Ileostomy for steroid-resistant acute graft-versus-host disease of the gastrointestinal tract

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Abstract
Steroid-resistant acute graft-versus-host disease (GVHD) of the gastrointestinal tract associates with important morbidity and mortality. While high-dose steroids are the established first-line therapy in GVHD, no second-line therapy is generally accepted. In this analysis of 65 consecutive patients with severe, steroid-resistant, intestinal GVHD (92% stage 4), additional ileostomy surgery significantly reduced overall mortality (hazard ratio 0.54; 95% confidence interval, 0.36–0.81; p = 0.003) compared to conventional GVHD therapy. Median overall survival was 16 months in the ileostomy cohort compared to 4 months in the conventional therapy cohort. In the ileostomy cohort, both infectious- and GVHD-associated mortality were reduced (40% versus 77%). Significantly declined fecal volumes (p = 0.001) after surgery provide evidence of intestinal adaptation following ileostomy. Correlative studies indicated ileostomy-induced immune-modulation with a > 50% decrease of activated T cells (p = 0.04) and an increase in regulatory T cells. The observed alterations of the patients’ gut microbiota may also contribute to ileostomy’s therapeutic effect. These data show that ileostomy induced significant clinical responses in patients with steroid-resistant GVHD along with a reduction of pro-inflammatory immune cells and changes of the intestinal microbiota. Ileostomy is a treatment option for steroid-resistant acute GVHD of the gastrointestinal tract that needs further validation in a prospective clinical trial.

Keywords Hematopoietic stem cell transplantation · Graft-versus-host disease · Refractory GVHD · Intestinal adaptation · Ileostomy-induced immune modulation · Intestinal microbiota

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Introduction

Despite numerous advances in allogeneic stem cell transplantation (aSCT) during the last decades, acute graft–versus-host disease (aGVHD) remains a major source of morbidity and mortality [1]. In particular, aGVHD of the gastro-intestinal tract (GI-aGVHD) is a major threat to allogeneic stem cell recipients and challenges physicians [2]. In steroid-resistant disease [3], treatment effects are insufficient, hospitalizations extend over months, and patient recovery is complicated by nosocomial infections. In the past, a long list of immune-modulating drugs has been investigated in order to enhance treatment results, with limited effect and no significant difference between treatments [4–8]. In retrospective analyses, antithymocyte globulin (ATG) [9] and ruxolitinib [10] were identified as most promising treatments; nevertheless, only a limited fraction of patients in these studies had GI-aGVHD. As a consequence, consensus guidelines recommend the use of high-dose steroids as a first-line treatment, while no standard second-line treatment is generally accepted [4, 11].

Ileostomy is an established surgical procedure, regularly performed in patients with colon cancer resection and in few cases of patients with inflammatory bowel disease. Pre-clinical studies indicated physiological alterations following ileostomy, such as increased intestinal absorption and mucosal proliferation, which might be of therapeutic use in GVHD. Intestinal adaptation [12] after ileostomy has been shown in animal models of surgery-induced short bowel syndrome [13] in piglets [14], zebrafish [15], and mice [16], resulting in mucosal hyperplasia through significantly greater crypt depth and villi length. In an ileostomy mouse model, adaptation processes resulted in decreased expression of secretory progenitor cells’ stem cell markers such as protein atonal homolog 1 [16]. In an intestinal resection model, the ileum had a higher adaptive capacity than jejunum, duodenum, or colon [17] and signaling pathways involving molecules, such as insulin-like growth factor [17] or epidermal growth factor receptor (EGFR), have been induced. In zebrafish, EGFR expression significantly increased after ileostomy [15]. EGFR can prevent apoptosis [18], is co-expressed on regulatory T cells [19], and may contribute to immune homeostasis. Little is known on the pathophysiology of ileostomy-induced immune modulation. Patients with inflammatory bowel disease after total proctocolectomy and primary ileostomy rarely develop de novo Crohn’s disease in their neo-small bowel [20]. However, surgery may also induce inflammatory bowel disease [21] or celiac disease in predisposed patients. Amphiiregulin, an EGFR agonist involved in autocrine growth stimulation, is weakly expressed in intestinal biopsies of GVHD patients [22] and present at elevated levels in the serum of patients with aGVHD- [22] and late-aGVHD [23]. The status of surgical interventions in GI-aGVHD is ill-defined. Cases included 2 young adults with great bowel resection for complications of aGVHD [25], 2 cases of small bowel resection in chronic GVHD [26], and one elderly patient with laparoscopic left hemicolectomy in late-aGVHD [27]. None of the above-cited reports actually intended to treat aGVHD with ileostomy, neither compared ileostomy to other therapeutic options. To our knowledge, this study is the first description of ileostomy for the treatment of steroid-resistant, GI-aGVHD. At the same time, our data report the largest aGVHD patient cohort with performed ileostomy surgery along with a matched stage 4 GI-aGVHD cohort.

Methods

Patients

We retrospectively analyzed clinical characteristics and laboratory parameters of patients with severe intestinal GVHD after allogeneic stem cell transplantation. All patients who underwent allogeneic hematopoietic stem cell transplantation (HSCT) in the Bone Marrow Transplantation units of the West-German Cancer Center at University Hospital Essen between September 2009 and December 2015 were included in this study. From all 1353 consecutive transplanted patients that were screened, 65 developed severe, intestinal GVHD. Patients were followed for up to 5 years after transplantation. Overall survival (OS) was calculated from transplantation to last follow-up visit or death of any cause.

Treatment

All patients received conventional GVHD prophylaxis regimen (Table 1). Medical therapy for steroid-resistant aGVHD was according to physician’s choice (Table 2). In the experimental cohort, 10 patients consented to undergo experimental, individual therapy and received additional ileostomy surgery as salvage treatment for severe-, refractory-, steroid-resistant aGVHD. Surgery was performed at the Department of General-, Visceral- and Transplantation Surgery at the University Hospital Essen. The surgical procedure was according to physician’s choice. The following procedures were performed: loop ileostomy (7 patients), loop jejunostomy (2 patients), and 1 patient received a loop ascendostoma. Patients of the conventional therapy cohort received second- and further-line treatments according to physician’s choice.

Assessments

GVHD was classified according to published criteria for aGVHD [28, 29]. In the experimental cohort, GI-aGVHD was further histologically confirmed according to the Freiburg criteria [30]. For inpatients, daily clinical assessment
and standard laboratory parameters were obtained. Fecal volumes were assessed 7 and 14 days before and after ileostomy and confirmed before discharge. For outpatients, clinical status and laboratory parameters were recorded weekly. Response to therapy and outcome were assessed according to published criteria [31]. A partial response (PR) in

| Characteristic                                      | Conventional therapy plus ileostomy | Conventional therapy |
|-----------------------------------------------------|-------------------------------------|----------------------|
| Total enrolled and treated                          | 10 100                              | 55 100               |
| Demographics                                        |                                     |                      |
| Median age at transplantation (range)               | 49 (2–53)                           | 57 (6–73)            |
| Female sex                                          | 3 30                                | 25 45                |
| Disease                                             |                                     |                      |
| Acute myeloid leukemia                              | 3 30                                | 22 40                |
| Myelodysplastic syndromes                           | 0 0                                 | 11 20                |
| Acute lymphoblastic leukemia                        | 1 10                                | 6 11                 |
| Bilinear acute leukemia                             | 0 0                                 | 1 2                  |
| Chronic lymphocytic leukemia                        | 1 10                                | 1 2                  |
| Chronic myeloid leukemia                            | 0 0                                 | 3 5                  |
| Chronic myelomonocytic leukemia                     | 0 0                                 | 2 4                  |
| Myeloproliferative disorders                        | 1 10                                | 4 7                  |
| Aggressive lymphomas                                | 1 10                                | 3 5                  |
| Hodgkin lymphomas                                   | 0 0                                 | 1 2                  |
| Dendritic cell neoplasia                            | 1 10                                | 0 0                  |
| Severe aplastic anemia                              | 1 10                                | 0 0                  |
| Congenital disorders                                | 1 10                                | 1 2                  |
| Viral serostatus prior transplantation               |                                     |                      |
| CMV positive donor/recipient                        | 10 100                              | 19 35                |
| EBV positive donor/recipient                        | 5 50                                | 55 100               |
| Conditioning and irradiation                        |                                     |                      |
| Myeloablative conditioning regimen                  | 10 100                              | 37 67                |
| Anti-thymocyte globulin containing                  | 5 50                                | 25 45                |
| Total body irradiation containing                   | 3 30                                | 15 27                |
| Transplant and donor constellation                  |                                     |                      |
| Unrelated donor                                     | 7 70                                | 42 76                |
| Sibling donor                                       | 3 30                                | 12 22                |
| Median CD34+ cells/kg [$\times 10^6$], range        | 5.4 3.7–17.3                        | 7.4 3.4–9.4          |
| Mismatch constellation                              |                                     |                      |
| HLA A mismatch                                      | 2 20                                | 8 15                 |
| HLA B mismatch                                      | 0 0                                 | 6 11                 |
| HLA C mismatch                                      | 1 10                                | 2 4                  |
| HLA DQ mismatch                                     | 1 10                                | 3 5                  |
| HLA DR mismatch                                     | 0 0                                 | 1 2                  |
| Male recipient female donor                         | 2 20                                | 7 13                 |
| Female recipient male donor                         | 2 20                                | 11 20                |
| Baseline immunosuppression                           |                                     |                      |
| Cyclosporin A                                       | 10 100                              | 49 89                |
| Methotrexate                                        | 9 90                                | 46 84                |
| Tacrolimus                                          | 1 10                                | 3 5                  |
| Mycophenolate mofetil                               | 3 30                                | 14 25                |

HLA human leukocyte antigen, CMV cytomegalovirus, EBV Epstein-Barr virus
aGVHD evaluation required organ staging improvement, without deterioration of any other organ, and a complete response (CR) required resolution of aGVHD manifestation in all organs. No response corresponded to any change and progression to a flare of GVHD after initial response. For responding patients, follow-up intervals were sequentially extended. Patient outcome was evaluated from date of transplantation until death of any cause or last follow-up. Adverse events were graded according to Common Terminology Criteria for Adverse Events (CTCAE) 4.0 [32]. At diagnosis of steroid-resistant GVHD, Eastern Cooperative Oncology Group (ECOG) general performance score and Charlson Comorbidity Index scores were obtained for all patients. Conditioning- and early transplant-related complications were included into comorbidity calculation.

Correlative studies

We collected blood serum and—in a subset of patients—stool samples from the experimental and conventional therapy cohort before and after ileostomy. Serum-soluble interleukin-2 receptor (s-IL2 R) levels were assessed according to laboratory standards [33]; fecal calprotectin analysis was performed as previously published [34]. All analyses were performed at the University Hospital Essen and its laboratories. Quantification of the gut microbiota from stool samples was performed in the Institute of Medical Microbiology. A four-quadrant sequential streak technique on agar plates was used for semi-quantitative analysis of bacteria and fungi. Stool samples were plated on C.L.E.D. agar (cystine lactose electrolyte-deficient agar) for aerobic bacteria and Beerens agar with sheep blood for anaerobic bacteria and fungi selective agar (all from Thermo Scientific, Waltham, MA). Quantification of grown colonies was routinely assessed after 24 h of culture for aerobic bacteria and fungi and after 48 h for anaerobic bacteria. Presence of bacteria and fungi was categorized as low, medium, or high according to culture counts. Bacteria and fungi were quantified as low for growth of 0 to < 5 colonies in sector 1, medium for growth of ≥ 5 colonies in sector 1 to < 5 colonies in sector 3, and high for growth of ≥ 5 colonies in sector 3 or in sector 4.

Peripheral blood mononuclear cells (PBMC) were isolated using an automatic red blood cell lysing system (TQ-Prep, Beckman Coulter, Brea, CA), washed with fluorescence-activated cell sorting (FACS) buffer and stained with surface markers. No intracellular staining was performed. FACS analysis of the patient’s immune status was performed on a FC500 and NAVIOS flow cytometer (Beckman Coulter) using the manufacturer’s software. A minimum of 15,000 lymphocytes were analyzed to ensure adequate subset analysis. Specific cell subsets within the CD45+ lymphocyte gate were characterized as following: T cells, CD3+; T helper cells, CD3+/CD4+; activated T cells, CD3+/HLA-DR+; regulatory T cells, CD3+/

| Characteristic                          | Conventional therapy plus ileostomy | Conventional therapy |
|----------------------------------------|-------------------------------------|----------------------|
| Total enrolled and treated             | 10 100                              | 55 100               |
| Overall GVHD grade                     |                                     |                      |
| III                                    | 0 0                                 | 6 11                 |
| IV                                     | 10 100                              | 49 89                |
| Gastrointestinal GVHD stage            |                                     |                      |
| 3                                      | 0 0                                 | 8 15                 |
| 4                                      | 10 100                              | 47 85                |
| Liver GVHD stage                       |                                     |                      |
| 1                                      | 3 30                                | 4 7                  |
| 2                                      | 1 10                                | 5 9                  |
| 3                                      | 4 40                                | 9 16                 |
| 4                                      | 2 20                                | 10 18                |
| Skin GVHD stage                        |                                     |                      |
| 1                                      | 1 10                                | 0 0                  |
| 2                                      | 4 40                                | 13 24                |
| 3                                      | 1 10                                | 20 36                |
| 4                                      | 2 20                                | 10 18                |
| GVHD biomarkers                        | Median Range                         | Median Range         |
| Serum s-IL2 R                          | 696 409–6960                         | 673 182–23997        |
| Fecal calprotectin                     | 3000 124–3079                        | 360 47–3010          |

GVHD graft-versus-host disease, s-IL2 R soluble interleukin-2 receptor
CD4+/CD25+/CD127−; cytotoxic T cells, CD3+/CD8+; naïve CD4+ T cells, CD3+/CD4+/CD45RA+; memory CD4+ T cells, CD3+/CD4+/CD45RO+; B cells, CD19+.

Statistical analysis

For statistical analysis, we used Statistical Package for the Social Sciences (SPSS) software (SPSS 23.0; SPSS Inc., Chicago, IL) and Matlab (Mathworks Inc., Nantick, MA). Clinical-pathological parameters were compared using chi-square tests. Overall survival (OS) was calculated from date of allogeneic stem cell transplantation until death from any cause. OS of surviving patients was censored at 60 months. Survival curves were obtained by using the Kaplan-Maier method and were compared by the log-rank test. All $p$ values are two-sided at the significance level of 0.05. Cox proportional hazards model was adopted to calculate the hazard rate. Multivariate and univariate Cox regression analysis was performed for competing risks and subgroup analysis using SPSS software. The statistical significance of changes, as a result of an intervention, was evaluated by testing before- and after-values with a paired $t$ test in Matlab.

Results

Patient characteristics

All 1353 consecutive patients receiving allogeneic stem cell transplantation at the University Hospital Essen between September 2009 and December 2015 were screened. A total of 65 patients were diagnosed with severe, GI-aGVHD. Underlying hematologic disease, donor constellation, viral serostatus, conditioning regimen, and baseline immunosuppression are detailed in Table 1. Of the total 65 patients with severe aGVHD, 10 underwent experimental surgical treatment for steroid-resistant GVHD in addition to conventional therapy, while the remaining 55 patients received only conventional medical therapy (Table 1). Patients of the experimental cohort were 8 adult and 2 pediatric patients. Differences between both cohorts involved diagnosis of myelodysplastic syndrome (0% versus (vs) 20%), myeloablative conditioning regimen (100% vs 67%), cytomegalovirus (CMV) risk profile serostatus (100% vs 35%), and Epstein-Barr virus (EBV) risk serostatus (50% vs 100%). Established GVHD risk factors were evenly distributed between cohorts, with unrelated donor stem cells (70% vs 76%) and HLA mismatch (40% vs 37%). Half of the patients in both cohorts received GVHD prophylaxis with ATG as part of their conditioning regimen. All patients of the ileostomy cohort and 85% of the conventional therapy cohort developed stage 4 GI-aGVHD (Table 2). Patients had GVHD of more than one site including stage 3–4 liver (60% vs 34%) and stage 3–4 skin GVHD (30% versus 54%). Comparison of ECOG performance and Charlson comorbidity scores at diagnosis of steroid-resistant GVHD between ileostomy and conventional therapy cohort revealed no significant difference (Supplementary Table 1).

Therapy and response

All 65 patients received a first-line GVHD treatment with high-dose steroids. Dosage of steroids was according to physician’s choice between 2 and 3 mg/kg bodyweight, with a weekly tapering following response. Patients’ first-, second-, and further-line GVHD treatments reflecting consensus recommendations and experimental approaches in refractory disease are detailed in Table 3. The majority of patients achieved a partial response (PR) through second-line therapy, except for their GI-aGVHD, which poorly responded or flared. In the experimental ileostomy cohort, all patients ($n = 10$) were pretreated with several second-line treatments, before being considered for salvage ileostomy. Ileostomy was performed at a median of 134 days (range 32 to 366) after allogeneic transplantation. Early surgery-associated morbidity was low (grade ≤2 CDCAE) and no intervention-associated mortality was recorded. All patients required limited red blood transfusions for moderate bleeding during and shortly after surgery. No early surgical intervention for bleeding was necessary. One non-responding patient required surgical intervention for wound dehiscence 2 months after ileostomy (grade 3 CDCAE). After ileostomy, median fecal volumes significantly declined by 50% (Fig. 1), despite a shortened resorption surface. At discharge, fecal volumes were significantly lower than in the early post-operative phase ($p < 0.005$). GVHD-associated gastrointestinal bleeding was reduced and patients reported a significantly improved individual pain assessment. Eight patients (80%) had a complete response (CR) of their aGVHD and could be discharged at a median time of 62 days after ileostomy (range 14–199). In 3 patients (30%), ileostomy was temporary and successfully removed after 5 months.

Accordingly with published results for patients with grade IV GVHD, 90% of patients deceased during the follow-up period of over 5 years (Fig. 2), but with important differences between both therapy cohorts. In the ileostomy cohort, the 5-year OS was significantly ($p = 0.002$) higher than in the conventional therapy cohort (30% vs 6%). The leading cause of death in the conventional therapy cohort was infectious disease (56%) including sepsis, aspergillosis, and respiratory failure, followed by uncontrolled GVHD (31%) (Table 3). Both aGVHD-related mortality (10% vs 31%) and mortality caused by severe infection (20% vs 56%) were significantly lower in the ileostomy cohort. All respective causes of death are detailed in Table 2. The majority of patients in both cohorts developed aGVHD in more than one site. Response of cutaneous aGVHD was regularly achieved through first- or
second-line treatments, while steroid-resistant liver aGVHD remained a challenge to treatment and accounted for 10\% of deaths in both cohorts. Cox regression analysis (Table 4) confirmed the OS benefit of ileostomy (hazard ratio (HR) 0.54 (95\% confidence interval (CI), 0.36–0.81; \( p = 0.003 \)). This result was also verified by a time-dependent Cox regression analysis (HR 0.36; 95\% CI, 0.16–0.80; \( p = 0.013 \)). After censoring all patients with early death (< day + 100 of
transplantation) of any cause, ileostomy still associated with longer OS (HR 0.63; 95% CI, 0.41–0.95; p = 0.029). Similar results were obtained when OS was evaluated from the date of GVHD diagnosis (HR 0.53; 95% CI, 0.36–0.82; p = 0.001). Multivariate analysis revealed no significant OS benefit for most second-line therapies (Table 4), likely due to limited patient numbers in each second-line therapy subgroup. ATG (50% vs 42%) and basiliximab (10% vs 16%) therapies were evenly distributed in both cohorts, while ruxolitinib (30% vs 5%) was underrepresented in the conventional therapy cohort, without resulting in significant OS differences, when stratified with respect to treatment (Table 4, Supplementary Table 2). The application of mycophenolate mofetil (MMF) associated with significantly longer OS (p = 0.014), but MMF use overlapped with baseline immunosuppression. Combination of second-line therapies had no significant OS advantage. Patients with GVHD response to any second-line therapy had a significant survival benefit (HR 0.30; 95% CI, 0.16–0.57; p < 0.0005). As expected for stage 4 aGVHD, involvement of all 3 organs associated with significantly reduced OS (HR 4.23; 95% CI, 1.22–14.7). Subgroup analysis of the ileostomy cohort revealed no significant difference regarding the time point of aGVHD diagnosis and of ileostomy surgery.

**Correlative studies**

With respect to tested biomarkers, the ileostomy cohort was representative of the whole study cohort with severe GI-

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**Fig. 1** Significant decline of fecal volumes after ileostomy. Change of fecal volumes per day was evaluated at days 14 and 7 before (n = 9) and after ileostomy (n = 8) and at discharge (n = 7). The significance of the fecal volume changes was evaluated within the paired sample t test. Fecal volumes after ileostomy were significantly lower as compared to volumes before ileostomy. Significance levels of fecal volume change between 14 days prior to ileostomy and 7 days after ileostomy were *p = 0.02 and **p < 0.01.

**Fig. 2** Comparison of overall survival of GI-aGVHD patients with additional ileostomy and conventional therapy alone. Survival from day of allogeneic stem cell transplantation until death of any cause. Data were censored after 60 months. Patients with GI-aGVHD were categorized into ileostomy (n = 10, hatched) and conventional therapy cohorts (n = 55, solid). Graphs were plotted with the Kaplan-Meier survival analysis. Cohorts were compared using the log-rank test. **p = 0.002
aGVHD. In both cohorts, mean fecal calprotectin values during aGVHD were significantly elevated compared to normal controls, reflecting the severity of inflammation (Table 2). Mean serum s-IL2 R levels of both cohorts were comparable and not predictive of GVHD. Flow cytometry analysis of patient’s immune reconstitution before and after ileostomy revealed changes in several T cell subsets. The absolute number of activated T cells significantly decreased by more than 50% (p = 0.04) after experimental surgery, while the number of regulatory T cells increased by 1.7-fold (p = 0.28) (Fig. 3). The total number of T cells and CD8+ T cells remained unchanged, while memory T cells increased 1.5-fold (p = 0.19).

In the experimental ileostomy cohort, stool samples recorded prior transplantation, during GVHD, and after ileostomy revealed an increased presence of aerobic, anaerobic bacteria, and fungi following ileostomy compared to samples obtained during GVHD (Fig. 4). Aerobic bacteria were predominantly present after ileostomy (9 of 9 evaluable patients). For 3 patients (30%), all with final ileostomy removal, high numbers of anaerobic bacteria were recorded after GVHD diagnosis and ileostomy. These data indicate that ileostomy induced clinical response in patients with steroid-resistant GVHD along with reduction of pro-inflammatory immune cells and changes of the intestinal microbiota.

### Discussion

This is the first study comparing ileostomy for the treatment of steroid-resistant aGVHD to conventional medical therapy in allogeneic stem cell recipients. Patients with stage 4 GI-aGVHD were treated with conventional therapy and received additional experimental ileostomy surgery in order to control GVHD. The majority of patients responded to ileostomy therapy and had a significant OS benefit compared to conventional medical care, alone. In the reported GI-aGVHD cohorts, prolonged immunosuppression together with infectious- and GVHD-associated complications limited long-time survival. The observed mortality rate was consistent compared to reported rates from studies involving GI-aGVHD patients [24, 35, 36], yet the proportion of stage 4 GI-aGVHD patients was higher. In the ileostomy cohort, enhanced control of intestinal aGVHD resulted in reduced exposure to immunosuppressive medication and may have limited infectious complications.

| Predictor | HR | 95% CI | p       |
|-----------|----|--------|---------|
| Ileostomy cohort | 0.54 | 0.36–0.81 | **p<0.003 |
| Ileostomy cohort (time-dependent analysis) | 0.36 | 0.16–0.80 | *p<0.013 |
| Time to GVHD (effect of 1 day increase) | 1.00 | 0.99–1.00 | 0.491 |
| Time to ileostomy (effect of 1 day increase) | 1.00 | 0.99–1.01 | 0.918 |

**Table 4 Cox regression analysis for HR of competing risks**

*HR hazard ratio, CI confidence interval, p significance as p value
* p < 0.05, **p < 0.01
Fig. 3 Ileostomy reduced activated T cells and increased regulatory T cells. Lymphocyte subsets in the peripheral blood were measured by flow cytometry before and after ileostomy. Specific cell subsets within the CD45+ lymphocyte gate were characterized as follows: T cells, CD3+(n = 7); T helper cells, CD3+/CD4+(n = 6); activated T cells, CD3+/HLA-DR+(n = 7); regulatory T cells, CD3+/CD4+/CD25+/CD127−(n = 7); naïve CD4+ T cells, CD3+/CD4+/CD45RA+(n = 5); memory CD4+ T cells, CD3+/CD4+/CD45RO+(n = 6); B cells, CD19+(n = 6). Fold change of T cells is expressed as a percentage of absolute cell numbers after ileostomy relative to that observed before ileostomy, and analyzed by the paired sample t test. p values are detailed in the figure. p < 0.05 was considered statistically significant. Data represents normalized mean values and the error bars represent the standard error of the mean.

Fig. 4 Ileostomy altered the fecal microbiota. Quantitative evaluation of cultured aerobic, anaerobic bacteria, and fungi from fecal samples. Patients were evaluated pre-transplantation (npre = 8), during GVHD (nGVHD = 9), and post-ileostomy (npost = 9). Microbiota are categorized into low, medium, and high categories according to culture plate counts. The proportion of categories at these time points are shown. a Quantitative evaluation of cultured aerobic bacteria pre-transplantation (npre = 8), during GVHD (nGVHD = 9), and post-ileostomy (npost = 9). b Quantitative evaluation of cultured anaerobic bacteria (npre = 8, nGVHD = 9, npost = 9). c Quantitative evaluation of cultured fungi (npre = 8, nGVHD = 8, npost = 9). d Quantitative evaluation of all cultured microorganisms (npre = 24, nGVHD = 26, npost = 27).
Due to the small patient number in the ileostomy cohort, we did not record a significant OS difference between early or late ileostomy (Table 4). In the conventional therapy cohort, the highest mortality was caused by infections, followed by GVHD. The association between GVHD and infectious—in particular invasive fungal [37]—complications has been previously well established, as GVHD associates inflammation, tissue damage, and severe immunosuppression [4]. The intestinal barrier function is disturbed [38], bacterial invasion eased [39], and immune cells are functionally impaired [40–42].

In GI-aGVHD patients, ileostomy resulted in a significant reduction of fecal volumes (Fig. 2), through processes involving, for example, intestinal adaptation, ileostomy-induced immune modulation, and changes of the intestinal microbiota. Post-ileostomy complications include high output [43] as a result of shortened gut surface [44], which may in particular occur in the early post-operative phase of ileostomy [45, 46], an observation that we shared in our study. Within 7 days from surgery, fecal volumes were reduced. Correlative studies in our patients before and after ileostomy support the hypothesis of ileostomy-induced immune modulation. Prior ileostomy, patients had elevated calprotectin values, which may be used as prognostic marker for GI-aGVHD [34, 47]. Patients’ cellular immune status also showed elements of inflammation, such as reduced levels of regulatory T cells and elevated levels of activated T cells. Reducing inflammation can contribute to limit fecal volumes [48]. T cell subset analysis revealed significantly reduced activated T cells after ileostomy, while the number of regulatory T cells and memory T cells increased (Fig. 3). In previous studies of successful GVHD treatments, T cell subset correlations associated with reduced activated T cells [10] or increased regulatory T cells [5, 49]. Due to the small patient number in the ileostomy cohort, changes of memory T cells and regulatory T cells remained below significance. Activated CD4+ T cells play a central role in GVHD-induced organ damage [50]. Their significant decrease as a result of ileostomy-induced immune modulation may help to explain why ileostomy proved successful in GVHD patients. Other previously described candidate pathways of ileostomy-induced immune modulation involve EGFR, whose expression was increased after ileostomy in zebrafish [15] and the mucosal cytokine interleukin-22 (IL-22). Reduced intestinal physical stimulation after ileostomy altered the expression of IL-22 [51] and 648 other intestinal genes. In IL-22-deficient recipient mice with GVHD, intestinal apoptosis and mortality were significantly increased [52], while IL-22-deficient donor T cells decreased mortality in GVHD mice [53].

A number of studies indicate that the intestinal microbiota plays an important role in aGVHD [54, 55] and a pilot study of fecal microbiota transfer induced complete response in 3 of 4 patients [56]. We detected changes of intestinal microbiota from pre-transplantation to GVHD and after ileostomy. During GVHD, the detectable number of cultured fecal microbes was lower compared to pre-transplantation samples (Fig. 4). In post-ileostomy fecal samples, the number of aerobic bacteria was higher than during GVHD. In small bowel transplants and ileostomy recipients, increased oxygen levels enhanced intestinal, microbial diversity from strict anaerobic to facultative anaerobic and aerobic bacteria [57]. High intestinal microbiota diversity has been associated with better survival of allogeneic stem cell recipients, due to reduced infectious and GVHD-associated mortality [58]. All 3 patients with later ileostomy removal finally achieved high numbers of anaerobic bacteria. Still, the effect of ileostomy on intestinal microbiota is controversial. Ileostomy may result in nutrient deprivation and less diverse microflora of the de-functioned ileum [59], but has also been associated with an enrichment of bacterial metabolites in the proximal ileum [57] and high inter-individual variation of microbiota [60]. We note the following limitations to this analysis. Data has been analyzed retrospectively and the absolute number of patients in the ileostomy cohort was limited. As patients in this study have been treated between 2009 and 2015, the extent of ruxolitinib use varied between cohorts, without resulting in a significant OS difference. Flow cytometric T cell subset analysis was conducted without intracellular staining.

Conclusions

Despite the limited patient number, our data show that ileostomy represents a promising treatment option for steroid-resistant GI-aGVHD. Correlative studies indicate induction of intestinal adaptation, immune modulation, and changed microbiota through ileostomy. When indicated, ileostomy should be performed before day +100, because hazards of GVHD-associated and infectious mortality increased in uncontrolled GVHD with time. A prospective, controlled, randomized trial comparing ileostomy to other second-line GVHD treatments should validate these results.

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Authorship. ATT, NKS, and DWB designed the study. EB and ATT performed statistical analysis. ATT, JY, JK, AT, and OB performed data collection. TB, JK, and TL participated in data acquisition and analysis. ATT, EB, and NKS wrote the manuscript. DWB, TL, and TB contributed to write the manuscript. All authors had access to primary clinical trial data, read, and approved the final manuscript.

Compliance with ethical standards

The study and data acquisition was conducted in accordance with German legislation and the revised Helsinki Declaration and evaluated...
by the ethical institutional review board of the University of Duisburg-
Essen (Protocol No. 18-8220-BO). Written consent to surgical-
therapy was obtained from all patients with ileostomy. We confirm
that no patient can be identified because of anonymized patient data.

Conflict of interest
ATT has received lecture fees from Jazz Pharmaceuticals and travel subsidies from Neovii Biotech outside the
submitted work. NKS received travel subsidies from MSD and Jazz,
DWB received travel subsidies from Medac, all outside the submitted
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