

Mapping the complexity and diversity of tertiary lymphoid structures in primary and peritoneal metastatic gastric cancer

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ABSTRACT

Background Tertiary lymphoid structures (TLSs) are thought to stimulate antitumor immunity and positively impact prognosis and response to immune checkpoint blockade. In gastric cancers (GCs), however, TLSs are predominantly found in GC with poor prognosis and limited treatment response. We, therefore, hypothesize that immune cell composition and function of TLS depends on tumor location and the tumor immune environment.

Methods Spatial transcriptomics and immunohistochemistry were used to characterize the phenotype of CD45⁺ immune cells inside and outside of TLS using archival resection specimens from GC primary tumors and peritoneal metastases.

Results We identified significant inpatient and interpatient diversity of the cellular composition and maturation status of TLS in GC. Tumor location (primary vs metastatic site) accounted for the majority of differences in TLS maturity, as TLS in peritoneal metastases were predominantly immature. This was associated with higher levels of tumor-infiltrating macrophages and Tregs and less plasma cells compared with tumors with mature TLS. Furthermore, mature TLSs were characterized by overexpression of antitumor immune pathways such as B cell-related pathways, MHC class II antigen presentation while immature TLS were associated with protumor pathways, including T cell exhaustion and enhancement of DNA repair pathways in the corresponding cancer.

Conclusion The observation that GC-derived peritoneal metastases often contain immature TLS which are associated with immune suppressive regulatory tumor-infiltrating leucocytes, is in keeping with the lack of response to immune checkpoint blockade and the poor prognostic features of peritoneal metastatic GC, which needs to be taken into account when optimizing immunomodulatory strategies for metastatic GC.

INTRODUCTION

Gastric cancer (GC) is a leading cause of cancer-related death,¹ mainly because the disease is often detected at a late stage in which systemic therapies are only minimally

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Tertiary lymphoid structures (TLS) in cancer are increasingly associated with improved patient survival and responsiveness to immune checkpoint blockade. However, in gastric cancer (GC), TLS are mostly present in patients with worse outcome, and the cause of this discrepancy is presently unknown.

WHAT THIS STUDY ADDS

⇒ This study uncovers a wide spectrum of TLS within GC-derived primary tumors and peritoneal metastases and pinpointed interpatient variation, maturation status and tumor location as underlying factors for diversity. In addition, tumors with mature TLS have increased numbers of infiltrating CD8⁺ T memory cells and plasma cells and express cancer cell apoptosis pathways, whereas tumors with immature TLS seem more immunosuppressive.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ These findings indicate that TLS diversity, and particularly maturation status, are key in shaping the tumor immune microenvironment. Future immunotherapeutic strategies should, therefore, take TLS maturation status into account.

effective. In search for biomarkers to select patients for treatment with immune checkpoint inhibitors, tertiary lymphoid structures (TLS) have recently gained increased attention due to their potential role in enhancing the antitumor immunity cycle.² Although this has never been directly demonstrated, this assumption is derived from the fact that TLS presence is (1) generally associated with a good prognosis^{3,4} and (2) associated with (PD-1) immune checkpoint blockade (ICB) response in multiple cancer types.^{3,5–7} TLS have shown great promise for predicting immunotherapeutic responses.⁸ TLS are de

novo formed and organized lymphoid aggregates (LAs) that are often observed at sites of chronic inflammation such as autoimmune disease, allograft rejection and cancer.^{9–12} These chronic inflammatory conditions stimulate and maintain the TLS by the production of various chemokines, recruiting the immune cells to form immune cell clusters that mimic lymph follicles as observed in lymphnodes.^{13–19} The inflammatory cues involved in lymphoid tissue formation have mostly been described for the development of secondary lymphoid organs and TLS in some inflammatory conditions, hence there may be other drivers in cancer. Most information on the phenotype and role of TLS in metastatic cancer comes from tumors with mature TLS and a favorable response to ICB. A negative effect of TLS on prognosis and recurrence rates is seen in few cancer types, despite the classical TLS characteristics.^{20–23} In a previous GC immunoprofiling study, TLS were mainly, but not exclusively found in GCs with a poor prognosis.^{24–26} This suggests that TLS function may be heterogeneous and may depend on the tumor immune environment in which TLS develop. In this study, we performed a detailed spatial transcriptomic analysis of tumor and immune cells in both GC primary tumors (PTs) and (matching) peritoneal metastases (PMs), which allowed us to define the contribution of the location and immune environment to TLS immune cell composition as well as its phenotypic characteristics.

METHODS

Patient cohorts

Formalin-fixed paraffin-embedded samples of GC-derived PT and PM were selected from two series of patients from the pathological archive in Amsterdam UMC, the Netherlands, and in Hospital Clínico Universitario in Valencia, Spain (online supplemental table S1). We chose PMs as a model for metastatic disease as the peritoneum is the most common metastatic site of GC. Furthermore, PMs are one of the few metastases that are occasionally resected instead of biopsied. The presence of TLS cannot be reliably assessed within biopsies because TLSs are distributed unevenly, and therefore, often missed in a biopsy. Archival material was used in compliance with the institution's ethical regulations. The first cohort (Amsterdam UMC) contained patients who underwent a resection of the PT (n=6) or of the PM (n=4). All tissues were treatment naïve. Tumors were selected based on the presence of LAs by an expert pathologist (NCTvG) using a H&E and CD20 staining. As PTs are not resected in the Netherlands in case of known PM, PT and PM are from different patients.

The second cohort consisted of patients who underwent a resection of both the PT and PM simultaneously (n=8) at Hospital Clínico Universitario in Valencia, Spain. In five patients both the PT and PM contained TLS. All tumors from both cohorts were microsatellite stable and none of the patients received neoadjuvant treatment.

Thus, a total of 26 samples (14 PT and 12 PM) from 18 patients were analyzed.

Immunohistochemistry

Chromogenic staining was performed with anti-CD20 (clone L26, Dako), anti-PNAd (clone MECA79, BD Biosciences), follicular dendritic cell (FDC) (clone can.42, Invitrogen), and anti-DC-lysosomal-associated membrane protein (LAMP (clone EPR24265-8, Abcam). Multiplex) (clone EPR24265-8, Abcam). Multiplex immunohistochemistry (IHC) was performed using the OPAL Polaris 7-Color IHC Detection kit (Akoya Biosciences, USA), anti-cytokeratin (CK; clone AE1/AE3, Dako), anti-CD8 (clone C8/144B, Dako), anti-CD3 (polyclonal, Dako), anti-FoxP3 (clone 236A/E7, Abcam), anti-CD163 (clone 10D6, Novocastra) and anti-Ki67 (clone SP6, Abcam). Stainings were performed following the manufacturer's instructions. Imaging was conducted with the Vectra Polaris microscope (Akoya Biosciences, USA). IHC data were analyzed using QuPath (V.0.5.0).

Spatial transcriptomics

Spatial transcriptomics was performed using the GeoMx Digital Spatial Profiler (Nanostring, USA) using the Cancer Transcriptome Atlas panel according to the manufacturer's instructions.²⁷ Per cohort, 95 regions of interest (ROIs) were chosen: CD45⁺ TLS (as confirmed by IHC), CD45⁺ tumor-infiltrating leucocytes (TIL), and CK⁺ cancer cells (online supplemental tables S2 and S3).

Spatial transcriptomic analyses

Data were analyzed using R (V.4.3.1). Quality control was performed according to GeomxWorkflows (V.1.5.0) using the GeomxTools (v3.2.0) package. The following quality settings were used within the *setSegmentQCFlags* function: minSegmentReads=1000, percentTrimmed=80, percentStitched=90, percentAligned=75, percentSaturation=50, minNegativeCounts=1, maxNTCCCount=9000, minNuclei=100, minArea=1000. ROIs that passed these requirements were used for downstream analyses. For the second cohort, this meant that patient-matched analysis was only possible for five out of the eight patients. Expression data was Q3 normalized using the *normalize* function of NanoStringNCTools (V.1.6.1) and subsequently log₂ transformed. Cell abundances were estimated by SpatialDecon (V.1.8.0)²⁸ and the associated SafeTME matrix.

Differential expression of genes and molecular pathways were determined using the GeomxTools *mixedModelDE* function using a random slope for patient effects. When combining the data from both cohorts, a random slope correcting for batch effects was included. Pathway lists were supplied with the CTA panel. Additional pathways were derived from Huang *et al.*²⁹ and the Reactome database via MSigDB. Gene set enrichment (GSA) was scored using the *gsa* package (V.1.46.0) according to the single sample GSA analysis (ssgea) method. Pathways containing three genes or less were excluded.

Statistical analyses

Wilcoxon tests were performed using *rstatix* (V.0.7.2). Unpaired and paired Wilcoxon tests were considered statistically significant when $p < 0.05$. Pairwise Wilcoxon tests, differential gene expression, and pathway analyses were considered significant when false discovery rate (FDR) < 0.05 . Spearman correlations were calculated using the *corr.test* function of *psych* (V.2.3.6) and deemed relevant when $p < 0.05$ and $\rho < -0.6$ or > 0.6 . Multivariate linear modeling was achieved using the base *lm* function and deemed significant when $p < 0.05$.

RESULTS

Higher interpatient than inpatient variability in TLS structure and cell content

To detail the structural and cellular features of GC-derived TLS, GC PT and PM were characterized with IHC using the following features: high endothelial venules (HEV; PNAd), mature DC (DC-LAMP), FDC, T cells (CD3), CD8⁺ T cells (CD8), regulatory T cells (Treg; FoxP3), macrophages (CD163) and tumor cells (CK; [figure 1A,B](#)). The number of TLS was based on CD20 staining and varied considerably between PT (range 15–57, 29 ± 17) and PM (range 4–25, 14 ± 11). FDC, DC-LAMP, and PNAd expression varied between patients but showed little inpatient variability ([table 1](#)). There was no difference between PT and PM regarding the number of TLS per mm² (online supplemental figure S1A). TLS in PM were surrounded by not only cancer and stroma cells but also by peritoneal adipose tissue ([figure 1C](#)). 76% of PT-derived TLS exhibited CD20⁺ inner zones and CD3⁺ outer zones while this organizational structure was present in only 41% of PM-derived TLS. However, the expression of PNAd⁺, FDC⁺ and DC-LAMP⁺ was similar between PT and PM ([table 1](#)). Overall, patient variability between the analyzed immune subsets was low, although the densities of CD8⁺ T cells and Treg were higher in TLS of PT ([figure 1D](#), online supplemental figure S1B).

Maturation status shapes the interpatient diversity of TLS

To comprehensively analyze GC-derived TLS, we characterized the immune compositions of TLS (n=53) using spatial transcriptomics ([figure 2A](#)). The predominant cell types in TLS were B cell subsets, including naive B cells (24.1%) and memory B cells (18.0%), T cell subsets such as CD4⁺ memory T cells (17.9%) and CD8⁺ memory T cells (10.7%), and macrophages (7.2%; [figure 2B](#)). Compared with PT, PM expressed significantly higher proportions of macrophages and Tregs (online supplemental figure S2A). Similar to IHC, the immune composition of TLS showed high concordance within patients while substantial heterogeneity was observed between patients (online supplemental figure S2B).

We then analyzed the TLS maturation state using the following maturation-associated and cell-associated genes: *CXCL13* (key TLS initiator), B cells (*MSA41/CD20*), FDC (*FCER2/CD23*, *CR2/CD21*), and germinal center B cells

(*BCL6*; [figure 2C](#)), as previously described.^{2 30–32} Expression of these markers was low in 12 ROIs (23%); these were classified as LA. In 11 ROIs (21%), the expression was moderate; these were considered ‘early TLS’. 17 TLS (32%) expressed high levels of *CXCL13*, *MSA41*, *CR2* and B memory cells, which were categorized as primary follicle-like (PF-like) TLS. Finally, due to either high expression of either *BCL6*, *CR2* and plasma cells, or *FCER2* and B naive cells, 13 TLS (25%) were annotated as secondary follicle-like (SF-like) TLS. LA and early TLS were considered as immature (iTLS); SF-like and PF-like TLS were annotated as mature (mTLS).^{2 30–32} Interestingly, 70% of the PT-derived TLS (26/37) exhibited a mature status, while this only applied for 25% of the PM-derived TLS (4/16). Moreover, TLS maturation states showed high intra-patient agreement. UMAP analysis confirmed a maturation-specific clustering of TLS transcriptomes ([figure 2D](#)).

In line with our annotation strategy, LAs were enriched with macrophages and Tregs, and early TLS with CD4⁺ T memory, CD8⁺ T memory, and CD8⁺ T naive cells. PF-like TLS were densely populated with B memory cells and SF-like TLS with B naive and plasma cells ([figure 2E](#)). Thus, iTLSs contain more macrophages and T cell subsets, whereas mature TLS harbor B cells subsets in various states of differentiation (online supplemental figure S2C). Reflecting these differences, iTLS showed upregulation of interleukin (IL) signaling, T cell-related pathways, lymphocyte trafficking and M2-like macrophage activation ([figure 2F](#)) while mTLS expressed more B cell-related pathways, MHC class II antigen presentation, and VEGF signaling.

Next, the expression of the previously postulated 12-chemokine signature, a signature that is strongly enriched in bulk transcriptome data from TLS-containing cancers, was determined, of which nine were available with our probe panel ([figure 2G](#)). These chemokines were not synonymously expressed. One cluster of mainly LA-expressed *CCL2*, *CCL18*, *CCL19*, and *CCL21*. A mixed cluster of mTLS and iTLS expressed *CXCL13*, *CCL4*, *CCL5*, *CXCL10*, and *CXCL9*, suggesting that these chemokines associate with distinct TLS states. Interestingly, these chemokines were exclusively expressed within CD45⁺ ROIs, and *CCL19*, *CCL21* and *CXCL13* even solely by TLS (online supplemental figure S2D). When correlating individual chemokines with subset proportions, we identified associations between *CXCL13* and B naive cells, *CCL2* and macrophages, and *CCL19* and CD4⁺ naive T cells (all $p < 0.001$, online supplemental figure S2E).

Tumors with mTLS have higher infiltrating CD8⁺ T memory cells and plasma cells

We then hypothesized that maturation-associated immunological TLS heterogeneity shapes the antitumor immune response. Due to the sample size, we were unable to perform meaningful differential pathway analyses between TLS and TIL within the same patient. Therefore, we analyzed the immune composition of two

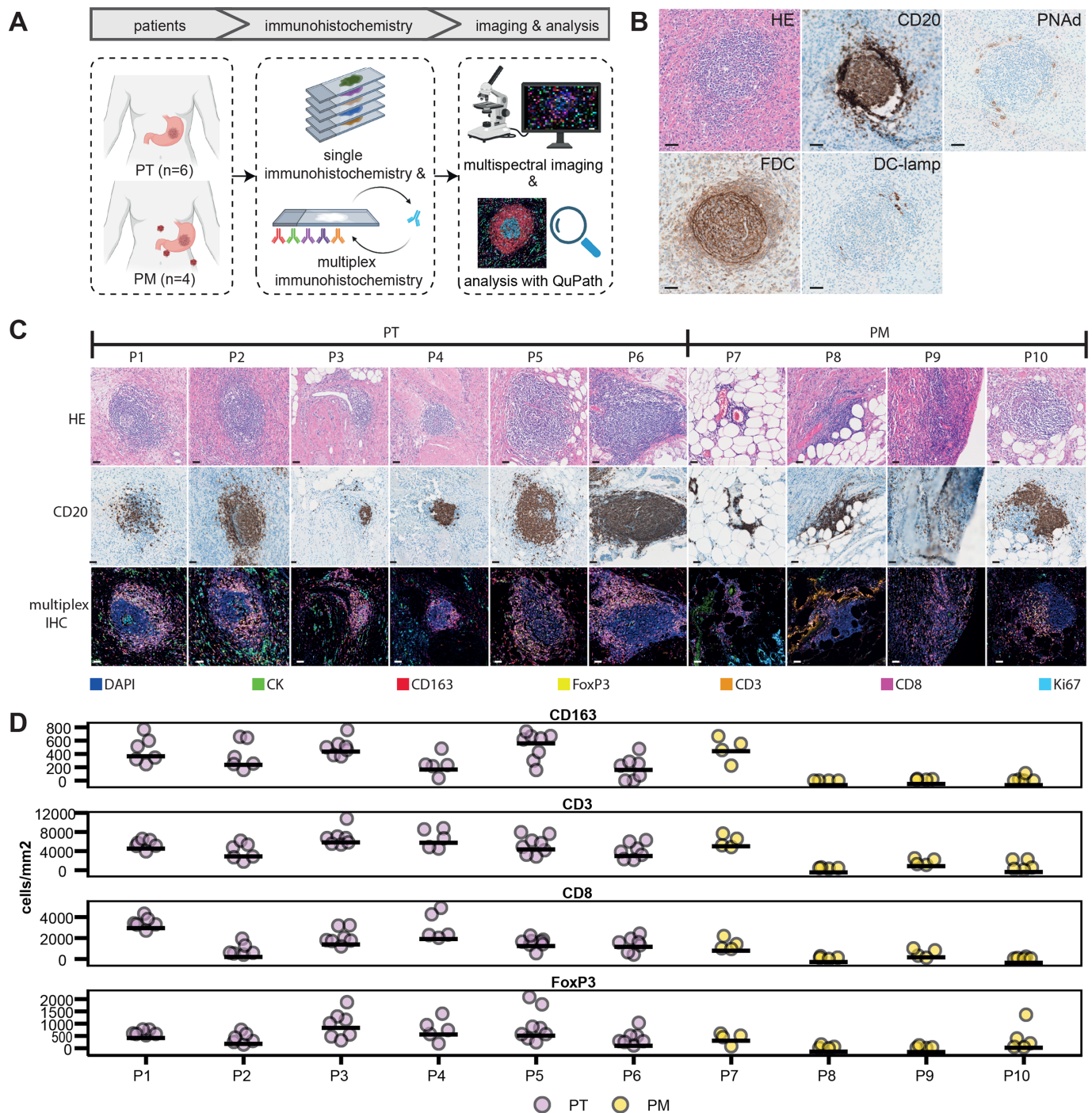


Figure 1 Structural and cellular characteristics of TLS in GC PTs and PMs. (A) Cohort of PT (n=6) and unmatched PM (n=4) were characterized using single IHC featuring HE, CD20, PNAAd, FDC and DC-LAMP and multiplex IHC using antibodies for CK, CD163, FoxP3, CD3, CD8 and Ki67 (B–C, scaling bar indicates 50 μ m). (D) Quantification of number of CD163⁺, CD3⁺, CD8⁺ and FoxP3⁺ cells within TLS per tumor (n=4–7 TLS analyzed per tumor). FDC, follicular dendritic cells; GC, gastric cancer; IHC, immunohistochemistry; PM, peritoneal metastases; PT, primary tumors; LAMP, lysosomal-associated membrane protein; TLS, tertiary lymphoid structures.

TIL-ROIs per tumor, which mostly consisted of macrophages (38.4%), CD8⁺ T memory cells (26.0%), and CD4⁺ T memory cells (12.5%; [figure 3A](#), online supplemental figure S2F). By correlating the immune cell compositions of TLS and TIL, we identified correlations between rates (proportions) of TLS-derived B cells and TIL-derived plasma cells and CD8⁺ T memory cells ([figure 3B](#)). In

addition, plasma cells in TLS associated with plasma cells in TIL (all $p < 0.05$ and $p > 0.6$).

Subsequently, the most common maturity state was assigned on a per-patient-basis when at least 80% of their TLS were only mature or immature ([figure 3C](#)). Thereby we identified that tumors with mTLS were enriched with tumor-infiltrating plasma cells and CD8⁺ T memory

Table 1 Immunohistochemistry data

Patient	Tumor location	Disease stage	CD20 ⁺ TLS	FDC ⁺ (%)	DC-LAMP ⁺ (%)	PNAd ⁺ (%)
P1	PT	III	26	62	89	94
P2	PT	III	57	85	83	43
P3	PT	III	14	86	90	75
P4	PT	II	40	97	14	73
P5	PT	II	20	100	71	47
P6	PT	II	15	100	62	71
P7	PM	IV	21	100	67	56
P8	PM	IV	5	60	67	67
P9	PM	IV	4	100	100	75
P10	PM	IV	25	75	76	72

CD20⁺ TLSs represent absolute TLS counts in the CD20 staining. FDC, DC-LAMP, and PNAd denote the percentage of positive TLS in the respective staining.

DC-LAMP, dendritic cell-lysosomal-associated membrane protein; FDC, follicular dendritic cells; PM, peritoneal metastases; PNAd, peripheral lymph node vascular addressin; PT, primary tumor; TLS, tertiary lymphoid structures.

cells (figure 3D). Conversely, in tumors with iTLS, CD4⁺ and CD8⁺ T naive TILs were more pronounced. When comparing immune pathway expression in TLS and TIL, we observed that within TLS, pathways related to lymphocyte trafficking, T cell activation and T cell differentiation were upregulated compared with TIL (all FDR<0.05; figure 3E) while the opposite was observed for cytotoxicity and MHC class I antigen presentation pathways which were most pronounced in TIL. These data confirm that TLS and TIL function as respective priming and effector sites.

TLSs in PM are immature and have immunosuppressive features

As indicated above, 70% of PT have mTLS, while 75% of PM contain iTLS (figure 2C), suggesting that TLS maturity has location specificity. We confirmed this using UMAP clustering (figure 4A). To rule out an effect of cancer stage (all PT were from patients with non-metastatic disease), we included a second cohort of patients with synchronously matched PT and PM samples for spatial transcriptomics (figure 4B). Also in this cohort, TLS from PT and PM clustered separately, although this was less pronounced compared with the first cohort (online supplemental figure S3A). When analyzing the cellular compositions, we found that PT-TLS were enriched with B naive and CD8⁺ T naive cells while TLS from PM harbored higher proportions of macrophages (figure 4C, online supplemental figure S3B) which confirmed that this difference is location and not disease stage dependent. Immune subset proportions were otherwise concordant among the cohorts (online supplemental figure S3C, D). We also compared the cellular compositions of the TLS within five patient-matched tissues and observed trends suggesting that PM-derived TLS contain more macrophages and less CD8⁺ T naive cells compared with TLS in the matching PT (figure 4D, online supplemental

figure S3E). No differences were observed for the TIL (online supplemental figure S2F).

Following our maturation annotation strategy, 15/18 (83%) of the PT TLS were classified as mature, in contrast to only 5/14 (36%) of PM derived TLS (figure 4E). Within the five matched samples that passed QC, maturation status was concordant between PT and PM (online supplemental figure S3G). Additional differential gene expression analyses identified that PM derived TLS had a higher expression of macrophage-associated genes, including *CD68*, *CSF1R* and *C1QA* and *VCAN* (figure 4F) while PT-derived TLS had an increased expression of B cell and BCR signaling, and Th17 differentiation (online supplemental figure S3H, $p<0.05$). In PM TLS, interleukin signaling, complement system and anti-inflammation pathways were upregulated. Collectively, these data confirm that tumor location contributes to the heterogeneity of TLS.

Antitumor effector pathways are more active in tumors with mTLS

As it is not immediately clear why some cancers contain mTLS and other cancers iTLS we followed the hypothesis that cancer cells can be the mediators of the TLS-maturation status. Therefore, we analyzed the transcriptional programs in cancer cells (CK⁺ AOI; figure 2A) in tumors with mTLS and iTLS from both cohorts (figure 4G) and identified that cancer cells in tumors with mTLS have specifically enhanced expression of apoptosis-related pathways. These include apoptotic cleavage of cellular proteins, TLR3-mediated, NIF-mediated and TRIF-mediated apoptosis and tumor necrosis factor alpha receptor binding, suggesting increased cytotoxic activity. Interestingly, also the Fcγ receptor (FCGR) pathway was upregulated, which is likely an indication of cross-linking of IgG antibodies with cancer cells as recently described.³⁰ Cancer cells from tumors with iTLS expressed various

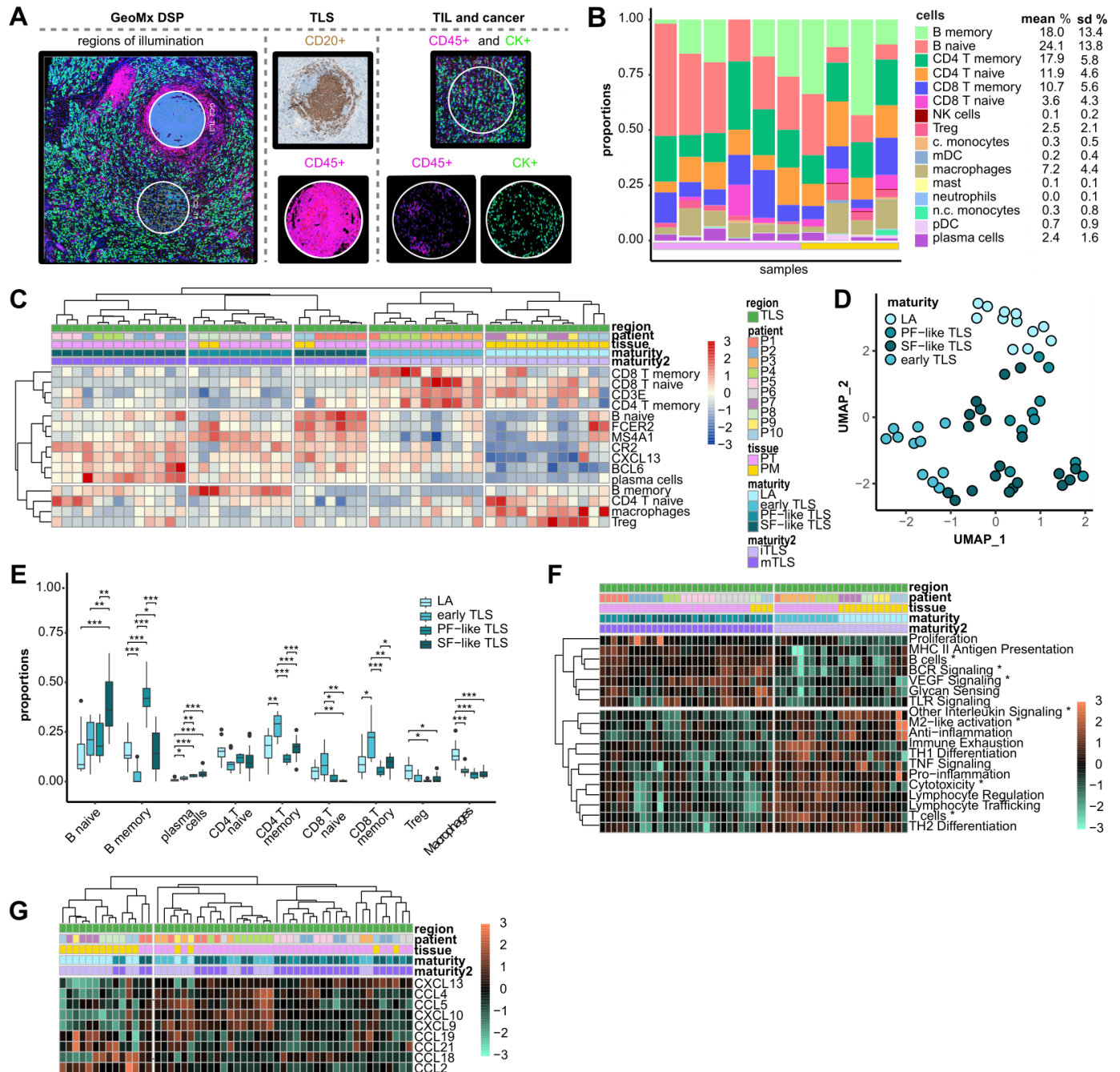


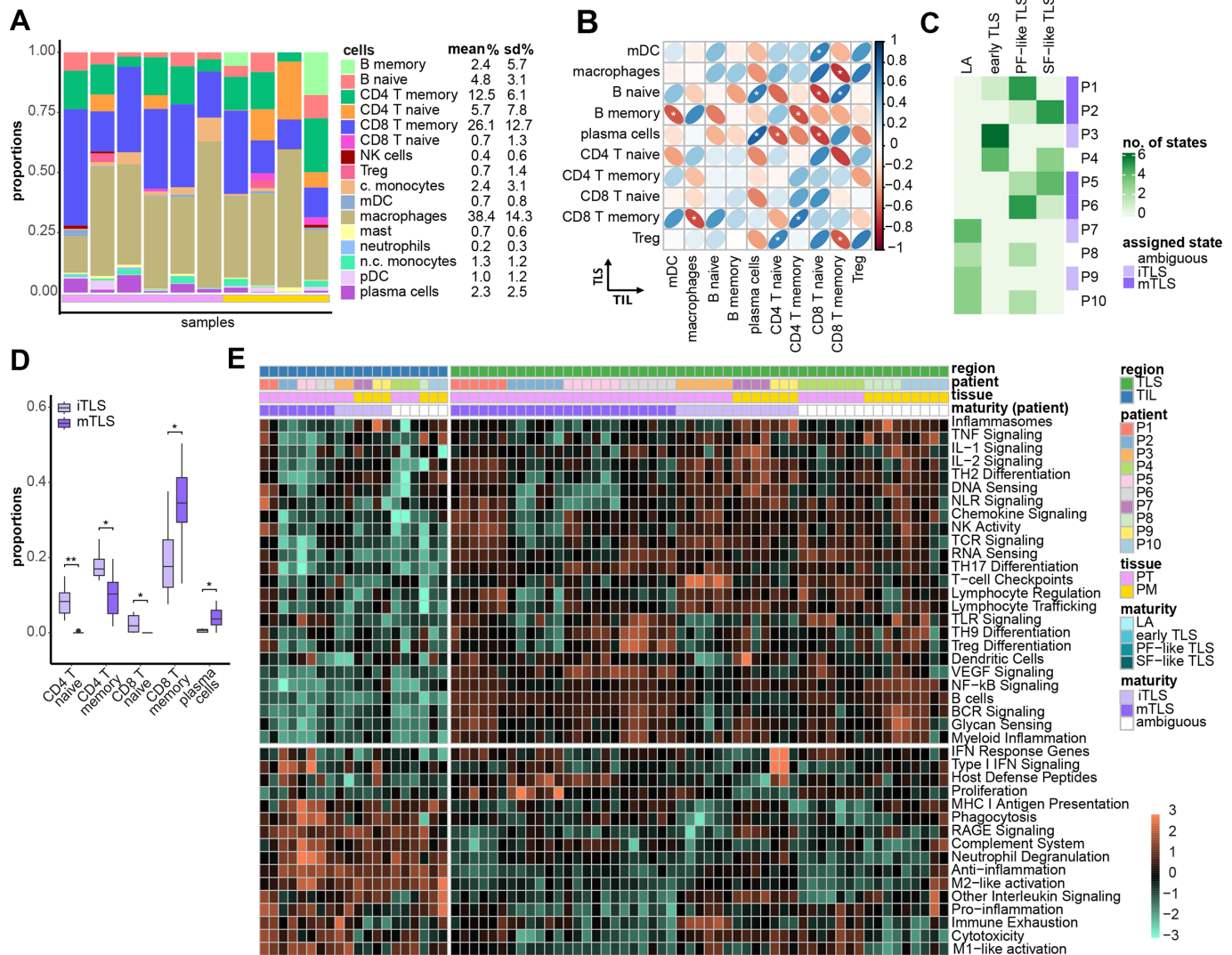
Figure 2 Transcriptomic characterization of TLS in PT and PM. (A) ROI selection of TLS, TIL and tumor using CD45 (pink) and CK (green). (B) TLS subsets, showing mean proportions per patient and annotations for PT (pink) and PM (yellow). (C) Supervised maturation clustering of TLS-associated markers and subset proportions (n=53). (D) UMAP of TLS-ROIs. (E) Differences in proportions of immune subsets across maturity subgroups (pairwise Wilcoxon, FDR correction, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). (F) Differential pathway analysis between mTLS and iTLS ($p < 0.05$; those with an * FDR < 0.05). (G) Chemokine signature expression among TLS. CK, cytokeratin; FDR, false discovery rate; iTLS, immature TLS; mTLS, mature TLS; n.c., non-classical; PM, peritoneal metastases; PT, primary tumor; ROIs, regions of illumination; TLS, tertiary lymphoid structure.

DNA repair-related pathways, including the homology-directed repair and mismatch repair pathways. In addition, cytokine pathways associated with immunosuppression were enriched, such as the IL-20, IL-6, IL-27 families and transforming growth factor beta (TGF β). Together, these data suggest that GC tumors with iTLS express more immune suppressive pathways which may

influence composition and function of TLS and TIL in the immune microenvironment.

DISCUSSION

TLS are promising biomarkers to stratify patients with cancer for immunotherapy, but whether this also



accounts for GC is unknown. The purpose of our study was to comprehensively characterize TLS in various stages of GC to inform immune-targeting studies about their contribution to the antitumor immune response. One of the main findings of the current study is that the immune cell composition of TLS in GC varies greatly between patients. In addition, maturity of TLS seemed to depend largely on the tumor immune microenvironment (TIME) in which the TLS developed, as iTLS were predominantly found in PM. Using spatial transcriptomics, we were able to associate TLS maturation status and the phenotype to the composition of TIL. This led to the understanding that GCs with iTLS are enriched for immune suppressive cell populations with limited evidence of cytotoxicity.^{33–35}

In agreement with former studies, we observed that mTLS were enriched for B cell populations in various

stages of differentiation, which are likely involved in antigen presentation and antibody production, as confirmed by differential gene pathway analysis.^{3 30} In contrast to mTLS, iTLS expressed pathways associated with M2-like macrophage polarization and Th2 differentiation, as well as T cell exhaustion pathways; all features of an immune suppressive microenvironment. Also in PT iTLS were infiltrated with Tregs, the presence of which within TLS has been shown to negatively impact T cell responses and patient outcomes in other cancer types.^{36–38} VEGF signaling was also upregulated in mTLS, suggesting increased development of HEV which are known for facilitating the influx of naive and memory B cells into TLS.^{3 39 40} iTLSs, however, harbored high proportions of naive T cells. In agreement, iTLS expressed lymphocyte trafficking pathways and specifically expressed *CCL19*,

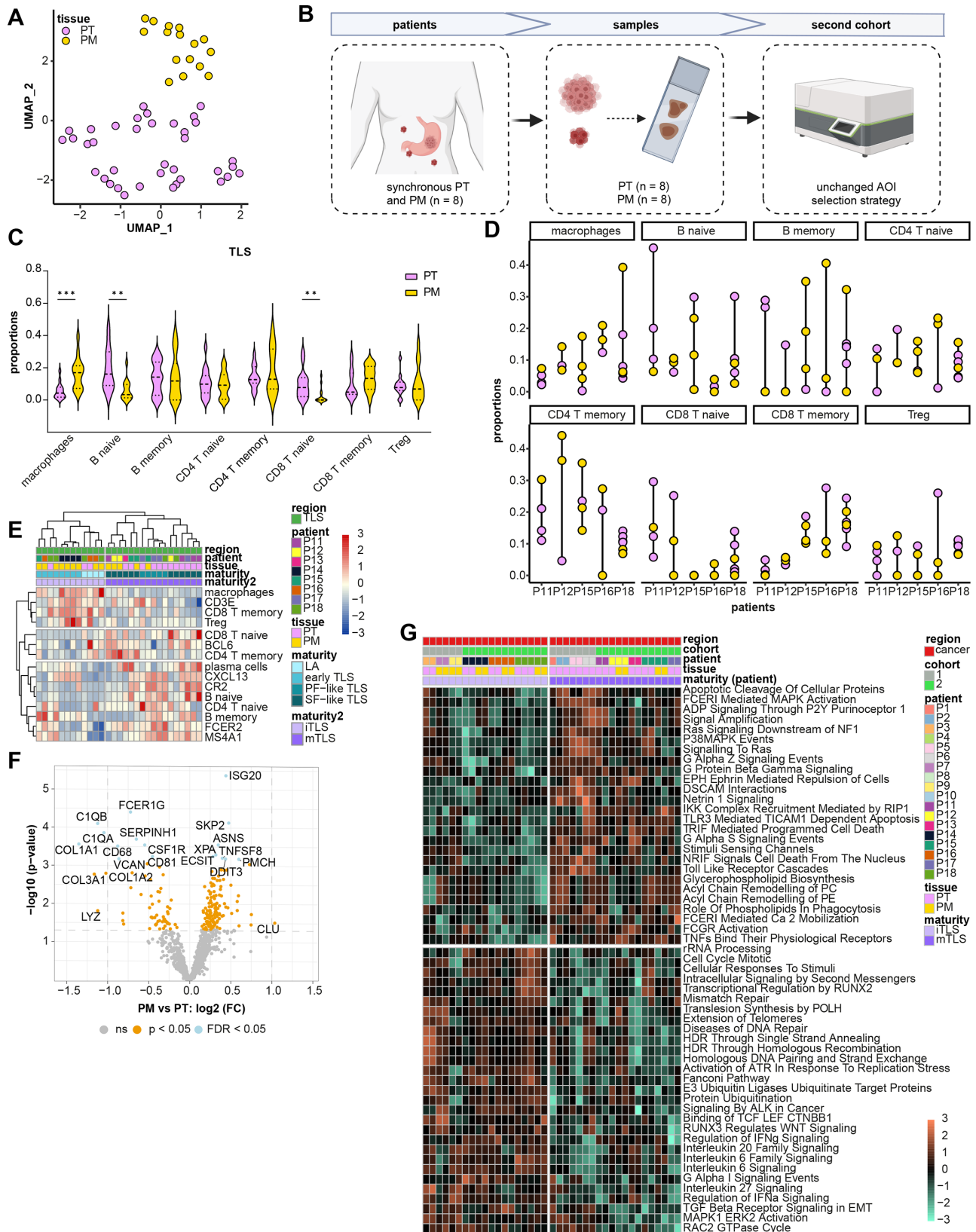


Figure 4 TLS in GC-derived PT and PM. (A) UMAP of TLS-ROIs from the first cohort (n=53). (B) Set-up of the second cohort containing matched PT and PM samples. (C) Subset proportions from TLS-ROIs from PT (n=15) and PM (n=14). **p<0.01, ***p<0.001. (D) Subset proportions from TLS-ROIs from matched PT (n=5) and PM (n=5). **p<0.01, ***p<0.001. (E) Maturation clustering of TLS-associated markers and subset proportions. (F) Differential gene expression of PT-derived and PM-derived cancer cell ROIs. (G) Differential pathway analysis contrasting cancer cell ROIs from tumors with mTLS vs iTLS. FDR<0.05. FC, fold change; FDR, false discovery rate; PM, peritoneal metastases; PT, primary tumors; ROIs, regions of illumination; TLS, tertiary lymphoid structure; UMAP, uniform manifold approximation and projection.

CCL21 and *CXCL13*, which are established chemoattractants of naive lymphocytes.^{41 42}

Though TLS are thought to serve as hubs for generating antitumor responses, evidence for this hypothesis is still scarce. For instance, TLS presence has been associated with increased numbers of TIL^{43–46} and recently, it was demonstrated that TLS-derived plasma cells migrated to tumor beds to secrete antibodies.^{3 30} In support of this, we demonstrate that tumors with mTLS were enriched for plasma cells and CD8⁺ T memory cells, both important players in antitumor immunity.⁴⁷ Moreover, TLS were enriched in T cell differentiation pathways while TIL were marked by increased effector functions, indicating complementary roles in priming and effector phases of the cancer immunity cycle.

Spatial transcriptome analysis allowed us to define the transcriptional program of cancer cells separately, which showed that cancer cells in tumors with mTLS had higher expression of the FCγ receptor (FCGR) pathway. As this pathway is activated when the Fc portion of IgG bind to an Fc-gamma receptor on the surface of cancers cells, this may indicate that indeed cancer cells are coated with IgG immune complexes. This is in agreement with a study of Meylan *et al*, who showed that TLS in renal cell cancer have higher expression of IgG complexes inside TLS and IgH complexes inside and outside TLS, suggesting that naive and memory B cells undergo affinity maturation into plasma cells and produce antibodies against cancer cells.³⁰ Interestingly, high levels of IgA-producing and IgG1-producing plasma cells associate with coating of tumor cells, eventually leading to improved overall survival.^{30 48–52} Most TLS-associated antibody responses are to tumor specific intracellular antigens that are released by dying cancer cells.⁵³ Indeed, we found upregulation of various apoptosis-related pathways in mTLS contrary to iTLS. Interestingly, tumors with iTLS cancer cell overexpress pathways associated with DNA repair such as mismatch repair pathways, homologous recombination, Fanconi pathway, and cell cycle pathways. The underlying biological mechanisms relating to these pathways and iTLS-presence should be further investigated but may simply signal tumor growth dominating over immune-related effector pathways.

It should be noted that the underlying cause of observed differences in TLS composition and/or maturation stage between PT and PM is not completely clear, yet it can be hypothesized that this relies on tumor stage, tumor-intrinsic features (aggressiveness and/or change in antigenic landscape) and/or the local microenvironment.

In conclusion, although the presence of TLS has been commonly correlated to improved patient prognosis and immunotherapeutic efficacy,² we here demonstrate that there are clear functional differences between mTLS and iTLS. We, therefore, propose that future studies should include maturation status and tumor location as a covariable in such analyses. In addition, our study demonstrates that TLS maturity may be driven by the location and thus the TIME in which TLS develop. Understanding the

mechanisms involved in TLS maturation may facilitate optimized ICB therapies to increase effective antitumor immune responses in (metastatic) GC.

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Contributors TSG-vS and RFF contributed equally to this work and shared the first authorship. SD and JG contributed equally to this work and share senior authorship. TSG-vS, RFF, ENB and SD planned the experiments. TSG-vS and ENB performed experiments. TSG-vS and RFF analyzed results and wrote the manuscript. RFF created the figures. TDdG, NCTvG, JG and SD contributed to study concept and design. TCF contributed to patient material. NCTvG performed pathological examination of the Amsterdam UMC samples. CM-C performed pathological examination of the INCLIVA samples. JSeidel, JSaris and DT provided input on the manuscript. TDdG, JG and SD provided supervision and corrected and approved the final version. Responsible for the overall content as the guarantor: SD. All authors contributed to a critical revision of the manuscript.

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Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by two medical ethical research committees. Amsterdamumc samples: medisch ethische toetingscommissie (METC) Amsterdamumc: 2022.0321; INCLIVA samples: Comité de Ética de la Investigación con medicamentos (CEIm) Hospital Clínico Universitario de Valencia: 2021/082. Participants gave informed consent to participate in the study before taking part.

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Data availability statement Data are available on reasonable request. The data sets that have been generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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