Akoya Biosciences, Marlborough, MA, USA), as previously described.[16] In brief, following the initial steps of deparaffinization, rehydration, and antigen retrieval, incubation with a primary antibody was performed (Supplemental Table S1). A horseradish peroxidase (HRP)-coupled secondary antibody (DISCOVERY OmniMap anti-Ms HRP: Roche Cat# 760–4310, RRID:AB_2885182, DISCOVERY OmniMap anti-Rb HRP Roche Cat# 760–4311, RRID:AB_2811043, Ventana Medical Systems) and an OPAL fluorophore (Akoya Biosciences) were subsequently applied (Supplemental Table S1). Next, the resulting antibody complex was removed using a heat-mediated stripping step, and five additional staining cycles were completed, each with different primary antibodies and fluorophores. To finalize the staining protocol, the tissues were counterstained with DAPI (catalog number D9542, Sigma-Aldrich, St. Louis, Missouri, USA) and mounted with Fluoromount-G® medium (RRID:SCR_015961SouthernBiotech, Birmingham, Alabama, USA). The respective antibody suppliers, incubation times and temperatures as well as the concentrations of the primary antibodies and OPAL fluorophores are listed in Supplemental Table S1. The slides were stored at 4 °C in the dark until image acquisition.

Image acquisition and analysis

Upon whole-slide scanning of the tissues using the Vectra 3.0 automated imaging system (Akoya Biosciences) at 100× magnification, regions of interest were defined on the basis of H&MampE sections using Phenochart™ Software (RRID:SCR_019156; Akoya Biosciences). Multispectral images (MSIs) were acquired at 200× magnification, followed by spectral unmixing with inForm software (RRID:SCR_019155; Akoya Biosciences). After the MSIs were exported as multichannel TIFF files, they were imported into QuPath software (RRID:SCR_018257),[17] where a pixel classifier, cell segmentation using the Stardist

QuPath extension,[18] and a composite object classifier were trained to phenotype all detected cells. Data obtained from QuPath were processed using packages from the *tidyverse* collection in RStudio and R v.2.2.2 (RRID:SCR_000432)[19] to assess the frequency and functional characteristics of the GEJ I/II-infiltrating T cell subsets. Subsequent analyses, correlations with clinical data, and visualizations were performed in GraphPad Prism version 10.4.0 (RRID:SCR_002798). Representative images were generated by processing unmixed multichannel TIFF files using ImageJ software (RRID:SCR_003070).[20]

Statistical analysis

The data are presented as the means ± SEM or medians. Two-tailed unpaired Student's *t* test or the Mann–Whitney *U* test was applied to determine statistical significance using GraphPad Prism version 10.4.0 (RRID:SCR_002798). $P \leq 0.05$ was considered statistically significant. OS was analyzed with the log-rank test and represented as Kaplan–Meier curves.

Results

Differential abundance of tumor-infiltrating CD8$^+$ T cells between primary resected and neoadjuvant treated gastroesophageal junction adenocarcinomas

Neoadjuvant chemotherapy with 5-fluorouracil, leucovorin, oxaliplatin, and docetaxel (FLOT) significantly improved response rates and overall survival in patients with resectable, locally advanced gastric cancer and gastroesophageal junction (GEJ) disease.[21] To investigate the impact of neoadjuvant chemotherapy (NAT) on T-cell infiltration in GEJ, a cohort of 72 patients, comprising 26 patients who underwent primary resection (PR) and 46 patients who received NAT prior to resection, was analyzed using mIHC (Table 1).[22] First, the presence of conventional T helper cells was investigated using a mIHC panel composed of the markers CD3, CD4, Tbet, GATA3, RORγt, and FOXP3 (Figure 1A), whereas a second mIHC panel included the markers CD8, Ki−67, GzmB, PD−1, LAG−3, and TIM−3 (Figure 1B) to identify cytotoxic T cells, which were both costained with DAPI and applied to the GEJ samples (Supplemental Table S1). A significant proportion of CD3$^+$ CD4$^+$ T cells were negative for the analyzed transcription factors ($P = 0.4789$; median PR of 63.22% vs median NAT of 68.34%; Figure 1C). Next, the proportions of the T helper cell subpopulations were examined (Figure 1D–H). The percentage of Th1 (Tbet$^+$) cells was very low in GEJ tumor tissue for both the PR and NAT cohorts ($P = 0.8928$; PR: median of 0.072% vs NAT: median of 0.089%; Figure 1D). However, the percentage of Th2 (GATA3$^+$) cells was greater in the NAT-treated GEJs than in the PR cohort (median PR: 1.618% vs median NAT: 7.082%), although this difference was not statistically significant ($P = 0.1066$; Figure 1E). Conversely, the percentage of Th17 (RORγt$^+$) cells was greater in treatment-naïve GEJs than in those that received NAT ($P = 0.2173$; PR median 6.717% vs NAT 2.586%, Figure 1F). Interestingly, Treg cells were the most prominent T helper subset, with 14.49% in the PR cohort and 11.40% in the NAT cohort ($P = 0.0752$; Figure 1G). The number of CD4$^+$ T cells that lacked FOXP3 expression was not significantly different between the two groups ($P = 0.4789$; Figure 1H). Following the evaluation of the T helper cell frequency in NAT GEJ tumors, the abundance and phenotype of CD8$^+$ T cells, which are capable of directly killing tumor cells,[23] were assessed. Compared with those in the PR cohort, the percentage of CD8$^+$ T cells that did not express any other analyzed marker was significantly greater in the NAT cohort (82.22% vs 72.63%, $^*P = 0.0169$; Figure 1I). To further characterize CD8$^+$ T cells, we evaluated their proliferation status and function by staining for Ki−67 and the cytotoxic serine protease GzmB. The proportions of Ki−67 and GzmB were lower in the GEJ cohort after NAT than in the PR cohort (Figure 1J, K). Notably, statistical significance was only observed for GzmB ($P = 0.0170$) and not for Ki−67 ($P = 0.1966$, Figure 1J, K). As tumor cells can evade immune recognition by upregulating immune checkpoints,[9,24] CD8$^+$ T cells expressing either PD−1, LAG−3, or TIM−3 were analyzed (Figure 1L–N). The proportions of CD8$^+$ PD−1$^+$ and CD8$^+$ LAG−3$^+$ T cells were lower in the NAT cohort than in the PR cohort (PD−1: 0.2781% vs 0.1841%, $P = 0.3891$; LAG−3: 7.368% vs 4.043%, $P = 0.0534$; Figure 1L, M), whereas the proportion of CD8$^+$ TIM−3$^+$ T cells was slightly greater in the NAT cohort (0.3907% vs 0.4362%, $P = 0.9838$; Figure 1N). Collectively, these data suggest that Tregs constitute the most prominent Th cell subset in GEJ tumors, despite the lack of difference in frequency between the NAT and

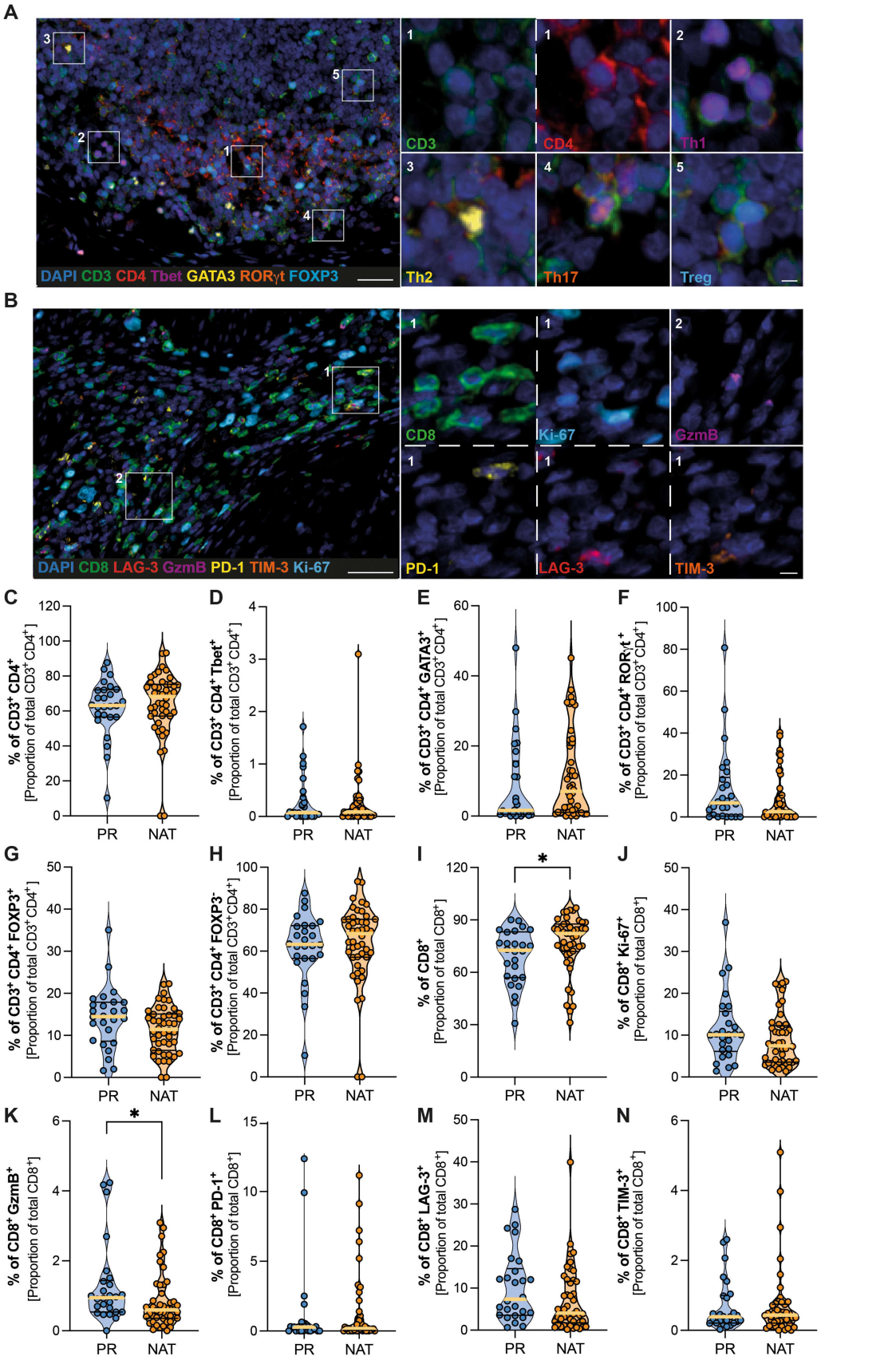

Figure 1. Differential abundance of tumor-infiltrating CD8$^+$ T cells between primary resected and neoadjuvant treated gastroesophageal junction adenocarcinomas. (A) Representative mIHC image of the T cell markers CD3 (green), CD4 (red), Tbet (purple), GATA3 (yellow), RORγt (orange), and FOXP3 (cyan) counterstained with DAPI (blue).

(caption on next page)

The image is from a tumor section from a treatment-naïve patient (PR). (B) Representative mIHC image of the T cell markers CD8 (green), LAG−3 (red), GzmB (purple), PD−1 (yellow), TIM−3 (orange), and Ki−67 (cyan) and counterstaining with DAPI (blue). The image is from a tumor section from a patient who received neoadjuvant chemotherapy prior to surgery (NAT). Scale bar in overview image: 100 µm, scale bar in magnifications: 10 µm for A and B. Violin plots of the median (C) proportions of $CD3^+CD4^+$ T cells alone, or coexpressing (D) $Tbet^+$, (E) $GATA3^+$, (F) $ROR\gamma t^+$, (G) $FOXP3^+$, (H) $FOXP3^-$, and (I) $CD8^+$ T cells, co-expressing (J) Ki−67, (K) GzmB, (L) PD−1, (M) LAG−3, and (N) TIM−3 of PR patients ($n = 26$; in blue) and NAT patients ($n = 46$; in orange). *P* values were calculated using the unpaired t test (Mann–Whitney U, MWU). $^*P \leq 0.05$.

PR cohorts. Furthermore, compared with the PR cohort, the NAT cohort presented lower proportions of $Ki{-}67^+$ and $GzmB^+$ $CD8^+$ cells, whereas no difference in the expression of immune checkpoints in $CD8^+$ T cells was observed.

A low proportion of intratumoral Tregs is associated with improved overall survival after neoadjuvant chemotherapy

The introduction of neoadjuvant FLOT chemotherapy significantly improved response rates and overall survival in patients with resectable, locally advanced gastric cancer, and GEJs.[21] The presence of $CD3^+$ $CD4^+$, and $CD8^+$ T cells across the analyzed tumor samples differed, as some surgical specimens presented lower numbers of tumor-infiltrating T cells than others did (Figure 2A). To determine which infiltrating T cell subsets might influence survival, Kaplan–Meier survival analysis stratified by the median infiltration levels of different immune cell subsets was performed for the PR and NAT cohorts. In the PR cohort, no significant difference in overall survival was observed between high- and low-infiltrating $CD3^+$ $CD4^+$ $FOXP3^+$ T cells ($P = 0.4399$; Figure 2B). Conversely, the NAT cohort showed a statistically significant association of lower $CD3^+$ $CD4^+$ $FOXP3^+$ T cell density with improved overall survival ($^{**}P = 0.0013$; Figure 2B). While investigating the frequency of $CD3^+$ $CD4^+$ $FOXP3^-$ T cells in both cohorts revealed no significant survival difference (PR: $P = 0.1702$, NAT: $P = 0.7178$; Figure 2C), PR patients with a higher frequency of $CD3^+$ $CD4^+$ $FOXP3^-$ T cells had better overall survival than did PR patients with a lower frequency of $CD3^+$ $CD4^+$ $FOXP3^-$ T cells. Furthermore, the infiltration of $CD8^+$ T cells was not associated with survival in either the PR or NAT cohort ($P = 0.1473$ and $P = 0.9165$, respectively, Figure 2D), although a high abundance of $CD8^+$ T cells alone in the PR cohort tended to improve survival, which could be important for surgical stratification. The proportions of the immune checkpoints PD−1, LAG−3, and TIM−3 also did not correlate with survival when patients were stratified into high and low expression groups (Supplemental Figure S1A–C). In summary, low intratumoral Treg infiltration after NAT is associated with increased survival.

Nonresponders to neoadjuvant chemotherapy display increased proportion of Treg cells and TIM−3-expressing $CD8^+$ T cells

Next, the response of infiltrating T cell populations to neoadjuvant treatment with FLOT was evaluated. Compared with nonresponders (NR, minor tumor regression grade 3), responders (R: classified here as major tumor regression grade 1 or moderate tumor regression grade 2) to NAT had significantly improved overall survival; ($^{***}P = 0.0001$; Figure 3A). Immune profiling of the infiltrating T cells revealed no significant differences in the proportions of $CD3^+$ $CD4^+$ T cells (Supplemental Figure S2A), including $CD3^+$ $CD4^+$ $Tbet^+$, $GATA3^+$ and $ROR\gamma t^+$ subsets, between responders and nonresponders (Supplemental Figure S2B–D). However, the proportion of Treg cells ($FOXP3^+$) was significantly greater in the NR cohort than in the R cohort (NR: 14.75% vs R: 10.00%; $^*P = 0.0121$, Figure 3B), whereas the proportion of $FOXP3^{+-}$ cells was similar in both cohorts (NR: 64.48% vs R: 69.49%; $P = 0.5321$, Figure 3C). The total $CD8^+$ T cell proportions were also similar between the groups (Figure 3D). Additionally, nonresponders to FLOT therapy presented increased levels of proliferating $CD8^+$ $Ki{-}67^+$ T cells ($P = 0.0516$, Figure 3E), whereas $CD8^+$ $GzmB^+$ cells presented no differences between R and NR ($P = 0.2139$, Figure 3F). Furthermore, the expression of the immune checkpoint markers PD−1 and LAG−3 on $CD8^+$ T cells was not significantly different between R and NR (PD−1: $P = 0.8464$; LAG−3: $P = 0.2774$; Figure 3G, H).

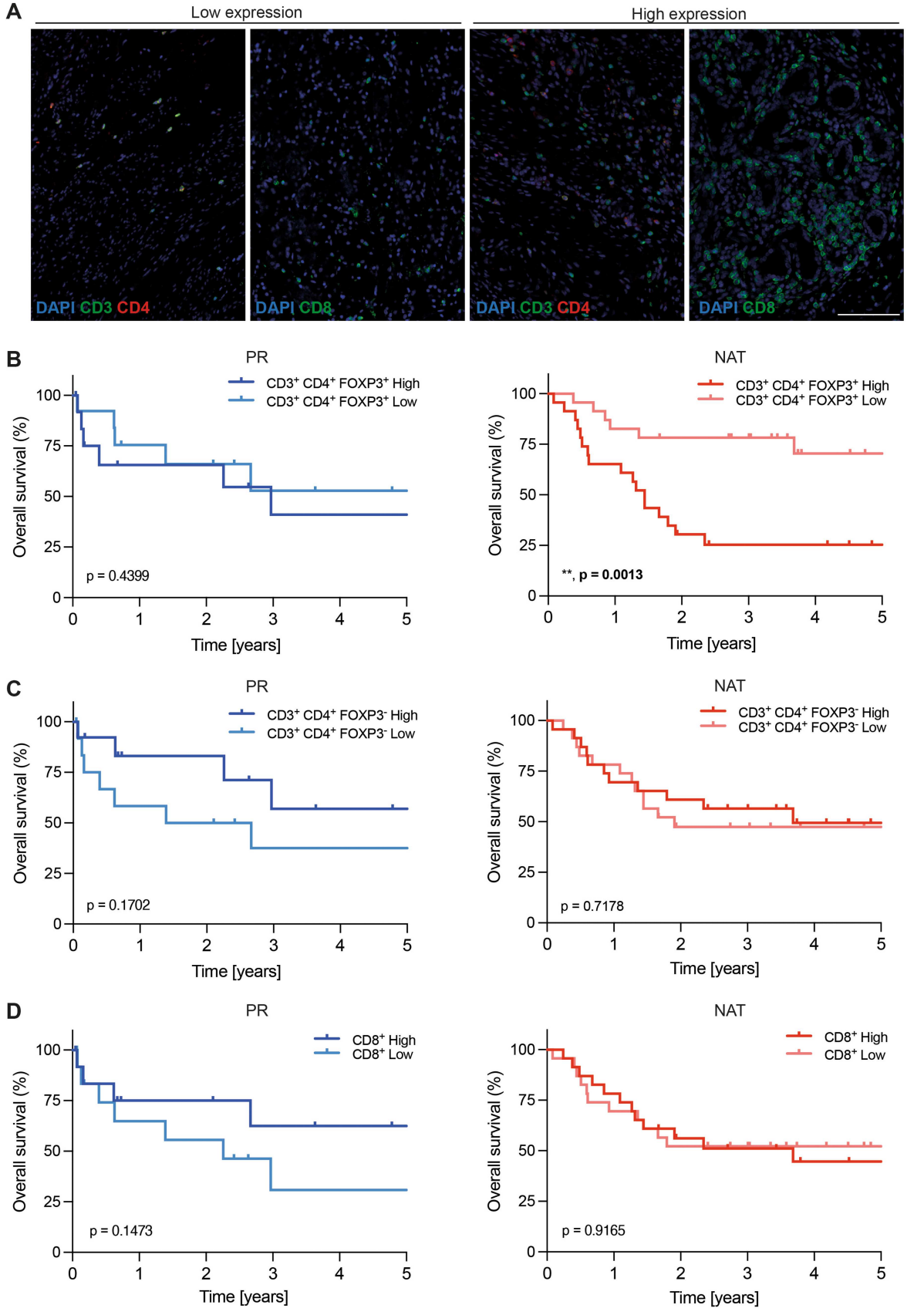

Figure 2. A low proportion of intratumoral Tregs is associated with improved overall survival after neoadjuvant chemotherapy. (A) Representative mIHC image of high- and low-frequency $CD3^+CD4^+$ Th cells and $CD8^+$ cytotoxic T cells, both of which were counterstained with DAPI. Scale bar: 50 μm. Images of $CD3^+CD4^+$ and high-$CD8^+$ cells were

(caption on next page)

obtained from NATs, whereas images of low-CD8$^+$ cells were obtained from a primary resected tumor. Kaplan–Meier analysis of overall survival of patients with GEJ, stratified by the median of the indicated proportions of (B) CD3$^+$CD4$^+$FOXP3$^+$, (C) CD3$^+$CD4$^+$FOXP3$^-$, and (D) CD8$^+$ T cells of PR and NAT patients. *P* values were calculated using the log-rank test. $^{**}P \leq 0.01$.

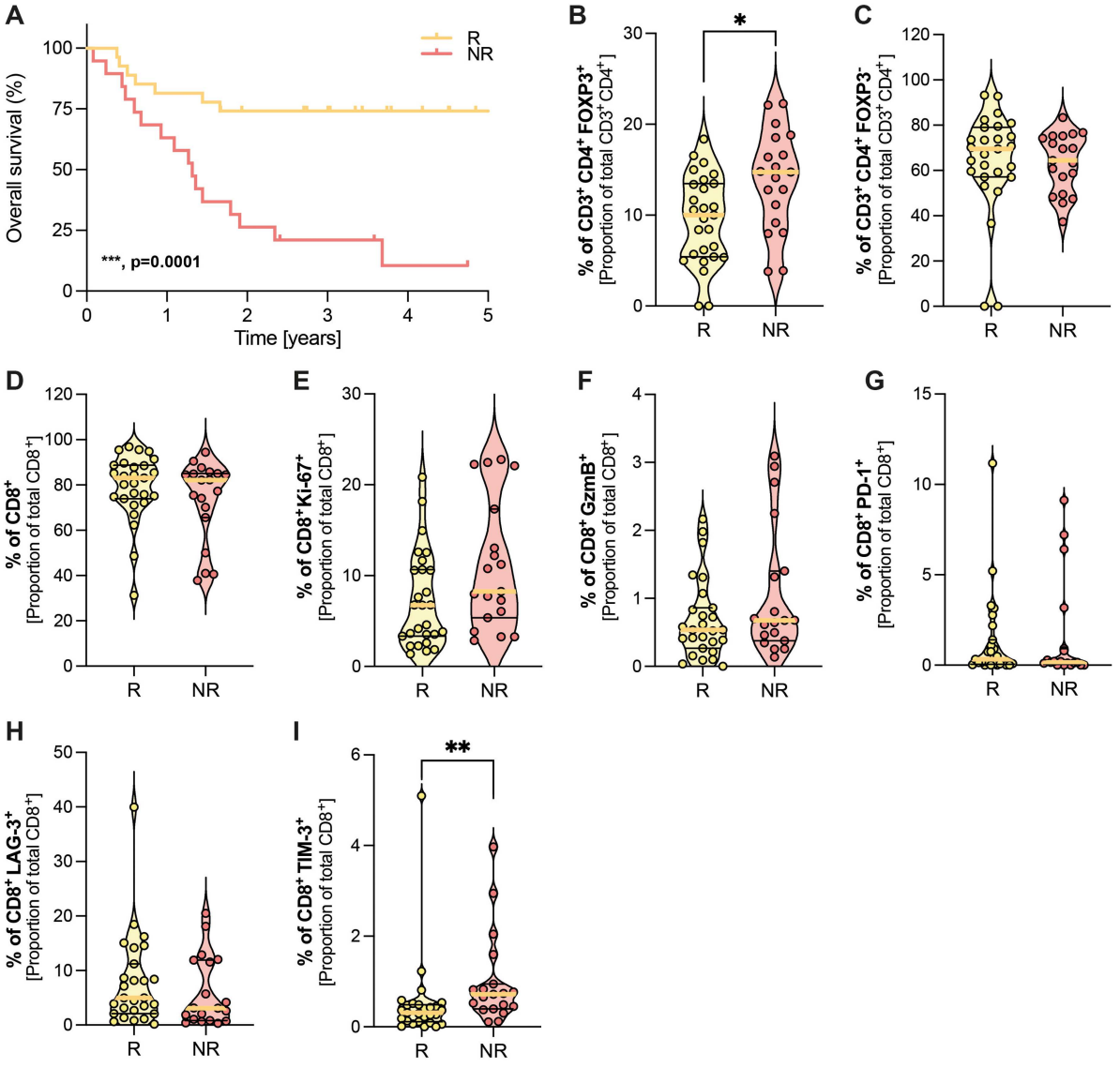

Figure 3. Nonresponders to neoadjuvant chemotherapy display increased proportions of Treg cells and TIM–3-expressing CD8$^+$ T cells. (A) Kaplan–Meier analysis of the overall survival of patients with GEJ according to treatment response to FLOT according to the pathological assessment. The responders (R) included major or moderate responses (TRG1/2, $n = 27$), and the nonresponders (NR) included minor responses (TRG3, $n = 19$). The *P* value was calculated using the log-rank test; $^{***}P \leq 0.001$. Violin plots of the median proportions of intratumoral conventional T cells CD3$^+$CD4$^+$ alone (B) or coexpressing FOXP3$^+$, (C) FOXP3$^-$, and (D) cytotoxic T cells CD8$^+$, or coexpressing (E) Ki–67, (F) GzmB, (G) PD–1, (H) LAG–3 or (I) TIM–3, according to response status. Mann–Whitney *U* test. $^{**}P \leq 0.01$, $^{*}P \leq 0.05$.

However, the proportion of CD8$^+$ TIM–3$^+$ T cells was significantly greater in the NR (NR: 0.7192% vs R: 0.3111%; $^{**}P = 0.0016$, Figure 3I). These findings suggest that the immune microenvironment in non-responders to NAT is characterized by the proliferation of not yet anergic CD8$^+$ T cells. Furthermore, CD8$^+$ T cells from nonresponders, particularly those expressing TIM-3, imply an altered immune response compared with those from responders.

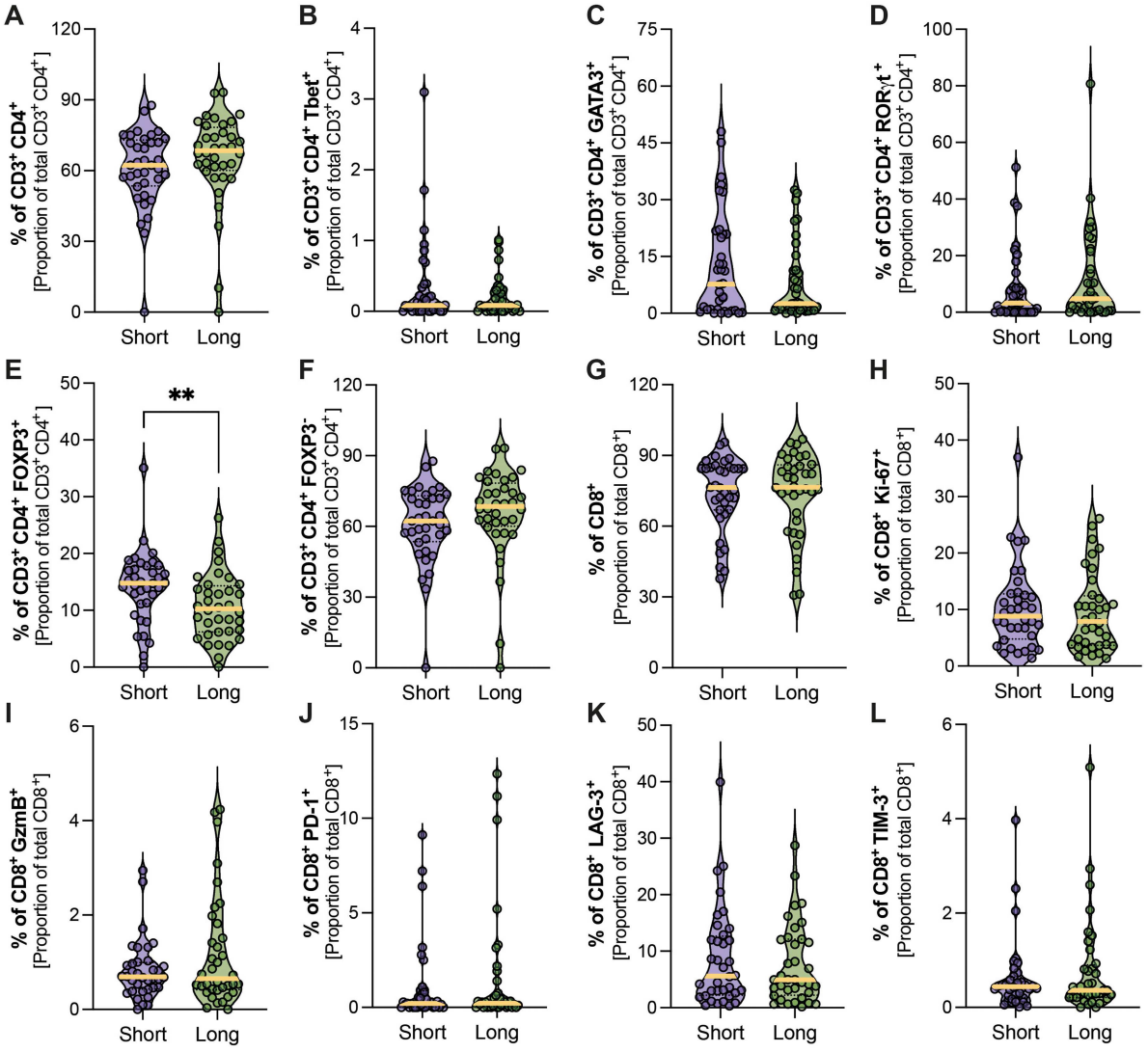

Figure 4. Increased intratumoral Treg cells predict short-term survival. Patients were stratified into short-term (<2y, $n = 36$) and long-term (≥2y, $n = 36$) survivors based on their overall survival. Violin plots of GEJ patients depict the median proportions of intratumoral T helper cells (A) $CD3^+CD4^+$ alone, or coexpressing (B) $Tbet^+$, (C) $GATA3^+$, (D) $ROR\gamma T^+$, (E) $FOXP3^+$, (F) $FOXP3^-$, and (G) cytotoxic $CD8^+$ T cells alone, or co-expressing (H) Ki−67, (I) GzmB, (J) PD−1, (K) LAG−3, and (L) TIM−3 compared between short-term (violet) and long-term (green) survivors. Mann–Whitney *U* test. $^{**}P \leq 0.01$.

Increased intratumoral Treg cells predict short-term survival

To evaluate the prognostic significance of overall survival in GEJ patients, the expression of immune response regulators on tumor-infiltrating T cells was assessed. Patients were stratified into short-term (<2y) and long-term (≥2y) survivors based on their overall survival (Supplemental Table S2). No difference in proportion was observed for $CD3^+CD4^+$ T cells, including $Tbet^+$, $GATA3^+$, and $ROR\gamma t^+$ subsets (Figure 4A-D). In contrast, the number of tumor-infiltrating $CD3^+CD4^+FOXP3^+$ T cells was significantly greater in short-term survivors than in long-term survivors ($^{**}P = 0.0072$; short: 14.79% vs long: 10.28%; Figure 4E). In contrast, no significant correlation was observed between overall survival and the proportion of $CD3^+CD4^+FOXP3^-$ T cells ($P = 0.1021$; Figure 4F) as well as cytotoxic T cells expressing CD8 alone ($P = 0.7753$; Figure 4G), or additional markers Ki−67 ($P = 0.6342$; Figure 4H), and GzmB ($P = 0.5891$; Figure 4I), or immune checkpoints PD−1 ($P = 0.8882$), LAG−3 ($P = 0.7839$) or TIM−3 ($P \geq 0.9999$; Figure 4J–L). Collectively, these results highlight the distinct prognostic implications of infiltrating T cell subsets in GEJ.

Discussion

GEJ cancer represents a growing challenge in clinical practice and research, with a rising global incidence and a tendency to be diagnosed at advanced stages owing to the lack of early symptoms.[25] The clinical management of locally advanced GEJ adenocarcinoma has been a topic of significant debate.[26,27] As GEJs are biologically distinct from both gastric and esophageal cancers, they complicate treatment strategies and necessitate a more personalized approach to care. Current standard-of-care neoadjuvant FLOT chemotherapy has demonstrated limited efficacy, as a significant proportion of patients exhibit resistance and poor pathological responses. Here, we examined the frequency, phenotype and function of tumor-infiltrating T cells in GEJ patients following NAT and assessed their associations with clinical outcomes. Tregs were the most prominent Th cell subset with a decreased frequency in the NAT cohort. Additionally, patients with lower Treg infiltration after NAT had improved overall survival. This has been reported by others, who reported that high Treg infiltration in the stromal compartment of esophageal squamous cell carcinomas,[28] melanomas and gastric cancers contributes to worse overall survival.[29] In contrast, NR had more Tregs, suggesting a potential role of these cells in the immune response and therapy outcome. One consideration could be whether patients with high Treg infiltration after NAT might benefit from additional immunotherapy.[30] Since Tregs play a suppressive role in the tumor microenvironment, targeting these cells or blocking their function may potentially increase antitumor immunity.[31] Strategies such as anti-CD25 or anti-CCR4 antibodies have shown promise in preclinical models.[25,26] However, clinical trials with daclizumab (anti-CD25) or mogamulizumab (anti-CCR4) confirmed that while Tregs were depleted, a reduction in effector T cells was also observed, thereby impairing antitumor immunity and causing autoimmunity.[32] Thus, the overall aim should be the identification of targets for specific intratumoral Treg depletion. Approaches, such as inhibition of PI(3)K p110δ, p300, or CoREST or genetic alteration of Treg-specific deletion of St2, an IL−33 receptor, or Cd36, have been shown to successfully deplete Tregs. This leads to increased $CD8^+$ T cell infiltration and a decreased tumor burden.[33-38] In addition, cyclophosphamide has also been shown to reduce intratumoral Tregs and prolong survival across tumor types,[39,40] supporting strategies that act locally within the TME. Furthermore, our data demonstrated that TIM−3 expression by $CD8^+$ T cells in GEJs is linked to a poor response to NAT. High TIM−3 expression has been correlated with nodal metastasis and advanced stage in non-small cell lung cancer,[41] and it is significantly associated with poor survival in gastric cancer patients.[42] A meta-analysis also confirmed its relevance as a negative prognostic marker across multiple solid tumors, including lung, gastric, and colorectal cancers.[43] These findings underscore the role of TIM-3 as a promising immunotherapeutic target. Anti-TIM−3 antibodies, such as TSR−022 (NCT02817633), have shown tolerability and preliminary efficacy both as monotherapies and in combination with PD−1 blockade,[44] while other trials (NCT03099109, NCT03066648) reported favorable safety and durable responses, particularly with chemotherapy.[45,46] Given its association with a poor response to NAT in GEJ cancer patients, integrating anti-TIM-3 therapy into treatment regimens may enhance chemotherapy efficacy and improve outcomes. In the era of immunotherapy and combination strategies with standard-of-care treatments, this approach has high priority.[47] Here, immunophenotyping and molecular profiling of GEJ tumors can help predict therapeutic responses, enabling more personalized and effective treatment strategies. A recent comprehensive immunophenotyping study analyzed 104 untreated GEJ cases, including 38 GEJs, by flow cytometry and mIHC.[48] The study revealed that tumors located in the esophagus exhibit more immunosuppressive features than those in the GEJ or stomach do. This may potentially explain the location-specific variability in response to immunotherapy in this disease. Biomarker identification and stratification in cancer are crucial for tailoring treatment strategies, as they help to predict therapeutic responses and patient outcomes, enabling more personalized and effective management of disease.[49] Furthermore, a recent study examined the evolutionary and immune TME changes in 27 GEJs using longitudinal sampling before, during and after neoadjuvant treatment.[50] Their integrative approach revealed that genetic clonal shifts are rare, indicating that resistance is driven by phenotypic plasticity rather than clonal evolution. Using T-cell receptor sequencing and mIHC, immune modulation was detected in the NR, leading to a more immunosuppressive environment associated with reduced cytotoxicity and poor treatment response.[50] These insights emphasize the need for therapies that target immune dynamics rather than genetic mutations. These studies and our work underscore the need for

continued investigations of GEJ cancers, particularly those focused on their immunological landscape, with an impact on therapeutic options. The integration of advanced therapeutic strategies with robust biomarker-driven approaches and/or immunophenotyping of the tumor using mIHC might hold the potential to significantly reshape the therapeutic landscape for GEJ cancer.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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ORCID

Franziska Baenke 0000-0001-9389-4688
Antonia Stammberger 0009-0005-1280-967X
Felix Merboth 0000-0003-2540-6421
David Digomann 0000-0002-2139-9183
Loreen Natusch Bufe 0009-0002-2144-552X
Luise Rupp 0000-0002-6610-326X
Daniela E. Aust 0000-0002-4867-8852
Jürgen Weitz 0000-0002-5760-6391
Daniel E. Stange 0000-0003-4246-2230
Marc Schmitz 0000-0003-3417-6736
Lena Seifert 0000-0003-4763-8127
Adrian M. Seifert 0000-0002-5329-3164

Data availability statement

The data generated in this study are available upon request from the corresponding author.

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