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Comprehensive immunophenotyping of gastric adenocarcinoma identifies an inflamed class of tumors amenable to immunotherapies

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ABSTRACT

Background Gastric adenocarcinoma (GAC) imposes a considerable global health burden. Molecular profiling of GAC from the tumor microenvironment perspective through a multi-omics approach is eagerly awaited in order to allow a more precise application of novel therapies in the near future.

Methods To better understand the tumor-immune interface of GAC, we identified an internal cohort of 82 patients that allowed an integrative molecular analysis including mutational profiling by whole-exome sequencing, RNA gene expression of 770 genes associated with immune response, and multiplex protein expression at spatial resolution of 34 immuno-oncology targets at different compartments (tumorous cells and immune cells). Molecular findings were validated in 595 GAC from the TCGA and ACRG external cohorts with available multiomics data. Prediction of response to immunotherapies of the discovered immunophenotypes was assessed in 1039 patients with cancer from external cohorts with available transcriptome data.

Results Unsupervised clustering by gene expression identified a subgroup of GAC that includes 52% of the tumors, the so-called Inflamed class, characterized by high tumor immunogenicity and cytotoxicity, particularly in the tumor center at protein level, with enrichment of *PIK3CA* and *ARID1A* mutations and increased presence of exhausted CD8+ T cells as well as co-inhibitory receptors such as *PD1*, *CTLA4*, *LAG3,* and *TIGIT*. The remaining 48% of tumors were called non-inflamed based on the observed exclusion of T cell infiltration, with an overexpression of *VEGFA* and higher presence of *TP53* mutations, resulting in a worse clinical outcome. A 10-gene RNA signature was developed for the identification of tumors belonging to these classes, demonstrating in evaluated datasets comparable clinical utility in predicting response to current immunotherapies when tested against other published gene signatures.

Conclusions Comprehensive immunophenotyping of GAC identifies an inflamed class of tumors that complements previously proposed tumor-based molecular clusters. Such findings may provide the rationale for exploring novel immunotherapeutic approaches for biomarker-enriched populations in order to improve GAC patient's survival.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Immunotherapy has been recently approved for the treatment of gastric adenocarcinoma, but a comprehensive understanding of the tumor microenvironment of this malignancy is lacking, which hinders improved clinical outcomes.

WHAT THIS STUDY ADDS

⇒ Immunophenotyping of gastric adenocarcinoma identifies an inflamed and non-inflamed class of tumors associated with specific somatic mutations, distinct clinical outcomes, and potential prediction capacity of response to immunotherapies.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study provides a comprehensive description of the intratumoral immunological milieu of gastric adenocarcinoma, while also revealing two main complementary immunophenotypes. The knowledge provided could contribute to a paradigm shift in precision immune-oncology for this prevalent disease.

INTRODUCTION

Gastric cancer is responsible for over one million new cases per year, representing the fourth-leading cause of cancer-related death worldwide.[1] Gastric adenocarcinoma (GAC), the predominant malignancy arising from the stomach, displays significant global variation as a result of differences in known risk factors such as *Helicobacter pylori* infection.[2] GAC is further classified into intestinal, diffuse, and mixed histological subtypes (Lauren classification)[3] and into chromosomal unstable (CIN), genome stable (GS), microsatellite instability (MSI) and Epstein-Barr positive (EBV) molecular subtypes (TCGA classification).[4]

Despite improvements in multidisciplinary therapies, 5-year survival rates for GAC remain unsatisfactory (~25%).[2] In advanced

clinical stages, cytotoxic chemotherapy is still the cornerstone of treatment.[5] In addition, targeted therapies have shown survival benefits: trastuzumab (anti-HER2) for those tumors overexpressing HER2[6] and ramucirumab (anti-VEGFR2).[7] More recently, immunotherapy with PD-1 inhibitors has also demonstrated clinical efficacy in GAC, with the addition of nivolumab/pembrolizumab to first-line chemotherapy being the new standard of treatment for tumors with overexpression of PD-L1.[8 9]

The use of PD-L1 expression by immunohistochemistry as a predictive factor of benefit to immune checkpoint inhibitors (ICIs) has been challenged by multiple factors, including the necessity of using different companion diagnostic assays for specific agents as well as the inter-assay variability in terms of both performance and cut-off points.[10] Indeed, data show that not only the cut-off for positivity is relevant but the cell that is expressing PD-L1 (stromal cells instead of tumorous cells).[11] All these factors explain the inconclusive results of the biomarker observed in GAC,[12] which may reflect the inherent complexity of the antitumoral immune response for a phenotypically heterogeneous disease.

The efficacy of ICIs is affected by a combination of factors involving tumor genomics and host germline genetics that determine specific features of the tumor microenvironment.[11] GAC is among the most immunogenic tumors after melanoma, bladder, and lung cancer as a result of a high tumor mutation burden (TMB) and the subsequent generation of neoantigens.[13] In terms of molecular subtypes,[4] the MSI (hypermutated genome) and EBV (enriched by *PIK3CA* mutations) groups have shown dramatic objective response rates (ORR) to PD-1 inhibitors.[14] In fact, pembrolizumab has been approved in a tissue-agnostic manner based on the presence of deficient DNA mismatch repair (dMMR) and the associated MSI.[15]

Anti-PD-1 therapies achieve some long-lasting clinical responses in cancer patients.[16] However, primary, adaptive, and acquired resistance usually emerge as a mechanism that prevents an effective antitumor response.[17] As these molecular mechanisms are elucidated, novel actionable strategies are designed. Treatment with anti-CTLA-4 agents has been reported to modulate regulatory T cell activity with a synergistic effect with anti-PD-1 blockade, which has already been tested in phase III investigations for GAC.[18] Beyond that, several other immune checkpoint targets such as LAG-3, TIM-3, and TIGIT are under clinical development and may represent future effective therapies for GAC.[19]

Taken together, although the understanding of mechanisms of efficacy and resistance to ICIs in GAC has advanced, the failure to identify appropriate predictive biomarkers to implement trial-enrichment strategies has limited the success of these therapies. With the aim to provide a comprehensive characterization of the tumor microenvironment of GAC, we have conducted an integrative molecular analysis of tumor-infiltrating immune cells in terms of type, functional status, and spatial distribution that have uncovered oncogenic programs governing specific immunophenotypes with potential sensitivity to specific immunotherapies.

MATERIALS AND METHODS

Patients and samples

A total of 82 GAC patients surgically resected between 2010 and 2020 without neoadjuvant treatment and with available frozen (covered with tissue freezing medium) and formalin-fixed paraffin-embedded tumor samples were retrospectively collected at Arnau de Vilanova University Hospital (online supplemental figure 1A).

Pathological diagnosis of GAC was confirmed by two independent gastrointestinal pathologists. Frozen samples were macro dissected to remove surrounding non-tumoral tissue and increase tumor purity. Clinical and pathological variables were collected, including demographics, risk factors, anatomical location, histology, AJCC pathological staging eighth edition, treatments received, and clinical outcomes in terms of recurrence and survival.

Detailed methods for multiplex RNA expression, whole-exome sequencing, multiplex spatial protein profiling, immunohistochemistry, and in situ hybridization are reported in the online supplemental material.

External validation

Two external GAC cohorts with available multiomics data were used to validate the molecular findings (online supplemental figure 1B). The TCGA cohort[4] included samples from surgically resected tumors in North America, Europe, and Asia without neoadjuvant treatment (N=295) with RNAseq (N=265), whole-exome sequencing (N=289) and copy-number array (N=293). The ACRG cohort[20] included samples from surgically resected tumors in Korea without neoadjuvant treatment (N=300) with RNA expression array (N=300), targeted-exome sequencing (N=232) and copy-number array (N=277).

Prediction of response to immunotherapies was first assessed in a cohort of 45 Asian patients with advanced GAC treated with pembrolizumab in second/third-line from a phase II trial (NCT02589496) that had available transcriptome data.[14] Beyond GAC, other available datasets[21] for advanced melanoma, renal cell carcinoma, and urothelial carcinoma with pretreatment biopsies and with at least 10 patients with radiological response (see references in online supplemental material) were used to compare the predictive power of response to immunotherapies of proposed and previously published biomarkers.[22 23]

Statistical analysis and plots

Associations between categorical variables were analyzed by Fisher's exact test. The t-test was used for the comparison of categorical and continuous variables. Correlations between two continuous data were analyzed by Pearson's

correlation coefficient (R). Kaplan-Meier estimates, log-rank test, and univariate and multivariate Cox regressions were used to analyze time-to-event variables. All reported p values were two-sided, a and p<0.05 was considered significant. The IBM SPSS V.26 (http://www.ibm.com/) was used for all analyses with the exception of data from multiplex spatial protein profiling that was analyzed using the GeoMx software V.2.5, allowing a linear mixed model adjusting for repeated measurements from the same sample with a Benjamini-Hochberg correction. Some plots for data visualization were performed by Flourish (https://flourish.studio).

RESULTS

Clinical, pathological, and molecular features of the GAC cohort

The study included a discovery dataset (HUAV) of 82 GAC patients with mostly early-stage disease (98% were AJCC I, II, or III) with a variety of anatomical locations (8% proximal, 33% body, and 59% distal) and histologies (70% intestinal, 18% diffuse, and 12% mixed) that were treated according to standard procedures (distal or total gastrectomy with lymphadenectomy followed by adjuvant chemotherapy and/or radiotherapy when indicated) and with a median overall survival of 72 months. A complete clinical and pathological description of the cohort is shown in online supplemental table S1 and online supplemental figure S2.

Tumors were reevaluated for biomarkers used nowadays for the identification of patients suitable for targeted therapies for this disease[24] (figure 1A and online supplemental table S2). 25% of the cases were MMRd, whereas the rest (75%) were proficient MMR (MMRp). HER2 overexpression/amplification was present in 14% of the samples, being all from the pMMR class. PD-L1 overexpression (combined positive score (CPS) ≥5), on the other hand, was observed in 35% of tumors, with an enrichment of the dMMR class (p<0.001). In terms of CLDN18.2 overexpression (≥75% moderate-high expression), it was detected in 29% of tumors, being mutually exclusive with PD-L1 overexpression (p=0.017). EBV was only identified in 4% of the cohort.

Whole-exome sequencing was applied in the cohort to identify somatic structural genetic alterations (figure 1B and online supplemental table S3). Single-nucleotide polymorphism was the more frequent variant type, originating mainly missense mutations (online supplemental figure S3). All MMRd tumors had a high MSI (online supplemental table S4) and a higher TMB than MMRp (median 1616 vs 471 variants per sample; p<0.001), as expected. In terms of frequently mutated driver genes described in GAC,[4] *TP53* was the more prevalent (41%), with a long tail of less common genes, including the actionable[25] *PIK3CA* (16%), *KRAS* (15%) and *ERBB2* (14%). Overall, the distribution of genetic events in the cohort and within MMR classes was consistent with previously published studies and portrays the known biological landscape of GAC.

Type, density, and location of tumor-infiltrating immune cells in GAC

Immune infiltrates, assessed by CD45 immunostaining, were heterogeneous in the GAC cohort (figure 2A): from tumors displaying a high and uniform pattern of infiltration to tumors with a global low degree of infiltration (online supplemental table S5). Of note, the Lauren histological classification qualitatively impacted the immune contexture of tumors, with the intestinal subtype producing a more arborescent infiltration surrounding tubular or glandular tumor formations, whereas the diffuse subtype producing a more isolated infiltration nearby to poorly cohesive cells and with a higher prevalence of lymphoid aggregates (p<0.001). Moreover, the region of the tumor determined the distribution of different types of immune cells present in GAC (figure 2B), with markers of T cells (p=0.023) being more expressed at protein level in the invasive margin when compared with the tumor center.

Immune subpopulations infiltrating tumors were then estimated by gene expression of immune metagenes and correlated with known histologies and biomarkers of GAC (figure 2C). Cytotoxic cells, the most powerful effectors in the anticancer immune response and the backbone of current successful ICIs, were enriched in MMRd (p=0.008) and PD-L1+ (p<0.001) tumors. However, the exhausted pattern of CD8 T cells was only observed in PD-L1+tumors (p=0.008). Conversely, NK cells were more present in the intestinal histology (p=0.007) as well as in MMRp (p=0.037) and HER2+ (p=0.007) subgroups. Diffuse histology was defined by a higher macrophage (p=0.041) and mast cell (p=0.002) infiltration. Finally, CLDN18.2 positivity determined lower amounts of CD8 T cells (p=0.049) and Tregs (p=0.033).

Several targets, including checkpoint molecules, were measured by multiplex protein profiling at the immune compartment of tumors (figure 2D). PD-L1 was overexpressed as expected in PD-L1+ (p=0.001) and MMRd (p=0.005) tumors. On the other hand, ICOS, a T cell activating costimulatory immune checkpoint, was downregulated in MMRd tumors (p=0.042). The enzyme IDO1, a proposed key factor of acquired immune tolerance,[26] was particularly upregulated in PD-L1+tumors (p=0.001), as well as in MMRd (p=0.012) and intestinal (p=0.020) subgroups. No significant associations were detected depending on HER2 status, whereas CLDN18.2 positivity was related to a higher expression of PD-1 (p=0.004), Tim-3 (p=0.036) and CTLA4 (p=0.002). These results reinforce the diversity of immunophenotypes present in GAC which warrants a rational use of immuno-oncology drugs.

Identification of the inflamed class of GAC by unsupervised clustering

Unsupervised clustering by tumor microenvironment gene expression (770 genes related to different features

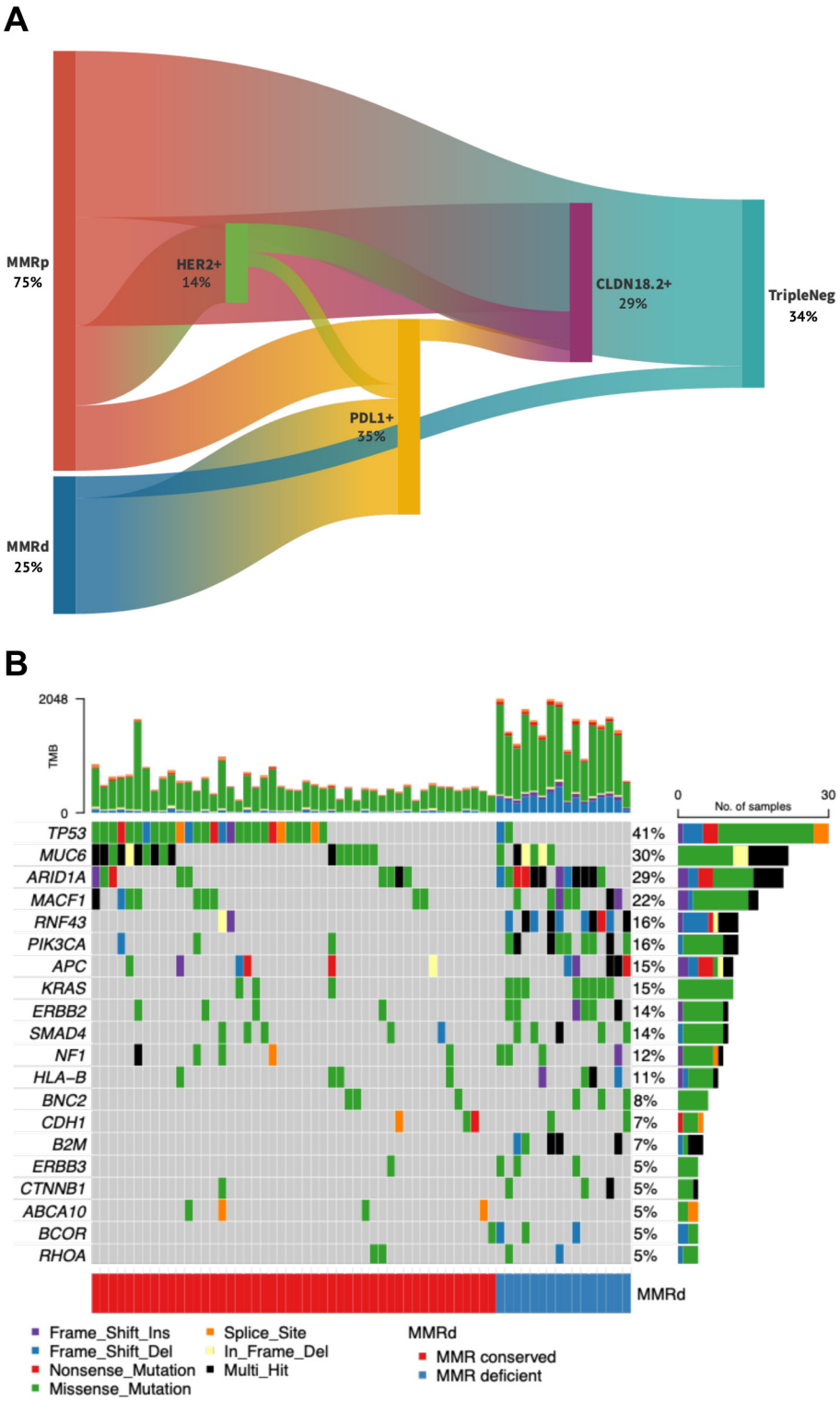

Figure 1 Biomarkers and genetic alterations in GAC. (A) Alluvial plot representing the prevalence and distribution of companion biomarkers (MMR, HER2, PD-L1 and CLDN18.2) assessed by immunohistochemistry and associated with active targeted therapies in GAC. (B) Heatmap representing the frequency and type of recurrent somatic mutations in GAC ranked by their prevalence and sorted by MMR class. MMRp (normal protein MLH1/MSH2/MSH6/PMS2 expression), MMRd (loss of protein MLH1/MSH2/MSH6/PMS2 expression), HER2+ (3+or 2+/FISHamp), PD-L1+ (CPS≥5), CLDN18.2+ (≥75% moderate-high expression), TripleNeg (negative for HER2, PD-L1 and CLDN18.2). GAC, gastric adenocarcinoma; MMRd, deficient mismatch repair.

of the antitumoral immune response as well as the composition of the immune cells infiltrating the tumor) proposed the presence of essentially two molecular classes of GAC (figure 3A). The first class, including 52% of the tumors, was named Inflamed class based on the enrichment of gene-expression signatures defining tumor

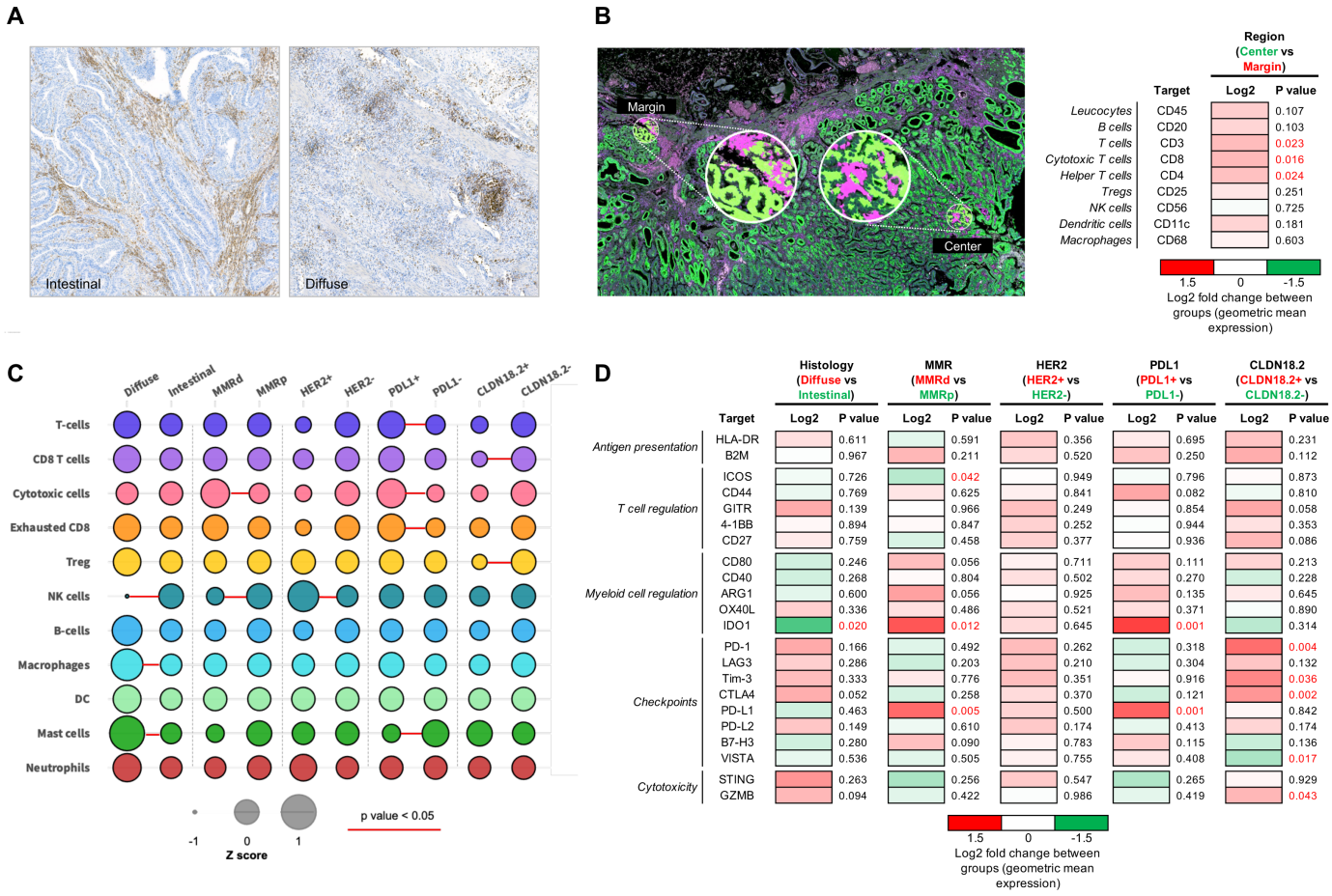

Figure 2 Landscape of tumor-infiltrating immune cells in GAC. (A) Illustrative example of the immune infiltration in intestinal and diffuse histological subtypes assessed by CD45 immunostaining. (B) Differential expression of immune cell markers by multiplex spatial protein profiling of immune cells (identified in the image in purple by CD45 immunofluorescence) depending on the region of the tumor (invasive margin vs tumor center). (C) Immune subpopulation abundance in tumors estimated by gene-expression deconvolution and their association with known histologies and biomarkers of GAC. Samples from the same molecular class were represented with a normalized enrichment score that is proportional to the size of the circle. (D) Protein expression at the immune compartment of tumors of immune-oncology targets including checkpoint molecules and T cell and myeloid cell regulators and their association with known histologies and biomarkers of GAC. GAC, gastric adenocarcinoma.

immunogenicity (ie, antigen presentation (p<0.001)) and antitumor immune activity (ie, interferon signaling (p=0.005)) (figure 3B). The second class, including the rest 48% of the tumors, was named Non-Inflamed based on the opposite gene-expression profile suggesting lower immunogenicity (presumably as a result of a more efficient DNA damage repair (p=0.020)) (figure 3B).

After interrogating 830 gene sets representing human cell types,[27] the pattern of expression of gastric immune cells[28] was more similar to the one depicted by the Inflamed class (online supplemental table S6 and online supplemental figure S4). Indeed, gene-expression deconvolution inferred increased amounts of a variety of immune cells in this class when compared with the non-inflamed class (figure 3C), particularly in terms of CD8 T lymphocytes with an exhaustion pattern (p<0.001). When analyzing 10 previously proposed ecosystems across human solid tumors,[29] we identified that the predominant multicellular community of the Inflamed class of GAC was proinflammatory, leukocyte-rich, and characterized by higher immunoreactivity (online supplemental figure S5). In terms of spatial protein profiling, tumors from the inflamed class were characterized by increased expression by immune cells of the major histocompatibility complex II molecule HLA-DR (p=0.020) as well as T cell regulation and cytotoxicity markers[30] such as STING (p=0.002), exclusively in the tumor center and not in the tumor margin (figure 3D).

Regarding clinical, pathological, and molecular correlates (figure 4), it was noticed that all tumors with diffuse histology were classified as Inflamed (p=0.112). On the other hand, no association was detected between the Inflamed class and the presence of MMRd (p=0.562) or PD-L1 overexpression (p=0.425). Despite not being significant, a higher prevalence of mutations in *PIK3CA* (30% vs 10%, p=0.152) and *ARID1A* (41% vs 24%, p=0.355) was observed in the Inflamed class. *TP53* mutations, on the contrary, were more common in the non-inflamed class (67% vs 52%, p=0.382). Patients with tumors from the Inflamed class had a better clinical

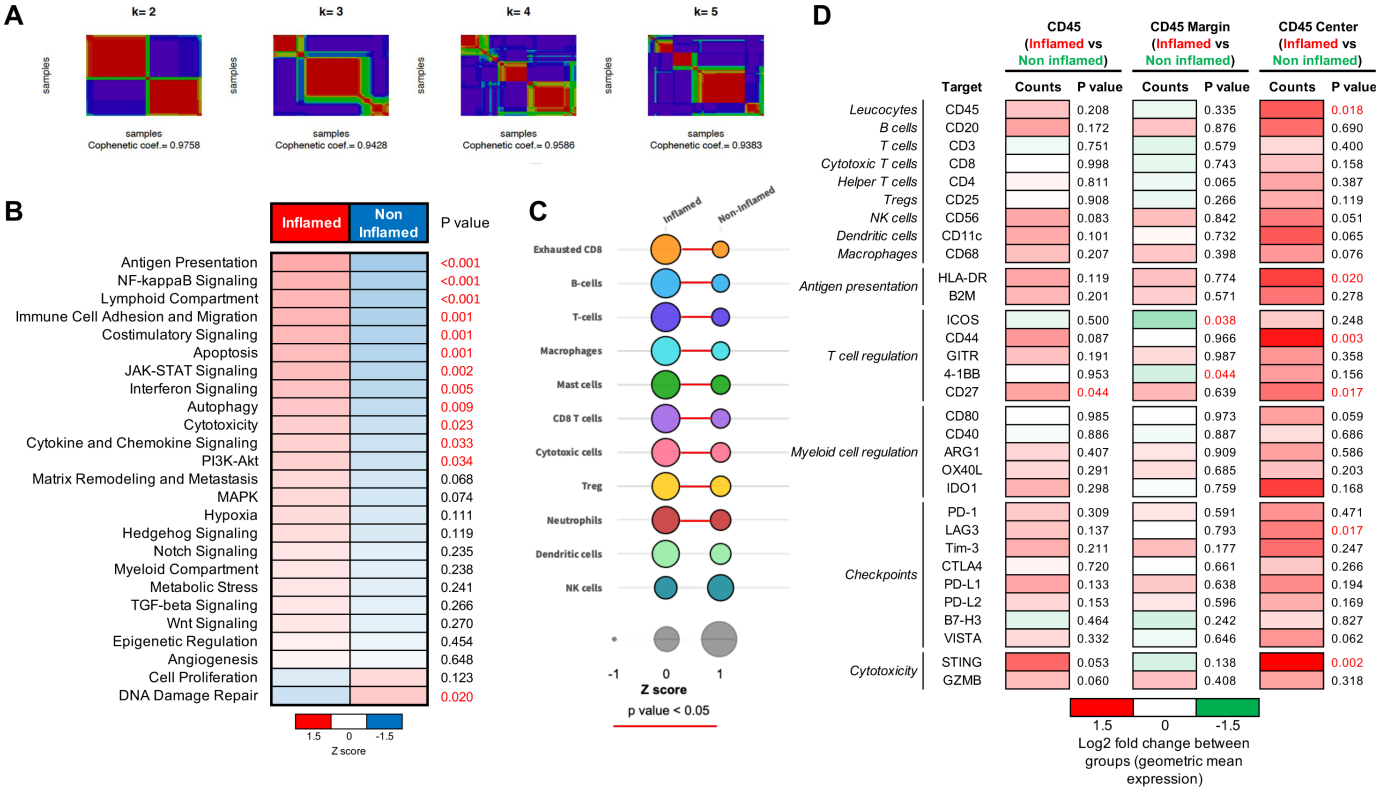

Figure 3 Identification and immunophenotyping of the GAC Inflamed class. (A) Solutions for gene-expression unsupervised clustering using non-negative matrix factorization are shown for k=2 to k=5 classes, with two being the number of classes with the highest cophenetic coefficient. (B) Gene signatures measuring biological variables crucial to the tumor-immune interaction (antigen availability, structural barriers to immune infiltration, inhibitory signaling, inhibitory metabolism, pro-immune signaling, killing of tumor cells, tumor receptiveness to immune signaling, tumor proliferation, and apoptosis) upregulated or downregulated in the inflamed and non-inflamed classes of GAC. Samples from the same molecular class were represented with a normalized enrichment score. (C) Immune subpopulation abundance inferred by gene-expression deconvolution in the inflamed and non-inflamed classes of GAC. Samples from the same molecular class were represented with a normalized enrichment score that is proportional to the size of the circle. (D) Spatial protein profiling of antitumoral immune response markers in the immune compartment (CD45+) of the inflamed and non-inflamed classes of GAC according to different regions of interest (invasive margin and tumor center). GAC, gastric adenocarcinoma.

outcome in terms of time to recurrence in comparison to the rest of patients (p=0.030) (online supplemental figure S6A). In summary, a novel inflamed class of GAC that captures a unique tumor microenvironment fingerprint has been identified.

External validation and prediction of response to immunotherapies of the inflamed class of GAC

In order to externally validate the above-described Inflamed class of GAC, we designed a 10-gene RNA signature (online supplemental table S7) that was able to identify GAC samples belonging to this class in the TCGA[4] (N=265) and ACRG[20] (N=300) external cohorts with a similar prevalence (46% and 54%, respectively) than in the discovery HUAV dataset (figure 5A). The same enrichment of the inflamed class of GAC in gene signatures defining tumor immunogenicity (p<0.001), antitumor immune activity (p<0.001) and high tumor-infiltrating lymphocytes with an exhaustion pattern (p<0.001) was validated in both external cohorts (online supplemental figure S7). Likewise, previously mentioned pathological and molecular associations were statistically confirmed for the Inflamed class of GAC when increasing sample size (figure 5A): First, the increased proportion of diffuse histology (p<0.001); second, the enrichment of *PIK3CA* (p<0.001) and *ARID1A* (p<0.001) mutations; and third, the lower proportion of *TP53* mutations (p<0.001). Finally, the better prognosis in terms of time to recurrence of the Inflamed class of GAC could be proved in the ACRG cohort (p=0.046) (online supplemental figure S6B). The median follow-up for recurrence of only 12.75 months in the TCGA cohort prevented further validation.

Next, we were interested in assessing if the proposed immunophenotypes of GAC reflected some of the proposed mechanisms of tumor immune evasion that modulate responsiveness to immunotherapies[23] (figure 4). Noticeably, the inflamed class of GAC was associated with the induction of T cell dysfunction (p<0.001) and with a predicted increased clinical response to ICIs (p=0.039), whereas the non-inflamed class of GAC was linked to the exclusion of T cell infiltration (p<0.001) associated with enrichment of myeloid-derived suppressor cells (p<0.001). In this regard, targetable co-inhibitory

Figure 4 Clinical and molecular characterization of the GAC Inflamed class. Heatmap representing clinical, pathological, and molecular features (companion biomarkers, recurrent somatic mutations, and tumor immune evasion signatures[23] of the inflamed and noninflamed classes of GAC. Each column represents one patient. MMRd (loss of protein MLH1/MSH2/MSH6/PMS2 expression), HER2+ (3+ or 2+/FISHamp), PD-L1+ (CPS≥5), CLDN18.2+ (≥75% moderate-high expression). CAF, cancer-associated fibroblasts; GAC, gastric adenocarcinoma; MDSCs, myeloid-derived suppressor cells; MMRd, deficient mismatch repair; TAM, tumor-associated macrophages.

receptors of the tumor-immune synapse such as *PD1* (p=0.039), *CTLA4* (p=0.048), *LAG3* (p=0.013) or *TIGIT* (p=0.002) were overexpressed in the inflamed class at RNA level (online supplemental figure S8). On the other hand, the also targetable *VEGFA* ligand was overexpressed in non-inflamed tumors (p=0.021) (online supplemental figure S8).

At last, we evaluated the predictive capability of response to immunotherapies of the Inflamed class of GAC in human cohorts. For advanced GAC, only one Asian cohort had available transcriptome data from 45 patients treated with pembrolizumab (PD-1 inhibitor) in second/third line[14] (figure 5B). The inflamed class of GAC was detected in up to 47% of the tumors of this cohort, which represents a similar performance of the gene signature compared with early clinical stages of GAC. The ORR was higher in patients from the inflamed class when compared with non-inflamed tumors (33% vs 21%), despite not being significant (p=0.501), representing a lower accuracy of the biomarker for this cohort in comparison to reported MSI (p=0.003), EBV (p=0.001) or PD-L1 CPS≥1 (p<0.001). Additional cohorts testing ICIs (PD-1, PD-L1 or CTLA4 inhibitors) or adoptive cellular therapies for other cancer types (melanoma, renal cell carcinoma and urothelial carcinoma)[21] were used to assess the cross-validity of the biomarker (figure 5C). Overall, after analyzing clinical responses to 994 patients, the average AUC for the inflamed class of GAC was 0.63, outperforming other gene-expression signatures such as the T-cell inflamed signature[22] (AUC 0.62) or the TIDE score[23] (AUC=0.57).

DISCUSSION

The initial molecular classifications of GAC were focused on epithelial malignant cells,[4 20] with a slightly shallow depth of analysis of the tumor microenvironment, which is essential for cancer progression.[31] Our study decodes this pluricellular dimension of GAC through a multiomics approach that seeks a comprehensive understanding

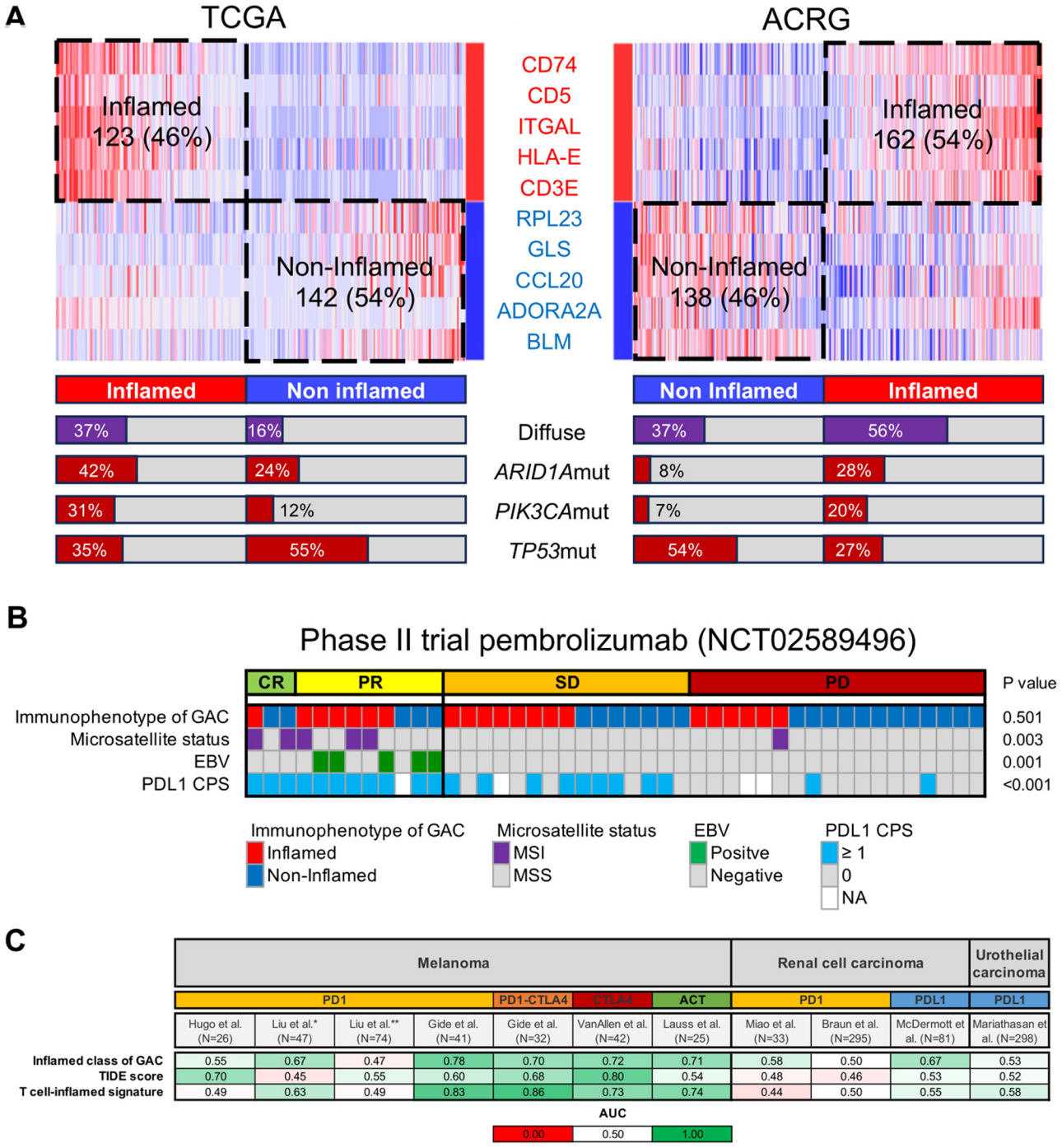

Figure 5 Validation and clinical utility of the GAC inflamed classifier. (A) Identification of the inflamed class of GAC through a 10-gene RNA signature in the TCGA[4] and ACRG[20] cohorts and validation of histological and mutational correlates. (B) Heatmap representing the association of radiological responses to pembrolizumab in a phase II trial for advanced GAC[14] with biomarkers potentially predicting sensitivity to ICIs, including the Inflamed class of GAC. Each column represents one patient. (C) Predictive capability of response to immunotherapies measured by AUC of gene-expression signatures (Inflamed class of GAC, T-cell inflamed signature[22] and the TIDE score[23]) in external cohorts[21] of melanoma, renal cell carcinoma and urothelial carcinoma. *Post ipilimumab progression. **Ipilimumab naïve. CPS, combined positive score; EBV, Epstein-Barr positive; GAC, gastric adenocarcinoma; ICIs, immune checkpoint inhibitors; MSI, microsatellite instability.

of this malignancy. In this regard, immunophenotyping of a discovery GAC cohort highlighted the diversity of immune subpopulations infiltrating tumors. These subpopulations were influenced not only by distinct histologies and biomarkers used for the clinical management of the disease, but also by the spatial localization of cells within the tumor, reinforcing the impact of the immune contexture in cancer.[32]

Integrative molecular analysis of 82 clinically annotated tumors unraveled two main molecular classes of GAC from the immune perspective. First, the inflamed class, including 52% of the tumors and characterized by high

tumor immunogenicity and cytotoxicity inferred by both gene-expression signatures and protein profiling that was more manifest in the center of the tumor than in the invasive margin. This class had increased amounts of exhausted CD8+T cells as well as co-inhibitory receptors such as *PD1*, *CTLA4*, *LAG3* and *TIGIT*, targets of current immunotherapies.[33] Second, the non-inflamed class, including the rest 48% of tumors, with a more efficient DNA damage repair and an exclusion of T cell infiltration possibly mediated by myeloid-derived suppressor cells. The overexpression of *VEGFA* in these tumors uncovers a potential role for antiangiogenic agents for the normalization of the tumor vasculature that can, in turn, increase the infiltration of immune effector cells.[34]

These novel immunophenotypes of GAC were externally validated in 565 tumors from the TCGA[4] and ACRG[20] cohorts through a 10-gene RNA signature, including a composition of immunosupportive[35] and immunosuppressive genes.[36] Notoriously, it was confirmed the association of the inflamed class with diffuse histology as well as with *PIK3CA* and *ARID1A* mutations, which complements previous observations linking these somatic alterations with increased therapeutic antitumor immunity in GAC.[37] The non-inflamed class, on the other hand, displayed a worse clinical outcome in terms of time to recurrence and was enriched in *TP53* mutations, a feature that has been also proposed to derive decreased clinical benefit from ICIs in GAC.[38] External validation and the subsequent minimization of false discoveries obtained through spatial protein profiling could not be fulfilled due to the absence of available data for this technology in previous studies for GAC.

Overall, the proposed clustering of GAC depending on tumor microenvironment features aligns well with the notion that tumors can present an immune hot, altered, or cold phenotype.[39] Nevertheless, other unsupervised transcriptome-based immune molecular classifications of GAC have been proposed. Cabeza-Segura *et al*[40] identified four different groups based on immune infiltration and function after analyzing a retrospective (N=31) and a prospective cohort (N=23). However, neither mutational status nor spatial protein profiling was conducted to enhance the findings. Conversely, other studies have published tumor microenvironment scores based on the association of gene expression with prognosis[41] or response to ICIs[42] in cohorts of GAC, although the usefulness of these signatures is dependent on the specific purpose of their design, not being a holistic descriptive analysis of the tumor microenvironment.

One of the potential clinical utilities of the inflamed class of GAC is the prediction of response to immunotherapies. Indeed, the finding of T cell dysfunction in this class suggested a potential benefit of anti-PD1 therapies, proposed to revive this dysfunctionality.[23] Nonetheless, only one small cohort of GAC with just 12 objective responses on pembrolizumab treatment was available for validating this hypothesis,[14] resulting the accuracy of the inflamed class of GAC inferior when compared with other biomarkers (MSI, EBV or PD-L1). Explorative analysis in other cancer types treated with a variety of immunotherapies suggested a comparable prediction capacity of the inflamed class of GAC than other gene-expression signatures designed for the same purpose.[22 23] However, the performance of the biomarker in these cohorts is still suboptimal for their recommendation as a tumor-agnostic biomarker. In this regard, a further functional immunoprofiling of live GAC cells exposed to immunotherapies, such as with ex vivo 3D organoids or PDX mouse models, would be instrumental to better characterize the potential predictive capability of the inflamed class.[43]

The consistent reproduction of the biology captured by the Inflamed class of GAC in external cohorts from different geographical regions confirms the robustness of the biomarker, surpassing eventual differences in tumor immunity between Asian and non-Asian GAC.[44] In addition, despite not using single-cell sequencing accounting for intratumoral cellular heterogeneity,[45] implementing RNA from the bulk of the tumor for the inference of the inflamed class of GAC may facilitate the clinical implementation of this biomarker. Of note, how the immunophenotype of GAC is maintained through the temporal evolution of the disease is something that needs to be confirmed in future adequately powered and prospective studies with on-treatment serial biopsies.[46]

Further research on biomarkers selecting GAC patients who benefit from the current standard of care with chemotherapy and immunotherapy combinations is eagerly awaited. For this purpose, in-depth companion translational studies of pivotal phase III trials[8 9] should be conducted. This could offer valuable insights on why some patients negative for PD-L1 expression obtain clinical responses from anti-PD-1 therapies[47] or why MSI is not always sufficient to elicit tumor immunogenicity.[48] Also, to provide new targets to overcome resistance mechanisms,[49] that in turn would require clinical studies with biomarker-enriching designs that no longer can rely on PD-L1 immunohistochemistry. In this context, multivariable prediction models that consider both tumor and T-cell-intrinsic mechanisms of response emerge as potential solutions.[50]

To conclude, our integrative molecular analysis of the tumor microenvironment of GAC provides a comprehensive description of the intratumoral immunologic milieu of these tumors, while also revealing two main complementary immunophenotypes that establish the rationale for exploring novel immunotherapeutic approaches for biomarker-enriched populations. Altogether, these results could have fundamental implications for advancing precision immuno-oncology for this prevalent disease in need of progress.

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