Dear editors and reviewers,

The authors are very grateful to the editors and technical reviewers for their careful reviews of the manuscript entitled "**Histological differentiation impacts the tumor immune microenvironment in gastric carcinoma: relation to the immune cycle**" (Manuscript NO.: 65300) and offering their insightful comments and suggestions to improve the quality of the article. We revised the manuscript according to your decision letter, and all the detailed correction were shown in our response letter text material.

The following responses have been prepared to address the Editor's and the reviewers' comments. The responses to the comments are marked as blue text. And the tracking version of manuscript is uploaded again. Thank you!

1 Reviewer

Thank you very much for your comments. Down below we report point by point our answers to your comments.

Comment 1: the cohort analysed does not include any case of mixed gastric cancer. It could be interesting to elucidate the immune infiltration pattern of this subtype. Are the different components of mixed gastric cancer distinct, in terms of immunophenotype, or similar, as they (supposedly) have a common clonal origin? Is the immune context more similar to diffuse or intestinal type gastric cancer? It could be interesting to add a small group of mixed gastric cancers to discuss these points.

Response: Thanks for the recommendation. Indeed in this study we did not include any mixed carcinomas. However, this is a great clue for further studies.

Comment 2: A table describing the general clinicopathological variable of the series is missing: age, sex, stage, histological type, grade (please remind that grading only applies to tubular and papillary subtypes), survival etc.

Response: Thanks for recommendation. The requested information was added.

Table 1. Clinical-pathological features of the patients

Characteristics	Intestinal GC	Mucinous GC	Diffuse GC	Total	p-value
Number	31	4	15	50	
Age	56.3±11.2	51.3±7.37	46.0±18.3	52.8±13.7	P = 0.174
Sex					P = 0.738

Males	18 (58.1%)	3 (75%)	8 (53.5%)	29 (58%)	
Females	13 (41.9%)	1 (25%)	7 (46.7%)	21 (42%)	
Stage					P = 0.33
Stage 2	2 (6.5%)	0	0	2 (4%)	
Stage 3	13 (41.9%)	1 (25%)	6 (40%)	20 (40%)	
Stage 4	16 (51.6%)	3 (75%)	9 (60%)	28 (56%)	
Grade		-	-		
G1	5 (16.1%)				
G2	11 (35.5%)				
G3	15 (48.4%)				
MMR status					
MSI	2	-	-	2	
MSS	29	4	15	48	

Comment 3: the clone used in this study to evaluate PD-L1 expression (E1L3N(R)) is not currently used in the clinical practice to select gastric cancer patients for immunotherapy. Please state this limitation in the study or, if possible technically, use the 22C3 antibody.

Response: The information about the limitation was added to the text of the manuscript.

Besides, the clone of antibodies used in the study to evaluate PD-L1 expression is not currently used in the clinical practice to select gastric cancer patients for immunotherapy.

Comment 4: Abstract - Conclusion: "These data help to clarify the links among tumor histogenesis, molecular profile and immunogenicity for a better understanding of GC biology and more tailored patient management." Please consider eliminating "molecular profile" from the text, as the molecular profile of gastric cancer was not fully elucidated in this article (except for MMR protein deficiency). The same apply for "Core tip": "These data help to clarify the links among tumor histogenesis, molecular profile and immunogenicity."

Response: Thanks for noticing this discordance. Amended

These data help to clarify the links between tumor histogenesis and immunogenicity for a better understanding of GC biology and more tailored patient management.

Comment 5: MATERIALS AND METHODS - Tissue processing and immunohistochemistry: Modify MLH2 to MSH2 and MLH6 to MSH6

Response: Thanks for noticing this discordance. Amended

deficiency was assessed using antibodies against MLH1 (Clone ES05, DAKO), MSH2 (Clone FE11, DAKO), PMS2 (Clone EP51, DAKO), and MSH6 (Clone EP49, DAKO)

Comment 6: MATERIALS AND METHODS - Methodology of tumor-host immunity assessment "The number of immunopositive cells was assessed as both continuous and dichotomized variables using cutoff values (84 cells per mm2 as a median)." It is not clear how the authors select the cut-off values. If the selection was based on the median value, should not the median value be different for each biomarker? Does 84 cells/mm2 refers to the CD8 counting? What about CD68 and CD163?

Response: we used cut-off for stratification according to lymphocytes density as their number was used for TIME evaluation according to immune cycle concept.

Comment 7: Please clarify - RESULTS - TIL and TAM densities varied in GC of different histological types "Notably, GCs with a poor prognosis (mucinous and diffuse type) demonstrated a considerably higher M1/M2 ratio (Table 1)." This result is not represented in Table 1.

Response: this paragraph was edited to express the idea clearer

The assessment of GC immune profiles revealed that CD3+ and CD8+ lymphocyte counts varied in GC of different histological types. However, CD8+ cell density did not correlate with tumor grade (P = 0.669) or stage (P=0.560). GCs of various histological differentiation types differed in the density of TILs (Figure 1, Table 2). The number of CD3+ and CD8+ lymphocytes in intestinal-type GC was significantly higher in intestinal GC than in diffuse and mucinous cancers (P < 0.001). Importantly, mucinous GCs demonstrated prominent heterogeneity of immune cell infiltration, with few cells within tumor clusters and a higher density around them.

In contrast to the common concept that TAMs correspond to M2 macrophages, in GCs, we found that M1 macrophages prevailed over the M2 type. There was no statistically considerable difference in CD68+ macrophage infiltration in peritumor stroma with regard to histological subtypes of GC (P = 0.471). However, the number of CD68+ cells within tumor cluster was higher in intestinal and diffuse GS when compared to mucinous GSs (P < 0.001).

Comment 8: RESULTS - Immunophenotyping GC of different histological types "We did not find any statistically significant relationship between TIME and tumor grade (P = 0.523) or

stage (P = 0.756).” Although there is not statistically significant difference, row data on tumour grade and stage should be presented, at least as supplementary file.

Response: Agree, thanks, these data are in Table 1 of the new version of the paper.

Table 1. Clinical-pathological features of the patients

Characteristics	Intestinal GC	Mucinous GC	Diffuse GC	Total	p-value
Number	31	4	15	50	
Age	56.3±11.2	51.3±7.37	46.0±18.3	52.8±13.7	P = 0.174
Sex					P = 0.738
Males	18 (58.1%)	3 (75%)	8 (53.5%)	29 (58%)	
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Grade		-	-		
G1	5 (16.1%)				
G2	11 (35.5%)				
G3	15 (48.4%)				
MMR status					
MSI	2	-	-	2	
MSS	29	4	15	48	

Comment 8: RESULTS - Immunophenotyping GC of different histological types "Inflamed TIME was more common for intestinal GCs, IE TIME prevailed in mucinous adenocarcinomas, and ID TIME was more typical for diffuse-type GC” This seems to be true looking at the row data. However, it would be great if the authors presented the statistical analysis for this assumption (see also table 3 – row data are presented, without a statistical comparison.

Response: data are given in the text

Comment 9: RESULTS - PD-L1 expression in GCs with different immunophenotypes "and CD163+ macrophages (P = 0,032)” please modify 0,032 to 0.032

Response: done

Comment 10: Figure 1. The quality of the images can be improved. Moreover, it would be better to present images representing the same magnification.

Response: We provided the decomposable figure of figures and organized them into a PowerPoint file

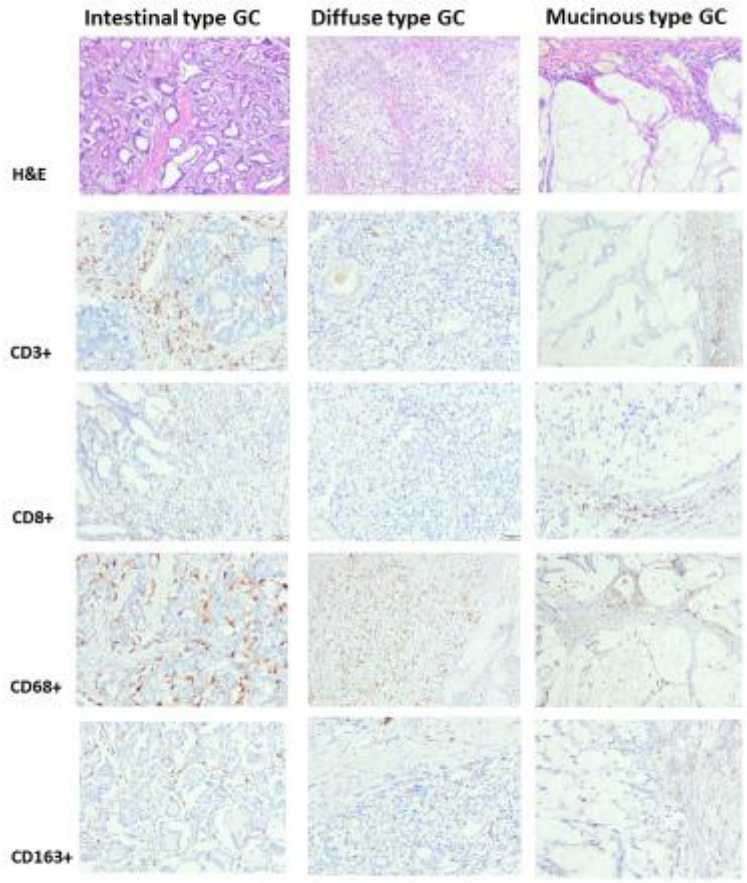

Figure 1. Density of immune cells in GC of different histological types. H&E and Immunohistochemistry. Figures demonstrate differences in infiltration of various histological type GC by immune cells, including entire population of T-lymphocytes (CD3), T-cytotoxic cells (CD8), M1 and M2 macrophages (CD68 and CD163 respectively).

Comment 11: Figure 3. In C and D, it would be useful to indicate the p-value of the comparison between the two groups. "E" and "F" images are not shown in the panel submitted.

Response: figure reorganized according to reviewer suggestion

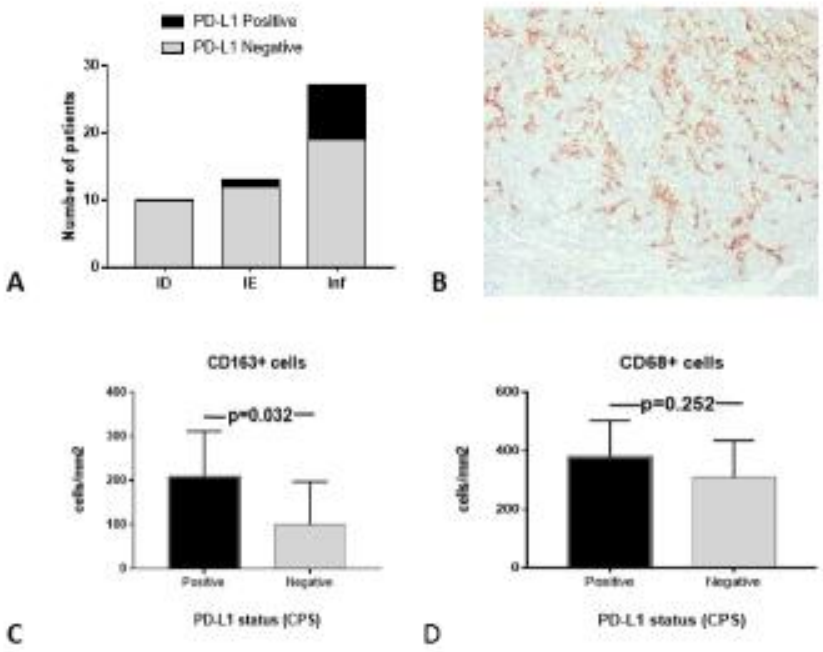

Figure 3. Relationship between PD-L1 expression, GC immunophenotype and number of M1 and M2 macrophages.
A – Frequency of PD-L1 expression in GC of different TIME. B – PD-L1 expression in tumor cells of Inflamed TIME GC; C and D – number of M1 (C) and M2-macrophages in GC regarding PD-L1 expression; B – IHC for PD-L1, ×50

Comment 12: Table 1. "Mucinous e". Please delete "e" - Table 1. "Shaded areas correspond to variables with statistically significant differences at the level P<0.05" Shaded areas are not visible in this table.

Response: corrected according to advice.

Table 2. Immune cells number in GC of different histological types and TIME

Characteristics	CD8+ cells		CD68+ cells		CD163+ cells	
	TC	TS	TC	TS	TC	TS
Histological type						
Intestinal	214±44.9 124-305	202±14.7 173-232	303±21.7 259-347	244±12.4 219-269	173±17.3 138-208	170±9.67 151-190

Diffuse	49.5±6.63 36.0-63.1	66.0±6.03 43.6-98.3	339±23.6 291-388	225±23.8 176-274	51.7±8.60 34.1-69.4	55.5±8.94 37.2-73.9
Mucinous	7.53±2.50 2.26-34.3	52,5±37,5 23.9-82.9	101±59.0 64-125	219±16,2 126-264	12.5±11.8 6.32-78.2	62.5±12.5 36-121
	P = 0.045	P = 0.059	P = 0.071	P = 0.471	P = 0.032	P = 0.011
TIME type						
ID	43.7±4.05 35.1-52.3	43.2±5.93 30.6-55.7	367.5±36.9 288-446	213.1±35.9 135-290	72.5±18.3 33.2-111	48.7±10.5 26.2-71.2
IE	19.4±3.43 12.2-26.7	112±15.2 80.1-144	157.3±29.6 94.5-220	266.6±25.8 211-321	55.5±13.6 26.5-84.4	119.5±16.3 84.9-154.1
Inflamed	229±44.9 138-319	190±16.5 156-223	352.8±15.6 321-384	232.6±13.7 204-260	165.6±18.2 128-202	151.3±12.1 126-175
	P = 0.042	P = 0.004	P = 0.674	P = 0.060	P = 0.024	P = 0.011
Status PD-L1 expression						
Positive	341±72.3 66.3-488	204±52.7 58.4-350	430±27.7 341-518	200±8.19 174-226	231±54.3 58.4-404	135±29.5 41.3-229
Negative	49.0±13.1 20.1-77.9	116±20.7 72.0-159	261±41.4 169-352	279±37.9 195-362	96.3±28.0 34.6-157	131±28.2 69.5-193
	P = 0.010	P = 0.075	P = 0.252	P = 0.715	P = 0.032	P = 0.260

Comment 13: Table 2. As stated above, the authors should present the comparison between different groups and respective p values

Response: corrections were made, data are given in the text.

Table 3. Relationship between immunophenotype and histological pattern of GC

Characteristics	ID TIME	IE TIME	Inf TIME	Total number
Histological types of GC				
Intestinal	1 (3,2%)	5 (16,1%)	25 (80,7%)	31 (62%)
Diffuse	8 (53,3%)	5 (33,3%)	2 (13,3%)	15 (30%)
Mucinous	1 (25%)	3 (75%)	0	4 (8%)
				P < 0.001
PD-L1 expression status				
PD-L1 positive	1 (5,6%)	0	8 (94,4%)	9 (18%)

GCs				
PD-L1 negative GCs	9 (21,9%)	13 (31,8%)	19 (46,3%)	41 (82%)
Total number	10	13	27	50
				P < 0.001

ANSWER to EDITORS'S COMMENT: 1. We replied point by point to reviewer's comments 2, We delete 2 self-cited reference 3, We provided the decomposable figure of figures and organized them into a PowerPoint file. We provided the text in figure(s) in text boxes. 4. We add the "Article Highlights" section at the end of the main text. 5, We uploaded all the files