The immune microenvironment landscape shows treatment-specific differences in rectal cancer patients

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Neoadjuvant therapy is the cornerstone of modern rectal cancer treatment. Insights into the biology of tumor responses are essential for the successful implementation of organ-preserving strategies, as different treatments may lead to specific tumor responses. In this study, we aim to explore treatment-specific responses of the tumor microenvironment. Patients with locally advanced adenocarcinoma of the rectum who had received neo-adjuvant chemotherapy (CT), neo-adjuvant radiochemotherapy (RCT), neo-adjuvant radiotherapy with a long-interval (LRT) or short-interval (SRT) or no neoadjuvant therapy (NT) as control were included. Multiplex-immunofluorescence was performed to determine the presence of cytotoxic T-cells (T-cyt; CD3+CD8+), regulatory T-cells (T-reg; CD3+FOXP3+), T-helper cells (T-helper; CD3+CD8-FOXP3-), B cells (CD20+), dendritic cells (CD11c+) and tumor cells (panCK+). A total of 80 rectal cancer patients were included. Treatment groups were matched for gender, tumor location, response to therapy, and TNM stage. The pattern of response (shrinkage vs. fragmentation) was, however, different between treatment groups. Our analyses reveal that RCT-treated patients exhibited lower stromal T-helper, T-reg, and T-cyt cells compared to other treatment regimens. In conclusion, we demonstrated treatment-specific differences in the immune microenvironment landscape of rectal cancer patients. Understanding the underlying mechanisms of this landscape after a specific therapy will benefit future treatment decisions.

KEYWORDS
immune microenvironment, response, rectal cancer, neoadjuvant treatment, immune landscape
1 Introduction

In recent times, the prognosis of patients with rectal cancer (RC) has improved immensely (1). Neoadjuvant therapy is the cornerstone of RC treatment, allowing for easier resections and better clinical outcomes (2, 3). The presence of various degrees of tumor response demonstrates organ-preserving treatments are possible in selected patients (4, 5). To fully exploit the organ-preserving potential, we need to fully understand this response and the role of the tumor microenvironment in this process.

Different types of treatments have different clinical effects. A simple comparison of pathological complete response (pCR) rates already illustrates this. Neoadjuvant radiochemotherapy (RCT) has increased pCR rates, with an incidence of approximately 14% of patients (6). Lower pCR rates are present in patients treated with 5x5Gy radiotherapy (RT), depending on the treatment interval; either short (SRT) (reported pCR of 0.3% but increasing after 4 week waiting period) or long wait (LRT) (pCR range 9.3%-10.4%) (7, 8). New clinical trials with different combinations of chemotherapy and, especially, wait intervals promise promising results, in some cases reaching 28% pCR rates (6). In general, longer waiting time after initial treatment and more intense local therapy are considered to improve pCR and several oncological outcomes (9).

Previous studies have shown the potential role of immune infiltrates in the prediction of radio-responsiveness to neoadjuvant RCT in rectal cancer (10). These improved outcomes might also be induced by changes in the immune cell and cytokine composition as a response to therapy, and may, therefore, be therapy-specific. The immune microenvironment of RC is complex, different from colon cancer, with variable prognostic impact of individual types of immune cells, such as tumor-infiltrating T-regs (11–17). The well-established role of CD3+ and CD8+ cells in colorectal cancer (i.e., the immunoscore (18)) has paved the road to investigate the impact of other immune cell subsets, such as tumor-infiltrating T-cyt (19–27). The approach allows us to compare the effects of specific therapeutic strategies in order to elucidate relevant differences in the microenvironment.

Here, we explore the effects and interactions of the different types of treatment on tumor cells and microenvironment and correlate this with a biologically meaningful and clinically relevant response scoring method.

2 Methods

2.1 Patient cohort and material used

From an original in-house cohort of 728 rectal cancer (RC) patients from the Radboud University Medical Center Nijmegen, a total of 80 patients with adenocarcinoma NOS (not otherwise specified) of the rectum were selected to be included in this study. Patients with known hereditary colorectal cancer were excluded. An opt-out system for ethical approval was in place. Patient material was obtained from the pathology archives of the Radboud University Medical Centre, Nijmegen, The Netherlands. Selected patients received one of four different treatment regimens between 2015 and 2019; neoadjuvant chemotherapy (CT), neo-adjuvant radiochemotherapy (RCT), neo-adjuvant radiotherapy with a long interval (LRT), or neo-adjuvant radiotherapy with a short interval (SRT), with no neo-adjuvant therapy (NT) serving as control. Per group, 16 patients matched (as much as possible) for gender, tumor location, and cTNM stage were included (details are shown in Table 1).

For each patient, two consecutive slides were cut from the most representative block of the primary tumor and were stained for H&E and an immunofluorescence (IF)-multiplex panel (33) (Figure 1).

2.2 Mutation analysis and MSI status

Sufficient material was present for 64 out of the 80 patients for molecular analysis. A targeted mutational panel (PATHv3) used in routine diagnostics was used to determine any mutations that could clearly influence the immune phenotype of patients (exact panel targets can be found in Supplementary Table 2).
2.3 Immunofluorescence stains

The multiplex IF staining protocol Opal™ 7 Tumor Infiltrating Lymphocyte (TIL) Kit (OP7TL3001KT, Akoya Biosciences, MA, USA), was modified and optimized for rectal 4µm thick FFPE tissue sections (34). In short, slides were sequentially stained using antibodies against CD8, CD20, CD3, Foxp3, CD11c, and pan-Cytokeratin AE1/3 using the BOND RX automated research stainer (Leica Biosystems, Wetzlar, Germany). The anti-CD45RO used in a previous study (35) was exchanged for CD11c (ab52632, Abcam, Cambridge, UK, RRID: AB_2129793). Concentrations of antibodies, retrieval steps, and corresponding Opal dyes were adjusted (details available in Supplementary Table 1) to ensure the best signal

Table 1: Relevant patient information.

| Variable                  | NT n(%) | CT n(%) | RCT n(%) | LRT n(%) | SRT n(%) | P       |
|---------------------------|---------|---------|----------|----------|----------|---------|
| Age, median (IQR)         | 65 (39-84) | 60 (29-78) | 64 (46-80) | 67 (49-79) | 74 (40-87) | 0.02    |
| Gender                    |         |         |          |          |          | 0.44    |
| Female                    | 7 (44%) | 7 (44%) | 8 (50%) | 4 (25%) | 4 (25%) | 0.02    |
| Tumor location            |         |         |          |          |          | 0.02    |
| Rectum                    | 7 (47%) | 12 (75%) | 14 (88%) | 15 (94%) | 14 (88%) |         |
| Rectosigmoid              | 7 (47%) | 4 (25%)  | 0 (0%)  | 0 (0%)  | 2 (12%) |         |
| LAR (low anterior res)    | 1 (6%)  | 0 (0%)  | 2 (12%) | 1 (6%)  | 0 (0%) |         |
| Differentiation grade     |         |         |          |          |          | 0.17    |
| Good                      | 16 (100%) | 16 (100%) | 15 (94%) | 15 (94%) | 13 (81%) |         |
| Poor                      | 0 (0%)  | 0 (0%)  | 1 (6%)  | 1 (6%) | 3 (19%) |         |
| (c)LN involvement         | 10 (100%) | 14 (100%) | 14 (100%) | 13 (100%) | <0.001  |         |
| Yes                       | 2 (20%) | 13 (93%) | 13 (93%) | 11 (85%) | 10 (77%) |         |
| No                        | 8 (80%) | 1 (7%)  | 1 (7%)  | 2 (15%) | 3 (23%) |         |
| Angioinvasion             |         |         |          |          |          | 0.13    |
| Yes                       | 10 (62%) | 6 (38%)  | 6 (38%) | 7 (44%) | 12 (75%) |         |
| No                        | 6 (38%) | 10 (62%) | 10 (62%) | 9 (56%) | 4 (25%) |         |
| (y*)pT category           |         |         |          |          |          | <0.001  |
| 1                         | 0 (0%)  | 0 (0%)  | 0 (0%)  | 0 (0%) | 2 (13%) |         |
| 2                         | 8 (50%) | 3 (18%) | 7 (44%) | 5 (31%) | 1 (6%) |         |
| 3                         | 7 (44%) | 7 (44%) | 9 (56%) | 10 (63%) | 13 (81%) |         |
| 4                         | 1 (6%)  | 6 (38%) | 0 (0%)  | 1 (6%) | 0 (0%) |         |
| (y*)pN category           |         |         |          |          |          | 0.07    |
| 0                         | 14 (88%) | 4 (25%) | 10 (63%) | 7 (44%) | 9 (57%) |         |
| 1                         | 1 (6%)  | 7 (44%) | 4 (25%) | 6 (38%) | 5 (31%) |         |
| 2                         | 1 (6%)  | 5 (31%) | 2 (12%) | 3 (18%) | 2 (12%) |         |
| (y*)pM category           |         |         |          |          |          | 0.11    |
| 0                         | 15 (94%) | 12 (75%) | 16 (100%) | 16 (100%) | 16 (100%) |         |
| 1                         | 1 (6%)  | 4 (25%) | 0 (0%)  | 0 (0%) | 0 (0%) |         |
| Downstaging               |         |         |          |          |          | 0.97    |
| Progression               | *       | 1 (6%)  | 0 (0%)  | 1 (6%) | 1 (6%) |         |
| No change                 | *       | 6 (38%) | 6 (38%) | 7 (44%) | 6 (38%) |         |
| Downstage                 | *       | 7 (44%) | 9 (56%) | 6 (38%) | 6 (38%) |         |
| Unknown                   | *       | 2 (12%) | 1 (6%)  | 2 (12%) | 3 (18%) |         |
| Regression                |         |         |          |          |          | 0.04    |
| Partial                   | *       | 13 (81%) | 16 (100%) | 12 (75%) | 16 (100%) |         |
| No                        | *       | 3 (19%) | 0 (0%)  | 4 (25%) | 0 (0%) |         |
| Pattern of response       |         |         |          |          |          | 0.02    |
| Shrinking                 | 8 (62%)* | 0 (0%) | 6 (37%) | 7 (64%) | 7 (50%) |         |
| Fragmentation**           | 5 (38%)* | 12 (100%) | 10 (63%) | 4 (36%) | 7 (50%) |         |

This table shows the clinical variables of our cohort. Distribution of patients per treatment group with data as n(%).

*Cannot be assessed because no therapy was given. **14 cases with No response were excluded.
intensity for each marker that would allow exposure times of 15-100 ms on multispectral regions. Slides were manually stained with DAPI and mounted using Fluoromount-G® (0100-01, Southern Biotech, ALA, USA, RRID: SCR_015961).

2.4 Immunofluorescence imaging and data processing

Imaging was performed using the VECTRA 3 Quantitative Pathology Imaging System (PerkinElmer) and Vectra V3.0.4 software (PerkinElmer®, Hopkinton, MA, USA). All standard epi-fluorescent filters were used; DAPI, FITC, CY3, Texas Red, and CY5. Whole slide scans were acquired using x4 magnification and subsequently scanned at x20 magnification for the multispectral regions of interest (Figure 1B). Images were first processed using the inForm software (V.2.4.8, Akoya Biosciences, MA, USA, RRID: SCR_019155) for cell and tissue segmentation and then processed through an in-house AI pipeline to phenotype immune cells, which were thereafter analyzed in FlowJo™ (Ashland, OR, USA, RRID: SCR_008520) as previously described (35, 36).
2.5 Patterns of regression

Slides were visualized and annotated by two independent researchers (C.G.M & S.K.O.) and unclear slides were resolved by consensus with an expert gastrointestinal pathologist (I.D.N.). Each sample was scored blindly following an externally validated classification diagram that we developed for patterns of tumor response in esophageal cancer (32) and rectal cancer (unpublished data). Tumor patterns of regression were divided into fragmentation (disintegration of the tumor mass in different sized and shaped fragments) or shrinkage (the tumor mass downsizes) (Supplementary Figure 1A). The pattern of response was assessed blindly for all patients, regardless if they had had neoadjuvant therapy or not.

2.6 Statistical analysis

Statistical analysis was carried out using RStudio version 3.6.2 (Boston, MA, USA, RRID: SCR_000432). One-way ANOVA was done for comparisons between more than two groups in a parametric setting, while Kruskal Wallis one-way ANOVA was used in the non-parametric setting. Student’s T-test was done for comparisons between two groups. Correlations between non-parametric variables were analyzed using Spearman’s rank order correlation test. When comparing categorical variables, a chi-square test was used. A principal component analysis was conducted using singular value decomposition. A P-value lower than 0.05 was considered statistically significant.

3 Results

3.1 Patient characteristics and treatment schedules

A total of 80 RC patients were included; despite matching, selection bias based on treatment indications remained between the groups (Table 1). Gender, differentiation grade, angioinvasion, pN, and pM stage were similar across therapy groups. However, median patient age, tumor location, regression angiogenesis, pN, and pM stage were similar across therapy groups (Table 1, p=0.02, Supplementary Figure 1B). Shrinkage was not present in any of the 16 patients treated with CT while being present in 6/16 patients, treated with RCT (Supplementary Figure 1B). When analyzing the immune spatial contexture of these patterns of response we did not observe any single or combinations of immune cells that could explain these patterns. However, we observed a tendency towards higher stromal T-cyt, T-reg, and T-helper cells in patients exhibiting a shrinking pattern of response compared to those with a fragmented pattern (Supplementary Figure 2).

3.2 Tumor response

An excellent interobserver agreement was reached (κ=0.84). Upon histological evaluation, 14 patients were categorized as "non-responders", as there was extensive tumor present and no evidence of regression such as fibrosis or mucin. Prevalence of shrinkage and fragmentation was significantly different across therapy groups (Table 1, p=0.02, Supplementary Figure 1B). Shrinkage was not present in any of the 16 patients treated with CT while being present in 6/16 patients, treated with RCT (Supplementary Figure 1B). When analyzing the immune spatial contexture of these patterns of response we did not observe any single or combinations of immune cells that could explain these patterns. However, we observed a tendency towards higher stromal T-cyt, T-reg, and T-helper cells in patients exhibiting a shrinking pattern of response compared to those with a fragmented pattern (Supplementary Figure 2).

3.3 Tumor characteristics

Targeted sequencing for microsatellite instable (MSI) markers and 47 cancer-related genes identified pathogenic mutations in 61 of the 64 patients (88%) and MSI in 3 of the 69 patients (4%). The percentages of TP53, KRAS, PIK3CA, and NRAS mutated cases were respectively 74%, 56%, 11%, and 7%. These percentages are in the range of other cohorts (37). The samples with mutations were equally distributed over the different therapies. We then analyzed the relation between the molecular and the immune phenotype, we observed that MSI patients have higher immune cell densities compared to MSS, especially significant are T-cytotoxic cells (p<0.0005), and B cells (p<0.0005)(Supplementary Figures 3A, 3B). TP53-mutated tumors have lower immune cell densities compared to TP53-wild type tumors. The most affected immune cells seem to be T-helper cells (p<0.0005) and dendritic cells (p<0.0005) (Supplementary Figures 3C, 3D, respectively). Patients with PIK3CA-mutated tumors had higher tumoral dendritic cell infiltration compared to wild-type tumors (p<0.05, Supplementary Figure 3E).

3.4 Treatment analysis

The relative distribution of stromal immune cells showed T-helper cells as the predominant immune cell populations across therapies (Figure 2A). Moreover, a significantly higher density of absolute distribution of immune cells was observed in the stroma compared to the tumor infiltration (Supplementary Figure 4A). Immune infiltration in the tumor region did not show significant differences among therapies (Supplementary Figures 4B-F).

Differences in the immunophenotype per treatment could be observed (Figure 2B, Supplementary Figure 4A). T-helper cells were the most predominant population in the stroma of CT, NT, RTL, and RTS treated patients. RCT-treated patients had lower stromal T-helper cells and, had a higher population of T-cyt cells compared to all other treatments. Furthermore, differences could be observed between the stroma and the tumor region, as DCs were one of the predominant types in the tumor region after T-
helper cells, something that was not observed in the stromal region.

The stromal T-reg population was less present after any form of neoadjuvant therapy compared to NT, regardless of the waiting time (LRT vs. SRT) (Figure 3A, p<0.001). Acute radiotherapy effects were evaluated by comparing the SRT-treated group with the LRT-treated group. The presence of stromal T-cyt cells was significantly lower in SRT-treated patients (Figure 3B, p<0.01), especially compared to LRT-treated patients (p=0.001). A tendency toward lower T-helper cell density in SRT-treated patients compared to non-treated patients was also found (Figure 3C, p=0.05).

The impact of CT was evaluated in two ways, first by comparison with the NT group and secondly, by comparison of the RCT group with the LRT-treated group. The CT-treated group was then compared with the RCT-treated group to determine a potential synergistic effect of CT. Compared to NT, CT showed a decrease in T-regs in the tumor and the stroma, (p=0.05 and p<0.01, respectively Supplementary Figure 4D and Figure 3A). Differences in the presence of T-
FIGURE 3

Mean stromal immune cell populations and representative immunofluorescence multiplex images. (A) Mean stromal T-reg density in different treatment settings. (B) Mean stromal T-cytotoxic cell density in different treatment settings. (C) Mean stromal T-helper density per treatment regimen. (D) Mean stromal B cells. NT, No therapy; CT, Chemotherapy; RCT, Radiochemotherapy; LRT, Radiotherapy long course; SRT, Radiotherapy short course.
regs were observed when comparing LRT with RCT. Additionally, the tumor microenvironment showed higher stromal T-helper cells in LRT compared to RCT treated patients \( (p=0.045, \text{Figure 3C}) \). There was also a trend towards lower stromal B cells \( (p=0.1, \text{Figure 3D}) \) and T-regs \( (p=0.1, \text{Figure 3A}) \) in RCT-treated patients, as well as higher tumoral DCs \( (p=0.1, \text{Supplementary Figure 4F}) \) compared to LRT-treated patients. Moreover, when comparing RCT and CT-treated patients significantly higher stromal T-cyt cells \( (p=0.05) \) and significantly lower stromal T-helper cells \( (p=0.03) \) were found in RCT-treated patients.

3.5 Heterogeneity of the immune response according to therapy

When conducting a principal component analysis (PCA) to reduce the dimensions of the high-plex data, different-sized ellipses of the different treatment groups could be observed \((\text{Figures 4A, B})\). Local treatment including RT led to a more homogeneous overall immune response (smaller ellipse) compared to that of patients receiving no treatment or CT (large ellipse) only. Combining dimensions one and two we can explain around 56% of the variance. By analyzing the contribution of each variable to this overall variance we observed that the key players were T-helper cells.

3.6 Interaction between the network of immune cells

By grouping all treatment groups together we were able to analyze the overall immune spatial contexture and interplay between immune populations. There was a consistent correlation between the presence of immune cell subsets in different regions, where immune cell populations were positively correlated in stroma and tumor \((\text{Figure 5})\). Moderate correlations were present between the stromal populations of DCs, T-helper, T-regs, and T-cyt cells, with the strongest correlation being between stromal T-helper cells and T-regs \((r=0.65)\). Weaker correlations were observed in the tumor infiltrate, where the main correlations were found between T-regs, T-helper, and T-cyt cells. For more complex interactions, we demonstrated that the presence of T-helper cells positively correlated with all other immune cell populations in all compartments except for tumor-infiltrating DCs, suggesting that these cells behave differently from the rest of immune cell types, with, in many cases, weak negative correlations \((\text{Figure 5})\).

3.7 Exploratory analysis

3.7.1 Sub-analysis including brachytherapy treatment

A small exploratory cohort of five patients treated with brachytherapy (endoluminal radiotherapy) was also studied to investigate the immunophenotype as a result of this intense local form of therapy. Since three out of the five patients achieved a complete pathological response, we were only able to compare the tumor microenvironment in these five patients to those of the differently-treated patients, as a sample size of two for the tumor region would be biased and, therefore, not a reliable comparison. Strikingly, stromal T-helper, T-cyt, and T-regs showed very low immune densities compared to patients with other treatment regimens \((\text{Supplementary Figures 5A-C})\).

4 Discussion

Many studies have attempted to study the tumor microenvironment in RC \((4, 10, 21, 24, 30, 38–41)\). One study \((17)\) even compared the tumor immune microenvironment...
between RC and CRC, where RC patients seem to have lower levels of immune activation. To the best of our knowledge, this is the first attempt to discover the particularities of the immune contexture in relation to treatment strategy and subsequent tumor response. By comparing different treatment groups, we are able to contribute specific effects to treatment modalities and timing. We have shown that T-regs are less present after any form of therapy, regardless of the treatment interval, suggesting a long and maybe permanent effect on the tumor microenvironment.

Furthermore, we have shown that acute RT (SRT) affects the presence of T-cyt and T-helper cell populations the most. Strikingly, this is less evident with LRT, suggesting repopulation takes place in the interval that distinguishes SRT and LRT, which is in line with previous models. Previously, Mezheveyuski has shown that memory T-helper, memory T-cyt cells, and macrophage counts decreased immediately after radiotherapy and were increased after longer treatment intervals. Following this idea, the sub-analysis including BT-treated patients evidenced that a higher dose of radiation depletes the T-cyt and T-helper cell populations further than other therapies. This is especially striking given that the waiting period from end of therapy to surgery is equal to that of LRT, so either this interval is not enough to repopulate the tumor microenvironment after such an intense dose, or the mechanisms that control the repopulation process have been damaged.

Moreover, considerably fewer T-helper and T-regs were found in the RT-treated group when it was compared to non-RT-treated. This occurrence had been previously reported and stated that T-cell levels begin to decrease after RT is given and do not reach unirradiated levels until approximately two weeks after completion of treatment. Perhaps due to this somewhat coordinated response after the refractory period following RT, the immune response in RT-treated patients seems to be more homogenous compared to that of non-RT-treated patients (as can be observed in Figure 4). Thus, taking into account these results and given that RT is a more localized treatment compared to CT, we hypothesize that the local microenvironment is more affected by RT and less affected by CT.

Finally, the synergistic effect of radiotherapy in combination with chemotherapy seems to increase the presence of T-cyt cells in the stroma and to decrease the presence of T-helper cells when compared to CT-only treated patients. Furthermore, differences in several immune cell populations were found when comparing LRT and RCT, despite the similar interval and RT regimen. Tumor-infiltrating DCs were found to be higher in RCT-treated patients compared to LRT-treated patients. RCT-treated patients also showed lower stromal T-helper, B cells, and T-regs compared to LRT-treated patients. This suggests that the synergistic effect of combination therapy induces proliferation of T-cyt cells but depletion of other immune cells in the stroma and induces DC tumor infiltration compared to LRT-treated patients.

To study spatial neighborhood relationships, we used multiplex immunohistochemistry, which allowed us to look at cell population interactions. High stromal densities result in more immune cells in the vicinity of the primary tumor, allowing infiltration. In addition, the study of multiple types of cells allows for the analysis of complex interactions. Indeed, as expected, the presence of T-helper cells was associated with all other immune cell populations (Figure 5). Recent studies have found many correlations between specific immune cell types in

**FIGURE 5**
Correlation matrix explains the interaction between the network of immune cells.
different regions (tumor and/or stroma) and prognosis in RC patients (21, 22). When we examined the interregional interactions, we confirmed the general premise that higher stromal cells correlate with higher immune infiltrate and we also found correlations between stromal DCs and T-cyt cells (Figure 5). It seems plausible that the reason for this correlation could be cross-priming, where DCs present in the tumor microenviroment activate T-cyt cells by cross-presenting antigens, generating anti-tumor immunity. Further research to confirm this is needed.

Further, we analyzed the spatial contexture of response in a broader sense, linking the histological classification of response with the immune microenvironment. We found that patterns of response could not be explained by a single or combination of multiple immune cell populations across different therapies. Nevertheless, a tendency towards higher stromal T-cyt, T-reg, and T-helper cells in patients exhibiting a shrinkage pattern of response compared to those with a fragmented pattern (Supplementary Figure 2) was observed. A plausible explanation could be that the coordinated presence of several types of immune cell lineages is necessary for a more effective cancer cell elimination, which seems to be the shrinkage pattern of response compared to the fragmented pattern.

Since our study included a heterogeneous group of patients (due to the different therapies given), outcome was not the main aim of our study and therefore we could not analyze immune populations for prognostic value. Nevertheless, a few studies had described a correlation between T-regs and improved survival (23, 24), which we could also observe in our cohort, regardless of treatment (data not shown).

There were several limitations to this study. Firstly, it was a retrospective study with relatively small patient groups from one single institution. Since RCT is the standard therapy for locally advanced RC, we could not expand the other treatment groups without introducing significant selection bias for patient and tumor characteristics. No outcome data could be reported since there was no randomization between the different treatment arms. Similarly, outcomes such as downstaging could not be compared since the different treatment groups did not have the same interval between therapy and surgery. The objective of this study was to explore the spatial and immune contexture after neoadjuvant therapy in rectal cancer to provide insight into their interplay as we move towards local and tailored treatments. Our goal was to unveil the unique immune infiltrate of the tumor and tumor microenvironment of differentially treated rectal cancer patients.

Further research should focus on unraveling the link between different patterns of response and the immune cell infiltrate. Potential therapeutic applications may arise as stimulating certain immune cell lineages could influence the pattern of response to treatment, which can be used to favor a shrinkage pattern environment instead of a fragmented or no response pattern. Moreover, the prognostic relevance of T-regs needs to be investigated in different regions of the primary tumor as its role in the stromal and tumor regions could be different.

In conclusion, we believe that this study is the first to report the differential effects of specific neoadjuvant therapies on the immune conexture of advanced rectal cancer. We have shown that many immune populations (including T-helper, T-cyt, and T-reg cells) are affected mainly by radiotherapy treatment. The re-emergence (or lack) of specific immune cell populations after treatment over time might be linked to tumor regression. Therefore, a better understanding of the reorganization of the immune contexture after therapy is important for the appropriate management of locally advanced RC patients.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

The following detailed Credit author statement is added by the corresponding author, who is responsible for ensuring that the descriptions are accurate and agreed by all authors:

CG: methodology, formal analysis, data curation, writing - original draft preparation, visualization, and writing - review and editing. YB: methodology, formal analysis, data curation, and writing - original draft preparation. SKÖ: methodology, validation, and writing - review and editing. MA: interpretation of data, original draft preparation, and writing - review and editing. MG: methodology, data collection, interpretation of data, and writing - review and editing. SV: methodology and writing - review and editing. CM: conceptualization, data curation, writing - review and editing, and supervision. IN: conceptualization, methodology, validation, data curation,
writing - review and editing, and supervision. All authors contributed to the article and approved the submitted version.

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Conflict of interest
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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