# NOVEL AND IMPROVED METHODS FOR THE PREVENTION AND TREATMENT OF GRAFT-VERSUS-HOST DISEASE (GVHD)

EDITED BY: Dietlinde Wolf, Evelyn Ullrich and Andreas Beilhack
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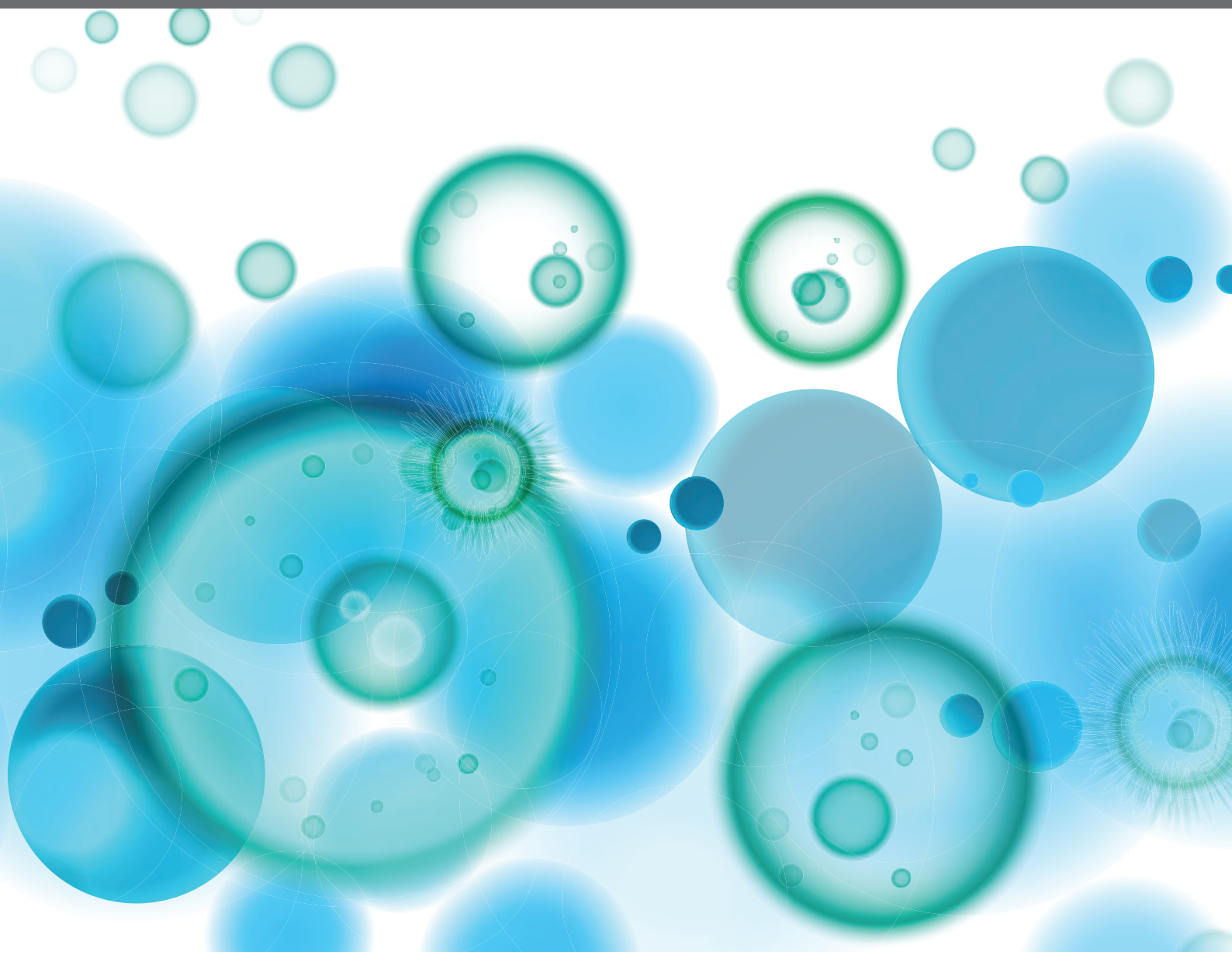

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# NOVEL AND IMPROVED METHODS FOR THE PREVENTION AND TREATMENT OF GRAFT-VERSUS-HOST DISEASE (GVHD)

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# Table of Contents

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EDITORIAL
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# Editorial: Novel and Improved Methods for the Prevention and Treatment of Graft-Versus-Host Disease (GVHD)

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**Keywords: allogeneic hematopoietic stem cell transplantation (aHSCT), acute and chronic graft versus host disease (a/c GVHD), GVL, metabolism, microbiome, cell therapy**

## Editorial on the Research Topic

## Novel and Improved Methods for the Prevention and Treatment of Graft-Versus-Host Disease (GVHD)

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For patients with a variety of severe diseases, including primarily hematopoietic malignancies, immunodeficiency syndromes, and genetic disorders, allogeneic hematopoietic stem cell transplantation (aHSCT) represents a potentially curative therapeutic approach. In this context, despite significant progress in the optimization of aHSCT, the development of graft-versus-host disease (GVHD) remains a challenge to long-term transplant success after aHSCT. It is associated with significant morbidity and mortality and is the major cause of non-relapse mortality.

GVHD occurs when donor T cells are primed by recipient antigens subsequently eliciting an inflammatory response against the host. Clinically, two types of GVHD are distinguishable: an acute form (aGVHD), and a chronic form (cGVHD). In brief, the main characteristics: aGVHD occurs in 30-50% of aHSCTs and is a multi-organ disorder resulting from inflammatory cytokines and donor T cells which primarily damage skin, liver, gastrointestinal tract, and eye. cGVHD, with a prevalence of 30-70% of aHSCTs, is induced by T and B cells resulting in a heterogeneous immunological complication affecting virtually every organ.

Traditionally, broad immune-suppressive drugs (with considerable toxicities) including calcineurin inhibitors (CNI) (cyclosporin or tacrolimus), together with methotrexate or mycophenolate mofetil (MMF), and mTOR inhibitors (Sirolimus/Rapamycin) are used as GVHD prophylaxis. But despite first success reports, significant GVHD still occurs with these drugs. Other prophylaxis strategies like pre-transplant anti-thymocyte globulin (ATG) are effective in reducing severe GVHD but have no survival benefits and steroids have serious side effects.

One of the most critical challenges in aHSCT is the development of less toxic and more targeted therapies that maintain the graft-versus-leukemia/tumor (GVL/T) effect but suppress GVHD while facilitating enhanced immune reconstitution relative to existing strategies. Recently, several prophylaxis strategies for GVHD have been developed and others are currently in development,

including, for example in the case of haploidentical HSCT, post-transplant cyclophosphamide (PTCy), which seem to be very promising.

In the frame of this specific Research Topic, we aimed to collect recent developments of innovative methods for both prevention and treatment of GVHD, without impairment of GVL. In 8 original research articles and 4 reviews, this edition provides a deep insight into the role of the microbiome and metabolism as well as recent advances of small molecule and cell therapy development.

We are glad, that experts in the field highlight the recent progress in the broad field of immune cell metabolism with two comprehensive reviews. Mohamed et al., summarize metabolic pathways contributing to GVHD and discuss metabolic targets for acute and chronic GVHD in immune and non-immune cell as well as the immunomodulatory function of microbial metabolites. Furthermore, they examine the metabolic effects of co-inhibitory pathway blockade (PD-1) and cellular therapies (Tregs/MSCs/Bregs) in aHSCT. The mini review by Karl et al., provides an overview of metabolic T cell alterations in GVHD and illustrates the impact of conventional GVHD therapy on T cell metabolism.

Recent studies have shown the association of microbiome dysbiosis and aGVHD. Here, primary research by Ghimire et al., investigates the role of G-protein coupled receptors (GPR43 and GPR109A) which engage microbial derived metabolites, like short chain fatty acids, in the mitigation of GVHD in intestinal biopsies from patients after allo-HSCT. A second study by Heidrich et al., describes an association of dental biofilm microbiota dysbiosis with the risk of aGVHD.

In the context of cell therapy development, the biological relevance of T helper cell lineage defining transcription factors as potential targets for GVHD therapy has been delineated in a review article by Campe and Ullrich. Moreover, Agbogan et al., explore the immunomodulatory effect of adoptively transferred CpG-activated B cell progenitors to alleviate GVHD symptoms. In addition, Scheurer et al., describe an *in vitro* generated sub-population of CD11b+CD11c+ myeloid-derived suppressor cells (MDSCs) as potent immune modulators leading to the prevention of GVHD without negatively affecting tumor cytotoxicity. Another innovative and attractive strategy using CRISPR/Cas9 has been described by Majumder et al., for genetical engineering of naïve T cells pre transplant as a method for GVHD prevention in a major murine mismatch model.

In addition, the recent therapeutic advances in the area of drug development, e.g. small molecules and antibodies, are also addressed. Braun and Zeiser thoroughly review the role of kinase inhibition as novel treatment strategies for acute and chronic GvHD after allo-HCT. Thangavelu et al., evaluate the efficacy of a novel agonist of the retinoic X receptor (RXR), IRX4204, to treat cGVHD in two complementary murine models with bronchiolitis obliterans or sclerodermatous manifestations. Primary research by Matos et al., analyzes a possible association of anti-thymocyte globulin (ATG) treatment and serum levels of 25-hydroxyvitamin D3 and 1,25-dihydroxyvitamin D3 in 4 HSCT cohorts with different vitamin D3 supplementation. Lastly, Hadjis et al., characterize post-transplant cyclophosphamide as superior in ameliorating pre-clinical GVHD compared to five other optimally dosed chemotherapeutics (methotrexate, bendamustine, paclitaxel, vincristine, and cytarabine) that vary in mechanisms of action and drug resistance.

Finally, this Research Topic makes us again aware of how complex the regulation of GVDH is and in which fragile balance between GVHD and GVL patients after aHSCT find themselves. We are aware that this issue can only compile a first selection of innovative findings and treatment strategies that are currently being developed for the prevention and treatment of GVHD.

GVHD biology and treatment remains a field that is always influenced by current research developments and new advances can be expected in a short time. Therefore, we will continue to monitor the field and provide updates to the authorship of Frontiers in Immunology.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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ORIGINAL RESEARCH
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# Dental Biofilm Microbiota Dysbiosis Is Associated With the Risk of Acute Graft-*Versus*-Host Disease After Allogeneic Hematopoietic Stem Cell Transplantation

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Acute graft-*versus*-host disease (aGVHD) is one of the major causes of death after allogeneic hematopoietic stem cell transplantation (allo-HSCT). Recently, aGVHD onset was linked to intestinal microbiota (IM) dysbiosis. However, other bacterial-rich gastrointestinal sites, such as the mouth, which hosts several distinctive microbiotas, may also impact the risk of GVHD. The dental biofilm microbiota (DBM) is highly diverse and, like the IM, interacts with host cells and modulates immune homeostasis. We characterized changes in the DBM of patients during allo-HSCT and evaluated whether the DBM could be associated with the risk of aGVHD. DBM dysbiosis during allo-HSCT was marked by a gradual loss of bacterial diversity and changes in DBM genera composition, with commensal genera reductions and potentially pathogenic bacteria overgrowths. High *Streptococcus* and high *Corynebacterium* relative abundance at preconditioning were associated with a higher risk of aGVHD (67% *vs.* 33%; HR = 2.89, P = 0.04 and 73% *vs.* 37%; HR = 2.74, P = 0.04, respectively), while high *Veillonella* relative abundance was associated with a lower risk of aGVHD (27% *vs.* 73%; HR = 0.24, P < 0.01). *Enterococcus faecalis* bloom during allo-HSCT was observed in 17% of allo-HSCT recipients and was associated with a higher risk of aGVHD (100% *vs.* 40%; HR = 4.07, P < 0.001) and severe aGVHD (60% *vs.* 12%; HR = 6.82, P = 0.01). To the best of our knowledge, this is the first study demonstrating that DBM dysbiosis is associated with the aGVHD risk after allo-HSCT.

**Keywords: oral microbiota, supragingival plaque, microbiome dysbiosis, acute GVHD, allogeneic HSCT, bone marrow transplant**

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## INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is the only curative treatment for several hematologic diseases. However, allo-HSCT recipients may experience potentially fatal complications, such as infections and graft-*versus*-host disease (GVHD) (1).

Acute GVHD (aGVHD) is a clinical syndrome characterized by maculopapular rash, hyperbilirubinemia, anorexia, diarrhea and abdominal pain (2). The incidence of aGVHD grade II-IV is 30-40% at day 100 (3). During transplantation, chemotherapy, radiotherapy, and infection can damage host cells, releasing sterile damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) into the extracellular milieu. DAMPs and PAMPs activate donor T cells leading to a proinflammatory state. Simultaneously, donor regulatory T cells, myeloid-derived suppressor cells and tolerogenic dendritic cells are activated, counterbalancing the inflammation as an anti-inflammatory response. An imbalance in these events towards the proinflammatory state may result in aGVHD (4).

In addition to the graft source and the intensity of the conditioning regimen (4), the intestinal microbiota (IM) composition was shown to be associated with the risk and intensity of aGVHD. Loss of IM diversity has been observed during the pre- and post-transplantation period (5), and low microbiota diversity at the time of stem cell engraftment has been associated with a higher risk of severe aGVHD (5) and transplant-related death (6).

Two non-exclusive ecological events can explain the link between loss of bacterial diversity and aGVHD risk: absence or loss of protective commensal bacterial species and sudden expansion (also known as bloom) of opportunistic pathogenic bacteria. Both events have been independently linked to aGVHD development. For instance, a higher abundance of commensal bacteria from the *Blautia* genus in the IM after allo-HSCT has been associated with reduced GVHD-related mortality and improved overall survival (7, 8). On the other hand, a shift in IM leading to the dominance of bacteria from the *Enterococcus* genus occurs more prominently in allo-HSCT recipients developing aGVHD (9), and it is associated with increased GVHD-related mortality (10).

Recent studies have shown that bacteria inhabiting the oral cavity can translocate to the gut (11) and drive IM dysbiosis (12). However, direct evaluation of the effect of allo-HSCT on the oral microbiota (OM) and the influence of OM dysbiosis on aGVHD risk have not been performed. To further understand the impact of gastrointestinal bacterial communities on aGVHD development following allo-HSCT, it would be crucial to extend the scope of these analyses to the OM.

The OM comprises over 700 bacterial species that stick to surfaces of the mouth, forming biofilms (13). The dental biofilm microbiota (DBM), in particular, is among the richest and most diverse and, like the IM, interacts with host cells and modulates immune homeostasis (14). In this study, we characterized changes of the DBM in patients during allo-HSCT and evaluated whether alterations in DBM diversity and composition could be associated with the risk of aGVHD.

## MATERIALS AND METHODS

### Sample Collection and Oral Care Protocol

Supragingival biofilm samples were collected from patients who underwent allo-HSCT. Samples were collected with sterile swabs at three phases during allo-HSCT: before the conditioning regimen (preconditioning), at aplasia and at engraftment. All patients were requested not to perform oral hygiene for at least 6h before sample collection. All patients were examined by an oral medicine specialist for potential infections and followed the same protocol for oral mucositis prophylaxis with photobiomodulation and oral hygiene with fluoride toothpaste and 0.12% chlorhexidine mouthwash. Informed consent was obtained from all participants prior to sample collection. The study was approved by the Institutional Ethics Committee (Protocol #1.414.217), in line with the Declaration of Helsinki.

### DNA Extraction and Sequencing

Bacterial cells were recovered from swabs by vortexing in TE buffer supplemented with PureLink RNAse A (Thermo Fisher Scientific, Waltham, MA, USA). DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Next, 12.5 ng of total DNA and pre-validated primers (15) were used to amplify 16S rRNA hypervariable regions V3–V4. Amplicons were sequenced as described elsewhere (16) on the MiSeq platform (Illumina, San Diego, CA, USA).

### Bioinformatics Analyses

Reads were demultiplexed and primer sequences were removed using the MiSeq Reporter software. Read processing was carried out within the QIIME 2 (*Quantitative Insights Into Microbial Ecology 2*) framework (17). Briefly, forward and reverse sequences were filtered for quality and bimeras, denoised, and merged into consensus sequences with the DADA2 pipeline (18), generating unique amplicon sequencing variants (ASVs). ASVs were further filtered for chimeric sequences using the SILVA database (19) and UCHIME (20), resulting in a total of 6 434 516 high-quality 16S rRNA sequences, with the median number of sequences obtained per sample being 58 867 (range: 2 153 - 240 734). Afterwards, ASVs were taxonomically assigned using the SILVA database and VSEARCH tool (21).

### Microbiota and Statistical Analyses

As determined by per sample alpha diversity rarefaction curves, <12 500 reads samples were considered defective and excluded. To adjust for differences in library sizes, the remaining samples were rarefied to 14 157 reads before calculating alpha diversity indexes (Shannon and Gini-Simpson indexes and the number of observed ASVs as a proxy for species richness) with the QIIME 2 *q2-diversity* plugin. Alpha diversity across transplantation phases was compared with the Mann-Whitney

U test. The relative abundance of each genus was calculated with the QIIME 2 *q2-taxa* plugin. Differentially abundant genera across transplantation phases were identified using ANCOM (22). ANCOM W represents the proportion of null hypotheses rejected when subtesting the differential abundance of a genus normalized by the abundance of each one of the genera in the dataset. W > 0.7 was considered as statistically significant. Cumulative incidence (CMI) rates for aGVHD (grade II to IV) and severe aGVHD (grade III and IV) were calculated with death as a competing event. Relative risks for developing aGVHD and severe aGVHD were estimated using the Fine-Gray risk regression model and adjusted for graft source and intensity of the conditioning regimen. Relative risks are presented as hazard ratios with 95% CIs and two-tailed P-values. R software (version 3.6.2) and the statistical package *cmprsk* (version 2.2.9) were used for statistical analyses.

# RESULTS

## Patient Characteristics

A total of 30 patients who underwent allo-HSCT for hematologic disorders at Hospital Sírio-Libanês between January 2016 and April 2018 were consecutively enrolled in our study. Patient clinical characteristics are summarized in **Table 1**. The most common underlying disease was acute leukemia (60%). The majority of patients received reduced-intensity conditioning (60%) and grafts from peripheral blood (67%).

**TABLE 1** | Clinical characteristics of study patients.

| | n = 30 |
|---|---|
| **Sex (Male)** | 16 (53%) |
| **Age in years (median, range)** | 50 (19-73) |
| **Underlying disease*** | |
| Acute leukemia | 18 (60%) |
| Other | 12 (40%) |
| **Conditioning intensity** | |
| Reduced intensity | 18 (60%) |
| **Total body irradiation** | 11 (37%) |
| **Pre-transplant T-cell depletion** | 15 (50%) |
| **Graft source** | |
| Bone marrow | 10 (33%) |
| Peripheral blood | 20 (67%) |
| **Donor** | |
| Matched sibling | 9 (30%) |
| Haploidentical | 10 (33%) |
| Matched unrelated | 9 (30%) |
| Mismatched unrelated | 2 (7%) |
| **GVHD prophylaxis** | |
| MMF + CsA | 11 (37%) |
| MTX + CsA | 10 (33%) |
| MMF + CsA + PTCy | 9 (30%) |
| **Follow-up in months (median, range)** | 37 (25-46) |

*HCT-CI, Hematopoietic cell transplantation-specific comorbidity index; MMF, mycophenolate mofetil; MTX, methotrexate; CsA, cyclosporin A; PTCy, post-transplant cyclophosphamide. *Acute leukemia: 11 acute myeloid leukemia and 7 acute lymphocytic leukemia cases; other: 5 non-Hodgkin lymphoma, 4 myelodysplastic syndrome, 1 chronic myeloid leukemia, 1 chronic lymphocytic leukemia and 1 multiple myeloma cases.*

The standard antimicrobial prophylaxis in our institution included oral levofloxacin, antiviral prophylaxis with acyclovir or valacyclovir, and antifungal prophylaxis with echinocandins or azoles according to the patient's risk of fungal infection. In addition, cephalosporin and antibiotics for anaerobic bacteria (metronidazole, meropenem or piperacillin/tazobactam) were administered to 70% and 57% of patients, respectively.

aGVHD was diagnosed and classified according to the Glucksberg grading system (23). Fifteen patients developed grade II-IV aGVHD and, of those, 6 developed severe aGVHD (grade III-IV). None of this cohort's clinical characteristics, including graft source, conditioning regimen, GVHD prophylaxis and antibiotics usage, was significantly associated with the risk of aGVHD (**Table S1**).

## Dental Biofilm Microbiota Dysbiosis During Allo-HSCT

Supragingival biofilm samples were collected for bacterial profiling at preconditioning, aplasia, and engraftment to characterize changes in DBM during allo-HSCT. Three engraftment samples were excluded from downstream analyses due to insufficient high-quality reads.

DBM alpha diversity was assessed using the Shannon index. We observed a statistically significant decrease in DBM alpha diversity during allo-HSCT, with engraftment samples presenting the lowest overall bacterial diversity (median at each collection phase: 4.15, 3.39, and 2.75, respectively; **Figure 1A**). A similar decrease in alpha diversity was observed when using the Gini-Simpson index (**Figure S1A**) or the number of observed ASVs as a proxy for species richness (**Figure S1B**).

Marked changes in DBM genera composition were observed for all patients during allo-HSCT (**Figure S2**). As expected, several dental biofilm commensal genera were detected at a high average relative abundance at preconditioning, including *Streptococcus* (19.5%), *Veillonella* (18.4%), *Actinomyces* (6.3%), and *Capnocytophaga* (6.1%) (**Figure 1B**). However, their average relative abundance decreased during allo-HSCT. Likewise, we observed an increase in the average relative abundance of potentially pathogenic genera, such as *Enterococcus* and *Lactobacillus* (**Figure 1B**).

For a more quantitative assessment of DBM changes during allo-HSCT, we compared genera abundances at preconditioning and engraftment using the ANCOM test (**Figure 1C**). The most statistically significant differences in abundance were observed for *Enterococcus*, *Lactobacillus*, and *Mycoplasma*, confirming the expansion of these potentially pathogenic genera in DBM during allo-HSCT. We also observed statistically significant (although less pronounced in terms of relative abundance change) decreases in commensal genera (**Figure 1C**).

## Dental Biofilm Microbiota Diversity and aGVHD Risk

Patients were stratified into two equal-sized groups (high and low-diversity groups) by the entire cohort's median alpha diversity value to evaluate the association between DBM diversity and aGVHD risk. Using the Shannon diversity index,

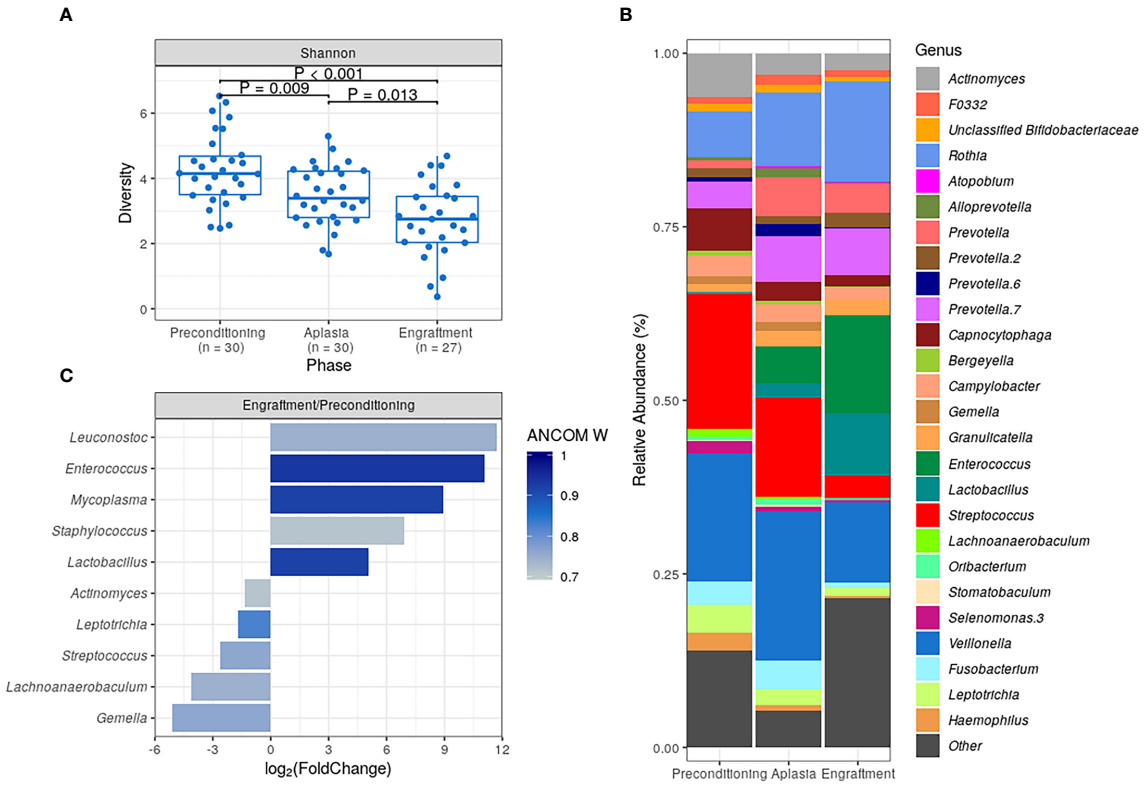


**FIGURE 1** | Characterization of dental biofilm microbiota (DBM) during allogeneic hematopoietic stem cell transplantation. **(A)** DBM alpha diversity (Shannon) boxplots at preconditioning (n = 30), aplasia (n = 30) and engraftment (n = 27). Mann-Whitney U test was used with the preconditioning as the reference for comparisons. The boxes highlight the median value and cover the 25th and 75th percentiles, with whiskers extending to the more extreme value within 1.5 times the length of the box. **(B)** Average DBM genera relative abundance composition across transplantation phases. Only genera with at least 0.1% relative abundance in at least 25% study samples are shown. Taxa are sorted based on taxonomic relatedness. **(C)** Significant genera relative abundance variations from preconditioning to engraftment according to ANCOM test (W > 0.7). $Log_2$(Fold Change) for the average relative abundance variation (Engraftment/Preconditioning) is shown.

DBM diversity showed no association with the risk of aGVHD at preconditioning, aplasia, or engraftment (**Figures 2A–C** and **Table 2**). Similar results were obtained when using the Gini-Simpson diversity index or the number of observed ASVs as a proxy for species richness (**Figure S3**).

## Dental Biofilm Microbiota Composition and aGVHD Risk

We then evaluated whether the abundance of specific genera at preconditioning, aplasia, or engraftment was associated with the risk of aGVHD (**Figure 3**). Only genera present at relative abundance ≥ 0.1% in at least 25% of the samples were considered for these analyses. Patients were stratified into two equal-sized groups (high and low relative abundance groups) by the median relative abundance observed in the entire cohort of each genus. *Veillonella*, *Streptococcus*, and *Corynebacterium* at preconditioning were significantly associated with the risk of aGVHD. We did not observe a similar association between the relative abundance of these or any other genus with the risk of aGVHD at aplasia or engraftment (**Figure 3A**).

Patients with high *Veillonella* relative abundance at preconditioning had a lower CMI of aGVHD (27% *vs.* 73%; HR = 0.24, 95% CI: 0.08–0.7, $P = 0.009$; **Figure 3B** and **Table 2**). This association remained significant after adjusting for graft source and intensity of the conditioning regimen (adjusted-HR = 0.21, 95% CI: 0.07–0.65, $P = 0.006$, **Table 2**). Patients with high *Streptococcus* or *Corynebacterium* relative abundance at preconditioning had a higher CMI of aGVHD (67% *vs.* 33%; HR = 2.89, 95% CI: 1.07–7.79, $P = 0.036$ and 73% *vs.* 37%; HR = 2.74, 95% CI: 1.05–7.15, $P = 0.04$, respectively; **Figures 3C, D** and **Table 2**). However, only *Streptococcus* remained significantly associated with the risk of aGVHD after adjusting for graft source and intensity of the conditioning regimen (adjusted-HR = 3.17, 95% CI: 1.12–9.01, $P = 0.03$, **Table 2**).

*Veillonella* and *Streptococcus* showed the highest average relative abundance at preconditioning (**Figure 1B**). Given their overall high relative abundance and an inverse association with the risk of aGVHD, we next evaluated whether the *Veillonella*/*Streptococcus* ratio at preconditioning was associated with the risk of aGVHD. Patients with a *Veillonella*/*Streptococcus* ratio >1 at preconditioning had a lower CMI of aGVHD (29% *vs.* 77%; HR = 0.23, 95% CI: 0.08–0.62, $P = 0.004$; **Figure 3E** and **Table 2**). Interestingly, the association between the *Veillonella*/*Streptococcus* ratio at preconditioning and aGVHD risk was

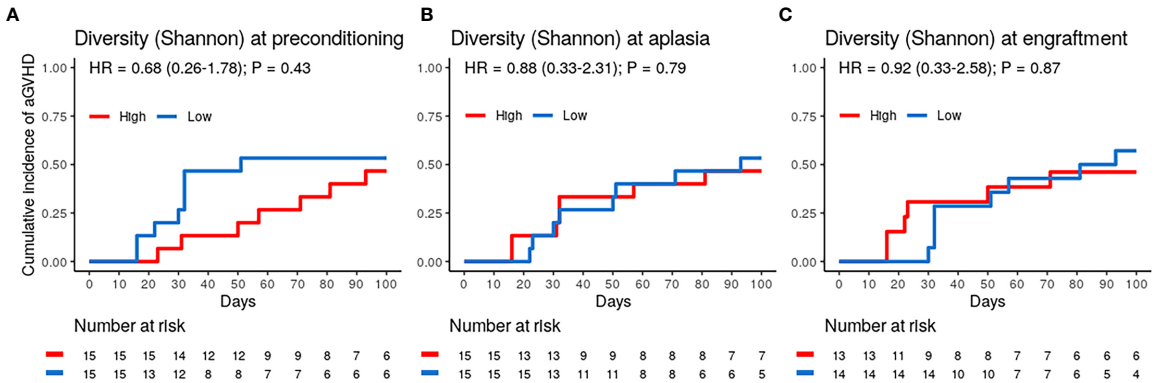


**FIGURE 2** | Dental biofilm microbiota alpha diversity is not associated with the risk of acute graft-versus-host disease (aGVHD). **(A–C)** Cumulative incidence of aGVHD with patients stratified by Shannon diversity index (High *vs.* Low) at preconditioning (**A**; n = 30), aplasia (**B**; n = 30) or engraftment (**C**; n = 27).

**TABLE 2** | Univariate (non-adjusted) and adjusted competing risk analyses for the association of acute graft-*versus*-host disease with relevant microbiota variables.

| | Non-adjusted | | Adjusted | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | ***Veillonella* at P** | | ***Streptococcus* at P** | | ***Corynebacterium* at P** | | **Ratio at P** | | ***E.faecalis* bloom** | |
| | **HR (95% CI)** | **P-value** | **HR (95% CI)** | **P-value** | **HR (95% CI)** | **P-value** | **HR (95% CI)** | **P-value** | **HR (95% CI)** | **P-value** | **HR (95% CI)** | **P-value** |
| Graft source (Bone Marrow) | 0.95 (0.35-2.63) | 0.92 | 1.42 (0.43-9.03) | 0.38 | 0.75 (0.23-2.46) | 0.64 | 1.42 (0.40-5.04) | 0.59 | 0.78 (0.25-2.46) | 0.67 | 1.63 (0.42-6.35) | 0.49 |
| Conditioning intensity (Myeloablative) | 0.74 (0.26-2.17) | 0.59 | 0.50 (0.11-2.32) | 0.37 | 0.79 (0.24-2.61) | 0.7 | 0.79 (0.20-3.04) | 0.73 | 0.92 (0.27-3.16) | 0.89 | 0.94 (0.24-3.61) | 0.92 |
| Diversity (Shannon) at P (High *vs.* Low) | 0.68 (0.26-1.78) | 0.43 | – | – | – | – | – | – | – | – | – | – |
| Diversity (Shannon) at A (High *vs.* Low) | 0.88 (0.33-2.31) | 0.79 | – | – | – | – | – | – | – | – | – | – |
| Diversity (Shannon) at E (High *vs.* Low) | 0.92 (0.33-2.58) | 0.87 | – | – | – | – | – | – | – | – | – | – |
| ***Veillonella* at P (High *vs.* Low)** | 0.24 (0.08-0.70) | **0.009** | 0.21 (0.07-0.65) | **0.006** | – | – | – | – | – | – | – | – |
| ***Streptococcus* at P (High *vs.* Low)** | 2.89 (1.07-7.79) | **0.036** | – | – | 3.17 (1.12–9.01) | **0.03** | – | – | – | – | – | – |
| ***Corynebacterium* at P (High *vs.* Low)** | 2.74 (1.05-7.15) | **0.04** | – | – | – | – | 2.79 (0.99-7.9) | 0.053 | – | – | – | – |
| **Ratio at P (>1 *vs.* ≤1)** | 0.23 (0.08-0.62) | **0.004** | – | – | – | – | – | – | 0.22 (0.08-0.64) | **0.005** | – | – |
| Ratio at A (>1 *vs.* ≤1) | 0.45 (0.16-1.23) | 0.12 | – | – | – | – | – | – | – | – | – | – |
| Ratio at E (>1 *vs.* ≤1) | 0.73 (0.27-1.98) | 0.54 | – | – | – | – | – | – | – | – | – | – |
| Any genus bloom (Yes *vs.* No) | 2.29 (0.63-2.36) | 0.21 | – | – | – | – | – | – | – | – | – | – |
| ***E. faecalis* bloom (Yes *vs.* No)** | 4.07 (1.82-9.14) | **0.0007** | – | – | – | – | – | – | – | – | 4.90 (1.66-14.5) | **0.004** |

*Each multivariate model adjusts for graft source and conditioning intensity. Statistically significant associations are marked in bold. HR, Hazard ratio; CI, Confidence interval; P, preconditioning; A, aplasia; E, engraftment.*

stronger than the association observed for each genus separately and remained significant after adjusting for graft source and intensity of the conditioning regimen (adjusted-HR = 0.22, 95% CI: 0.08–0.64, *P*=0.005, **Table 2**). The *Veillonella*/*Streptococcus* ratio at aplasia or engraftment was not associated with the risk of aGVHD (**Table 2**).

## *Enterococcus faecalis* Bloom and aGVHD Risk

Finally, we analyzed whether the blooming of potentially pathogenic genera observed during allo-HSCT was associated with the risk of aGVHD. For these analyses, bloom was defined as the sudden expansion of a particular genus from near absence

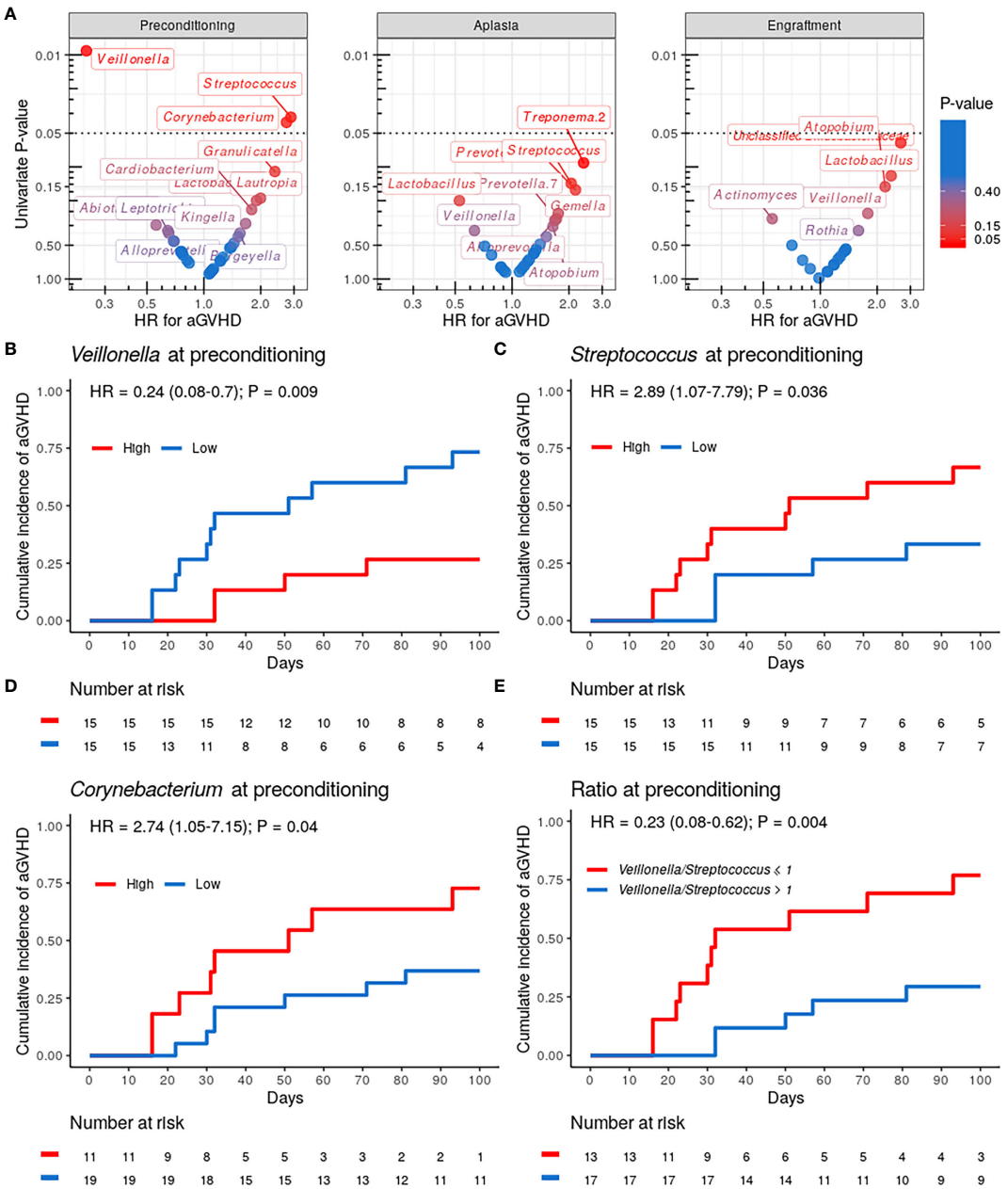


**FIGURE 3** | Specific genera relative abundance at preconditioning are associated with the risk of acute graft-*versus*-host disease (aGVHD). **(A)** Volcano plot for the univariate competing risk analysis for the association of aGVHD with genera relative abundance (hazard ratio *vs.* P-value) at preconditioning (left), aplasia (center) and engraftment (right). Only genera with ≥0.1% relative abundance in at least 25% of samples at a given phase were evaluated. Genera with P-value < 0.4 for the association are indicated explicitly. **(B–D)** Cumulative incidence of aGVHD with patients (n = 30) stratified by either *Veillonella* **(B)**, *Streptococcus* **(C)** or *Corynebacterium* **(D)** relative abundance at preconditioning (High *vs.* Low). **(E)** Cumulative incidence of aGVHD with patients (n = 30) stratified by *Veillonella*/*Streptococcus* relative abundance ratio at preconditioning (>1 *vs.* ≤1).

(relative abundance <1% at preconditioning) to dominance (relative abundance ≥30% at aplasia or engraftment). Analyzing variations in genera relative abundance during allo-HSCT, we observed 23 blooms, involving 12 different genera and affecting a total of 20 patients. Three patients experienced more than one blooming event (**Figure S4**). Patients experiencing any genus bloom (n = 20) did not have altered aGVHD risk (**Table 2**). *Enterococcus* bloom was the most frequent event (**Figure 4A**), observed in 20% of the patients undergoing allo-HSCT. For all patients experiencing *Enterococcus* bloom except one, the phenomenon was attributed exclusively to *Enterococcus faecalis* expansion (**Figure 4B**). There was no association

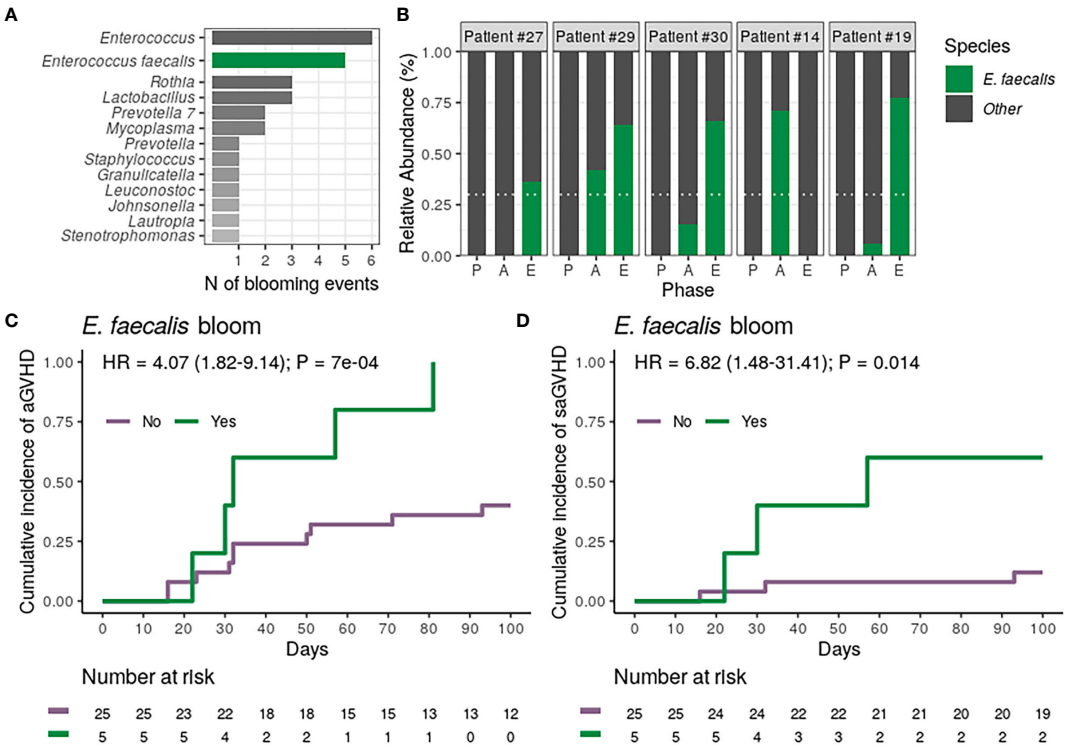


**FIGURE 4** | Dental biofilm *Enterococcus faecalis* bloom during allogeneic hematopoietic stem cell transplantation is associated with a higher risk of acute graft-*versus*-host disease (aGVHD) and severe aGVHD (saGVHD). **(A)** Number of observed blooming events per genera in all patients (n = 30). The number of *Enterococcus* blooms caused exclusively by *Enterococcus faecalis* is indicated. **(B)** Relative abundance of *Enterococcus faecalis* across transplantation phases for all patients experiencing *Enterococcus faecalis* bloom (n = 5). Patients are sorted based on the highest *Enterococcus faecalis* relative abundance observed per patient. White horizontal dashed line indicates dominance threshold. P, Preconditioning; A, Aplasia; E, Engraftment. **(C, D)** Cumulative incidence of aGVHD **(C)** or saGVHD **(D)** with patients (n = 30) stratified by *Enterococcus faecalis* bloom occurrence (No *vs.* Yes).

between *E. faecalis* bloom and cephalosporin (Fisher's exact test, $P = 0.29$) or antibiotic for anaerobic bacteria usage (Fisher's exact test, $P = 1$).

We next tested whether the occurrence of *E. faecalis* bloom was associated with the risk of aGVHD. All patients experiencing *E. faecalis* bloom developed aGVHD, and *E. faecalis* bloom was strongly associated with a higher CMI of aGVHD (100% *vs.* 40%; HR = 4.07, 95% CI: 1.82–9.14, $P = 0.0007$; **Figure 4C** and **Table 2**). This association remained significant after adjusting for graft source and intensity of the conditioning regimen (adjusted-HR = 4.90, 95% CI: 1.66–14.50, $P = 0.004$, **Table 2**). Notably, CMI of severe aGVHD (grade III-IV) was higher in patients experiencing *E. faecalis* bloom (60% *vs.* 12%; HR = 6.82, 95% CI: 1.48–31.41, $P = 0.014$; **Figure 4D**; **Table 2**), revealing a direct association between DBM *E. faecalis* bloom and aGVHD risk and grade.

## DISCUSSION

In our study, we describe, for the first time using high-throughput 16S rRNA sequencing, changes in DBM diversity and composition in 30 patients undergoing allo-HSCT. As observed for IM, DBM dysbiosis during allo-HSCT was marked by a gradual loss of bacterial diversity, with engraftment samples presenting the lowest overall bacterial diversity. Like for the IM, we also observed significant changes in DBM genera composition, with a decrease in the abundance of commensal core DBM genera, such as *Streptococcus* and *Actinomyces* (the only genera that can adhere to the tooth surface to start ordinary DB formation) (24), and overgrowths of potentially pathogenic bacteria, such as *Enterococcus*, *Lactobacillus*, and *Mycoplasma*. Most importantly, we observed that DBM genera relative abundance at preconditioning and changes in DBM composition during allo-HSCT (namely, *E. faecalis* bloom) were both predictive of aGVHD risk after allo-HSCT. There was no association between these aGVHD-associated microbiota variables and other allo-HSCT outcomes, including chronic GVHD (**Table S2**), as diagnosed in accordance with the NIH 2014 consensus (25).

aGVHD is a major cause of non-relapse mortality following allo-HSCT, with a one-year survival rate for patients developing severe aGVHD of only 40% (26). First-line therapy for aGVHD is based on corticosteroids, with response rates that vary between

40 and 70% (27). In this scenario, identifying biomarkers capable of predicting aGVHD risk and developing preventive therapies are critical.

Recently, the IM composition has been analyzed as a biomarker for clinical outcomes in allo-HSCT recipients, including the development of aGVHD (5, 7). Moreover, microbiota-based therapeutic interventions, including microbiota-driven antibiotics selection, alternative dietary regimens (including probiotics/prebiotics usage) and fecal microbiota transplantation have been proposed to prevent and treat aGVHD (28–32).

Like the IM, the OM plays an essential role in maintaining local and systemic health. Dental biofilm (DB) bacteria, as opposed to other shedding surface-living bacteria in the oral cavity, can adhere to hard surfaces and coaggregate (33), allowing the assembly of an organized three-dimensional structure, which confers DBM its distinctive ecological properties. The DBM interacts directly with host immune cells and modulates immune homeostasis (14). Moreover, DBM can also act as a microbial reservoir for systemic diseases. DBM dysbiosis can trigger local inflammation, destruction of surrounding periodontal tissue, and systemic translocation of oral microbes (24). The influence of the OM in systemic diseases such as colorectal cancer (34) and arthritis (35) has been increasingly studied. However, in the allo-HSCT context, studies are still limited and have focused mainly on the saliva and the tongue microbiota (36–39).

Loss of bacterial diversity in the salivary microbiota of patients undergoing allo-HSCT has been previously described and associated with oral mucositis (36). Likewise, a steep decline in the tongue microbiota diversity was observed in severe aplastic anemia patients from preconditioning to the day of transplantation (37). On the other hand, no appreciable changes in OM during allo-HSCT were observed in an additional study evaluating 4 different oral sites (buccal mucosa, saliva, tongue, and DB) (38). However, this latter study used a low-resolution methodology (microarray) for microbiota characterization in a small number of patients (n = 11). Noteworthy, a single study evaluated the association between OM and allo-HSCT outcomes (39). Allo-HSCT recipients showed a less diverse and distinct tongue microbiota on the day of transplantation than that of community-dwelling adults. In this study, the presence of the non-commensal bacteria *Staphylococcus haemolyticus* and/or *Ralstonia pickettii* in the tongue microbiota was significantly associated with lower overall survival after allo-HSCT, but not with aGVHD.

Out of the many allo-HSCT outcomes evaluated so far (40), aGVHD onset has the clearest causal connection to the IM (28, 29, 40). Briefly, it has been shown that the loss of commensal bacteria (especially SCFA-producing Clostridia species) during the conditioning regimen reduces the intestinal concentration of butyrate and indole-3-aldehyde (41, 42). Low levels of these metabolites compromise mucosal integrity (42, 43), promoting extravasation of bacterial lipopolysaccharide and activation of donor reactive T cells (40). Additionally, *Enterococcus faecalis* might contribute to aGVHD development *via* production of metalloproteases that impair barrier function (44) and by stimulating macrophages to secrete TNF (45). Accordingly, low IM diversity at the time of stem cell engraftment (6, 7), low abundance of commensal bacteria from Clostridia class (7, 8), and intestinal enterococci dominance during allo-HSCT (10) have been all associated with worsened aGVHD-related outcomes in studies evaluating stool specimens from allo-HSCT recipients (28, 29, 40).

In our study, DBM diversity was not associated with the risk of aGVHD in any transplantation phase evaluated, which is in line with a recent IM study that did not find differences in IM diversity between aGVHD groups neither pre- nor post-transplantation (46). Also, despite the presence (as expected (47)) of many Clostridia genera in DBM (such as *Oribacterium*), we did not find DBM Clostridia class members significantly associated with the risk of aGVHD. However, as for the IM, we observed a decrease in the relative abundance of several DB commensal genera during allo-HSCT, such as *Streptococcus*, *Veillonella*, *Actinomyces*, and *Capnocytophaga*, and an increase in the relative abundance of potentially pathogenic genera such as *Enterococcus* and *Lactobacillus*. Most importantly, high *Streptococcus* and high *Corynebacterium* relative abundance at preconditioning were associated with a higher risk of aGVHD, while high *Veillonella* relative abundance at preconditioning was associated with a lower risk of aGVHD.

Streptococci, corynebacteria, and veillonellae are part of the core DBM (48) and represent the 1st, 2nd and 10th most important genera in terms of relative abundance in healthy volunteers DBM, respectively (47). In our study, streptococci and veillonellae showed the highest average relative abundance at preconditioning and were both associated with the risk of aGVHD. Given their overall high relative abundance and the relative nature of the data, higher *Veillonella* relative abundance imposes lower *Streptococcus* relative abundance and vice versa. Hence, it is not possible to determine whether both genera are genuinely associated with the risk of aGVHD. Interestingly, the association between the *Veillonella/Streptococcus* ratio at preconditioning and aGVHD risk, independently of the conditioning regimen and graft source, was stronger than the association observed for each genus separately, suggesting a partial role for both genera in the observed effect.

During DB formation, bacterial early colonizers, after adhering to teeth salivary pellicles, coaggregate with other early and late colonizers, and a repeatable microbial succession takes place on the tooth surface (33). Streptococci are the most abundant microbe in DB, representing a predominant early colonizer with broad coaggregation partnerships. Streptococci and veillonellae are in close physical contact during the early phases of DB maturation (33, 49) and can grow together in a metabolic cooperation-dependent manner (33, 49). Since this interaction occurs in the early phases of DB formation (and therefore are instrumental for DB maturation), the ratio *Veillonella/Streptococcus* might be a marker of early DBM disruption associated with a higher risk of aGVHD.

Corynebacteria bridge the early biofilm members to late colonizers (48). In contradiction with the documented in the

aforementioned healthy volunteers study (47), we did not observe a high corynebacteria average relative abundance in any of the allo-HSCT phases evaluated. It is possible that the overall lower relative abundance of corynebacteria in detriment of early colonizers (such as streptococci and veillonellae) in our study may be indicative of a basal DBM disruption afflicting all allo-HSCT recipients. Alternatively, the lower relative abundance of corynebacteria may be explained by the stricter oral hygiene protocol recommended to our patients.

Finally, in our study, *E. faecalis* bloom in the DBM was observed in 17% of allo-HSCT recipients and was significantly associated with a higher risk of aGVHD and saGVHD. Noteworthy, despite recent *in vitro* evidence suggesting that high-dose of cephalosporin may promote *E. faecalis* biofilm formation (50), there was no association between cephalosporin usage and DBM *E. faecalis* bloom in the evaluated cohort.

During allo-HSCT, intestinal enterococci expansion is well documented and is linked to both aGVHD development (10) and subsequent bacteremia (51). Notably, *E. faecalis* alone exacerbates aGVHD severity in gnotobiotic mouse models (10). Our study reveals an additional site with enterococci expansion that might have systemic impacts after allo-HSCT. We can speculate that, during allo-HSCT, the dysbiotic DBM may act as an enterococci reservoir, triggering translocation to the gut and intestinal enterococci domination. This possibility is corroborated by the fact that there is intense oral bacteria translocation to the gut in hepatic cirrhosis patients (52) and that such translocations in colorectal cancer patients are negatively correlated with intestinal Clostridia bacteria presence (34). Indeed, oral bacteria translocation to the gut has been described in allo-HSCT recipients, and the presence of oral Actinobacteria and oral Firmicutes in stool samples of these patients was positively correlated with subsequent aGVHD development (5). Alternatively, DBM enterococci may have an intestinal origin, since the injury to Goblet cells during conditioning regimen was shown to induce dissemination of dominant intestinal bacteria (28). Further studies evaluating synchronously IM and DBM are necessary to decipher whether IM and DBM enterococci bloom are linked and which event precedes the other. Importantly, enterococci are present in small amounts in the healthy OM (47) but may overgrow in pathogenic/dysbiotic settings, including after solid organ transplantation (53), in a biofilm-dependent manner (54). This may explain why previous microbiota studies on soft oral sites have not reported the expansion of *Enterococcus* in allo-HSCT recipients.

Our study has many limitations. As a pioneering and exploratory work, it is single-centered and has a limited sample size. Besides, the study patients analyzed are heterogeneous and encompass several underlying diseases. Therefore, validation cohorts and multicentric prospective studies are needed to confirm our findings. We also emphasize that the associations reported herein are correlative, so that further studies on DBM during allo-HSCT that include synchronous fecal sampling and metabolomics analyses are needed to associate DBM dysbiosis with aGVHD pathophysiology.

Although patients usually receive rigorous oral health care during allo-HSCT (55), OM dysbiosis has been overlooked. Common oral care protocols already used in allo-HSCT patients to prevent and counteract oral health decay can also be used to directly (e.g. chlorhexidine mouthwash) or indirectly (e.g. photobiomodulation) modulate the OM. However, as the role of oral microbes in allo-HSCT outcomes become more prominent, complementary odontologic/pharmacologic interventions targeting specific sites and bacteria of the OM will be necessary. For instance, DBM dysbiosis could be managed by antimicrobial photodynamic therapy, which can eliminate pathogens with no risk of the emergence of drug-resistant strains (56). DBM dysbiosis could also be countervailed with the use of nanoparticles that alters DBM composition by interfering in fundamental biofilm properties such as adhesion and quorum-sensing (57, 58). These innovative approaches will be instrumental to evaluate whether early interventions to correct DBM dysbiosis can prevent aGVHD onset.

In conclusion, to our knowledge, this is the first study evaluating the DBM during allo-HSCT using a high-resolution technique. We identified markers of DBM dysbiosis during allo-HSCT. Most importantly, we showed that DBM composition during allo-HSCT may be predictive of aGVHD onset after transplantation, providing a simple and reproducible protocol for collection and analysis of allo-HSCT recipients microbiota before transplantation that may substitute fecal sampling when evaluating gastrointestinal dysbiosis and *Enterococcus* bloom.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: European Nucleotide Archive (ENA), PRJEB42862.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Hospital Sírio-Libanês (Protocol #1.414.217). The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

AC and EF designed the study. FK, PA, VH, JB, and WM-S performed the sequencing. VH and AC developed the bioinformatics pipeline. VH, JB, VM, CA-R, EF, and AC contributed to the analysis and interpretation of data. VH, JB, VM, CA-R, and AC contributed to writing the manuscript.

All authors contributed to the article and approved the submitted version.

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A manuscript regarding this work has been previously submitted to medRxiv as a preprint (59).

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.692225/full#supplementary-material

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# Rapid and Efficient Gene Editing for Direct Transplantation of Naive Murine Cas9$^+$ T Cells

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Gene editing of primary T cells is a difficult task. However, it is important for research and especially for clinical T-cell transfers. CRISPR/Cas9 is the most powerful gene-editing technique. It has to be applied to cells by either retroviral transduction or electroporation of ribonucleoprotein complexes. Only the latter is possible with resting T cells. Here, we make use of Cas9 transgenic mice and demonstrate nucleofection of pre-stimulated and, importantly, of naive CD3$^+$ T cells with guideRNA only. This proved to be rapid and efficient with no need of further selection. In the mixture of Cas9$^+$CD3$^+$ T cells, CD4$^+$ and CD8$^+$ conventional as well as regulatory T cells were targeted concurrently. IL-7 supported survival and naivety *in vitro*, but T cells were also transplantable immediately after nucleofection and elicited their function like unprocessed T cells. Accordingly, metabolic reprogramming reached normal levels within days. In a major mismatch model of GvHD, not only ablation of NFATc1 and/or NFATc2, but also of the NFAT-target gene IRF4 in naïve primary murine Cas9$^+$CD3$^+$ T cells by gRNA-only nucleofection ameliorated GvHD. However, pre-activated murine T cells could not achieve long-term protection from GvHD upon single NFATc1 or NFATc2 knockout. This emphasizes the necessity of gene-editing and transferring unstimulated human T cells during allogenic hematopoietic stem cell transplantation.

**Keywords: CRISPR/Cas9, gRNA-only, GvHD, metabolism, NFAT, naive T-cell gene editing, T-cell transfer, IRF4**

## INTRODUCTION

Until today, immunological studies depend on mouse models, which provide a manipulable systemic approach. Hence, in order to understand cause and consequence of gene function in health and disease, multiple transgenic mice have been created. This is tedious, although modern *Clustered Regularly Interspaced Short Palindromic Repeats-associated protein 9 nuclease* (CRISPR/Cas9)-mediated techniques have improved the procedure enormously (1, 2). Some insights can also be gained if transgenic cells are analyzed *in vitro* or transferred to new mice. Lately, models of T-cell

transfer additionally serve translational purposes. Prominent examples are Major histocompatibility complex (MHC, in humans also known as Human leukocyte antigen/HLA) mismatch models for graft-versus-host disease (GvHD), because they depict the inherent odds of allogeneic hematopoietic stem cell transplantation (allo-HCT). Any manipulation of T cells in GvHD models represents an idea to avoid or limit GvHD in the clinic.

To circumvent the creation of transgenic animals for such experiments, one could envisage an *in vitro* manipulation of primary T cells. However, in that case an efficient gene targeting is mandatory. Primary lymphocytes are difficult to transfect, which proves almost impossible for primary mouse T cells (3). For any success with electroporation or viral infection, usually activation is necessary. Yet, pre-activation directs the T cells towards a certain status before the transgene is expressed or an endogenous gene is inhibited and, importantly, before the T cells face the *in vivo* situation. Thus, the possibility for gene targeting of naive murine T cells is desirable, especially, when one wants to study genes involved in T-cell activation and differentiation. This, however, should be so effective that one can transfer the transgenic T cells without any further selection, enrichment and expansion.

Just recently, a technical breakthrough for efficient gene editing of primary T cells has been published (3, 4). The authors apply CRISPR/Cas9 technology. CRISPR/Cas9 is an RNA-guided endonuclease technique derived from a microbial defense system (5, 6). Target detection by base pairing ensures an extraordinary specificity. The RNA chaperone Cas9 consists of two nuclease domains, then generating a blunt-ended double strand break (DSB), the nucleation point for mutations. It allows the induction of 'indel' mutations caused by error-prone non-homologous end-joining repair (NHEJ). Indel mutations are insertions and deletions leading to frameshifts within a given coding region and consequently loss of the respective protein. In the technical paper mentioned (4), the authors transfect Cas9/target gene–specific CRISPR RNA (crRNA)/transactivating crRNA (tracrRNAs) ribonucleoproteins (RNPs) into human and mouse primary T cells. With this, they are even able to transduce non-activated T cells with high efficiency (3). Alternatively, pre-stimulated T cells from Cas9 transgenic mice can be mutated by guide (g) RNAs, the combination of crRNA and tracrRNA, which are delivered *via* retroviral transduction (7, 8). Since studies have shown that at least sole mRNA can be successfully electroporated into T cells (9), we attempted to make use of Cas9 transgenic mice (10) and to develop efficient gRNA-only delivery into naive T cells using a nucleofection technique.

If naive T cells shall be adoptively transferred after manipulation, Cas9-mediated knockout has to occur *in vivo*. In fact, T-cell receptor (TCR)-transgenic $CD8^+$ T cells were nucleofected with RNPs to target surface proteins, immediately transplanted and mice infected to challenge the transgenic TCR. This resulted in efficient knockdown of targeted proteins (11). Nevertheless, for many mouse models it will be necessary to transfer more than one subtype of T cells to understand their interplay *in vivo* as well as to mimic their involvement in human diseases. For example, T cell-mediated GvHD obstructs allo-HCT, while T cell-mediated graft-versus-leukemia effect (GvL) limits relapses of leukemia, lymphoma or multiple myeloma. Here, the ratio of CD4/CD8 T-conventional cells (Tcon) and especially the dominant suppression of GvHD over GvL by Treg cells is decisive (12, 13). We had shown with a major mismatch model for allo-HCT and GvHD that co-transfer of total $CD3^+$ T cells from NFAT-deficient mice shifts the balance towards $CD8^+$ T cells and that Tregs function well in the absence of one or two NFAT members (14, 15). NFAT (nuclear factor of activated T-cells) is a transcription factor family, which is primarily activated by TCR signaling *via* the phosphatase calcineurin and therefore restrained by the calcineurin inhibitors cyclosporin A or tacrolimus applied to patients receiving allo-HCT (16, 17).

Here, we develop a method for sole nucleofection with a combination of RNAs, i.e. 1-3 chemically modified synthetic crRNAs and one tracrRNA into murine primary naïve T cells, isolated from Cas9-expressing mice. Such manipulated T cells survived rather well, preserved their naïve phenotype, were almost indistinguishable in their metabolic reprogramming, and importantly, presented with a high knockout efficiency. Our B6.*Cas9.Cd4cre.luc*.CD90.1 mice express Cas9 from double-positive $CD4^+CD8^+$ thymocytes on in all $CD4^+$ – including $CD4^+CD25^+$ Tregs – and $CD8^+$ T cells. In addition, due to the luciferase transgene, cells from these mice are trackable by bioluminescence after transfer and recognizable by the congenic marker CD90.1. Accordingly, we performed allo-HCT together with manipulated T cells to induce acute GvHD (aGvHD). Assuring, only gRNA-transfected and CRISPR/Cas9-edited primary naïve murine T cells behaved as if naïve T cells were isolated from NFAT-deficient mice and directly transplanted. With this, we provide an easy and cost-effective method to create transgenic T cells for any adoptive T-cell transfer model, but especially to study the needs and obstacles during allo-HCT.

# MATERIAL AND METHODS

## crRNA Design and gRNA Assembly

crRNAs were selected using DESKGEN or CHOPCHOP (*Irf4*) online platform. The target area was limited to the first ~40 % of the coding sequence. Guides targeting common exons between isoforms with the highest on-target and off-target scores were selected. crRNAs were ordered from Integrated DNA Technologies in their proprietary Alt-R format. crRNA and Alt-R CRISPR-Cas9 tracrRNA (IDT) were mixed in equimolar concentration (10 µl each) in nuclease-free PCR tubes, heated at 95°C for 5 min and then cooled at RT for 10 min to anneal.

## BM and T Cell Isolation

BM cells were isolated by flushing femur and tibia bones of $Rag1^{-/-}$ mice with PBS containing 0.1 % BSA and passed through a 70-µm cell strainer. Spleens and lymph nodes were directly passed through a 70-µm cell strainer, washed with PBS containing 0.1% BSA and enriched with Mojosort Mouse CD3 T cell Negative Isolation kit (Biolegend) according to the

manufacturer's instructions (14). $CD4^+$ or $CD8^+$ T cells were isolated using negative Isolation kit (Biolegend) according to manufacturer's instructions.

## T-Cell Culture and Stimulation

T cells were cultured in RPMI media with 10% FCS (Gibco), 2 mM l-alanyl-l-glutamine (GlutaMAX; Gibco), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 55 μM β-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM Hepes (Invitrogen). Purified T cells were stimulated with plate bound 5 μg/ml anti-CD3, and soluble 1 μg/ml anti-CD28 and 10 ng/ml IL-2 for 72h in 1 million cells per ml density. Naïve $CD3^+$ T cells were either cultured with 5 ng/ml IL-7 for overnight before nucleofection or immediately nucleofected after purification.

## Nucleofection

T-cell culture media was pre-warmed in a $CO_2$ incubator. T cells (2.5 million) were washed with Ca/Mg-free PBS to remove traces of FBS and resuspended in 100 μl of Ingenio Electroporation Solution. Cells were added on 3 μl (150 pmol) crRNA-tracrRNA duplex (gRNA) in a nuclease free tube, mixed gently by pipetting and incubated at RT for 2 min. When targeting two genes, 2 μl (100 pmol) of each gRNA was used. The cell RNA mix was then transferred in a cuvette and nucleofection was performed using Lonza Nucleofector™ IIb and X-001 preset program or using Lonza 4D Nucleofector and CM137 (for stimulated cells) or DS137 (for naïve T cells). Post nucleofection, pre-warmed media was added to the cells slowly and cells were carefully transferred to the 12-well plate. Plates were incubated in the $CO_2$ incubator up to 3 days before transplantation into mice for a GvHD major mismatch model.

## Allogenic Hematopoietic Stem Cell Transplantation

BALB/c host mice ($H\text{-}2^d$ $CD90.2^+$) were conditioned by myeloablative total body irradiation (TBI) at a dose of 8.0 Gy using a Faxitron CP-160 X-ray system. Two hours after irradiation they were injected retro-orbitally with sex- and age matched $5 \times 10^6$ C57BL/6 BM cells from *Rag1*$^{-/-}$ mice ($H\text{-}2^b$ $CD90.2^+$) with or without $2.5 \times 10^6$ stimulated or $0.3\times 10^6$ naïve CRISPR/Cas9-edited T cells from B6.*Cas9.Cd4cre.luc*.CD90.1 ($H\text{-}2^b$ $CD90.1^+$) mice. Mice were given antibiotic (Baytril, Bayer) for one week to avoid opportunistic infections. Transplanted mice were assessed daily for weight loss and clinical aGvHD score adapted from Cooke et al. (14, 18, 19).

## *In Vivo* and *Ex Vivo* Bioluminescence Imaging

Mice were anesthetized by i.p. injection of 80 mg/kg body weight ketamine hydrochloride (Pfizer) and 16 mg/kg xylazine (CP Pharma). Together with anesthetics, mice were injected with 150 mg/kg D-luciferin (Biosynth). After 10 min, BLI signals of the anesthetized mice were recorded using an IVIS Spectrum Imaging system (Caliper Life Sciences). For *ex vivo* imaging of internal organs 6 d after allo-HCT, mice were injected with D-luciferin and sacrificed 10 min later. Internal organs were removed and subjected to BLI. All pictures were taken with a maximum of 5-min exposure time and analyzed with the Living Image 4.0 software (Caliper Life Sciences) (14, 19).

## Flow Cytometry Staining

Cells were washed once in FACS buffer (PBS containing 0.1% BSA) before blocking with anti-FcγRII/FcγRIII (2.4G2, BD Pharmingen). Staining of surface molecules (all Biolegend) was performed on ice using FITC-conjugated CD4 (RM4-5), CD8α (53-6.7), and CD90.1 (OX-7); PECy7-conjugated CD44 (IM7); PE-conjugated α4β7 (LPAM-1, DATK32), CD4 (RM4-5), CD8α (53-6.7), CD62L (MEL-14), CD44 and CD25 (PC61); APC conjugated CD90.1 (OX-7). Intracellular Foxp3 (FJK-16s, APC-conjugated; eBioscience), NFATc1 (anti-NFATc1 PE 7A6, Biolegend), IRF4 (3E4, APC or PB-conjugated; Biolegend) staining was performed using the Foxp3 staining kit (eBioscience) according to the manufacturer's instructions. Antibodies (all Biolegend) for intracellular cytokine staining were APC-IFN-γ (XMG1.2), FITC-TNF-α (MP6-XT22) and PE-IL-2 (JES6-5H4). Cytokine detection was performed after a 6 h *in vitro* restimulation with 12-O-tetradecanoylphorbol-13-acetate (TPA; 10 ng/mL, Sigma) plus ionomycin (5 nM, Merck Biosciences) in the presence of GolgiStop and GolgiPlug (both BD Pharmingen) using the IC Fixation Buffer kit (eBioscience). Viable cells were detected with the Zombie Aqua™ Fixable Viability Kit (Biolegend). Data were acquired on a FACSCanto II (BD Biosciences) flow cytometer and analyzed with FlowJo software (Tree Star).

## Quantitative qRT-PCR

RNA was extracted from cultured cells using Trizol (Ambion/Life Technologies) followed by cDNA synthesis with the iScript II kit (BioRad). Quantitative RT-PCR was performed with an ABI Prism 770 light cycler with the appropriate primer pairs, Sequences available in **Table S2**.

## Indel Detection

Genomic DNA was extracted from cultured cells using Omega E.Z.N.A DNA/RNA Isolation kit. PCR (initial denaturation 95°C, 3 min, denaturation 95°C, 15 sec, annealing 55°C, 30 sec, elongation 72°C, 2 min, 40 cycles, final elongation 72°C, 10 min) was performed to amplify gRNA target regions using specific forward and reverse primer. The amplified product was gel purified and cloned in TA cloning vector kit (Promega) and transformed in DH5α E.coli (Invitrogen). Colonies were individually grown in LB broth and plasmid DNA was isolated. Presence of insert was confirmed by PCR. Clones were sequenced by Sanger sequencing using Hi-Di and ABI Prism Genetic Analyzer from Applied Biosystems. For performing 'Tracking of Indels by Decomposition' (TIDE), amplified target regions were gel purified and sequenced by Sanger sequencing. Sequencing files were uploaded in TIDE DESKGEN website along with gRNA sequence and analyzed using the TIDE tool. Primer sequences available in **Table S2**.

## Metabolism Study

*Compounds:* Glucose was purchased from Agilent/Seahorse Bioscience. Oligomycin, 2-deoxyglucose (2DG), Trifluoromethoxy

carbonylcyanide phenylhydrazone (FCCP), antimycin A, and rotenone were purchased from Sigma Aldrich; Media for MST: XF Base Medium (Agilent Technologies), 2 mM sodium pyruvate, 10 mM Glucose, 2 mM L- Glutamine pH 7.4 +/- 0.05 (Sigma-Aldrich). Media for GST (All from Sigma-Aldrich): DMEM, 2 mM L- Glutamine pH 7.35 +/- 0.05. *Extracellular flux analysis:* Bioenergetics were determined as previously described (Böttcher et al., 2018). Briefly, one day prior measurements, Seahorse XFe96 culture plates (Agilent/Seahorse Bioscience) were coated with Corning™ Cell-Tak Cell and Tissue Adhesive (BD) with 0.1 M $NaHCO_3$ (Sigma Aldrich) according to the manufacturer's recommendations. A Seahorse XFe96 cartridge (Agilent/Seahorse Bioscience) was loaded with XF Calibrant solution (Agilent/Seahorse Bioscience) and incubated overnight at 37°C in a $CO_2$-free atmosphere. The next day, cells were harvested from the culture, washed in assay-specific medium according to the manufacturer's recommendations and viable cells were automatically counted on a Muse® Cell Analyzer (Luminex Corp.). The cells were seeded at a density of $2.4 \times 10^5$ T-cells per well. The ports of the Seahorse cartridge were loaded with appropriate dilutions of the following compounds (final concentrations in brackets): glucose (10 mM), oligomycin (1 μM), and 2DG (100 mM) for the GST and oligomycin (1 μM), FCCP (1.5 μM), and antimycin A/rotenone (3 μM each) for the MST. After sensor calibration, assays were run as detailed in the manufacturer's manual by recording ECAR (extracellular acidification rate) and OCR (oxygen consumption rate). Metabolic parameters were obtained from the XF Wave software (Agilent/Seahorse Biosciences) and calculated using Microsoft Excel.

## Luciferase Reporter Assay

EL-4 cells were cultured in complete RPMI containing 5 % FCS (15). They were transiently transfected with an *Irf4* promoter luciferase-reporter construct. A 836 bp Irf4 fragment, generated by PCR with *Irf4*-Pr_s 5' TTT GCT AGC CAT GAT TGA AAC TTT GGG G 3' and *Irf4*-Ex1-Nco_a 5' TTT CCA TGG TCC CAA GTT CAA GTG GTG 3', was cloned into pGL3 *via* NheI/NcoI restriction sites, thereby matching the translational start site of IRF4 with that of luciferase. Plasmids expressing constitutive active HA-NFATc2 (20) or Flag-IRF4 were co-transfected by standard DEAE Dextran. 36 h post transfection, luciferase activity was measured from the cells that were left untreated or treated with TPA (10 ng/ml, Sigma), ionomycin (5 nM, Merck Biosciences) o/n and relative light units were corrected for the transfection efficacy based on total protein concentrations. Normalized mean values of at least 3 independent experiments are depicted in relative light units as fold activation over empty vector control.

## Immunoblot

T cells of spleen and LNs were harvested from *Nfatc1*$^{caaA}$ mice (21) crossed to dLckcre (22) and activated by 2.5 μg/ml ConA (C0412 Sigma). Whole cell extracts were resolved by 10% SDS-PAGE followed by immunoblotting (23). The primary antibodies used were rabbit anti-NFATc1/αA (IG-457, ImmunoGlobe), goat anti-IRF4 (sc-6059, Santa Cruz), and mouse anti-β-actin (C-4, Santa Cruz biotechnology).

## Statistical Analysis

Figures were prepared using GraphPad Prism 5 and Corel Draw software. Different groups were compared by Unpaired Student's t test or Mann Whitney test using GraphPad Prism 5 software. Differences with p values of less than 0.05 were considered significant: *p<0.05; **p<0.005, and ***p<0.001. Replicates, as indicated, are individual mice or experiments.

# RESULTS

## Nucleofection of gRNAs Into Cas9$^+$ T Cells Is as Effective as That of RNPs in WT T Cells

To eliminate the need of using recombinant Cas9 protein, we have bred *Rosa26-floxed STOP-Cas9* to *Cd4cre*-expressing mice (10, 24), which led to a deletion of the STOP cassette in CD4$^+$ and CD8$^+$ T cells. For subsequent transfer studies, these mice were crossed to L2G85.CD90.1 transgenic mice, which express firefly luciferase and CD90.1 as a congenic marker (14, 25). Thus, we generated B6.*Cas9.Cd4cre.luc*.CD90.1 (Cas9$^+$) mice to isolate CD3$^+$ T cells from spleen and lymph nodes. Now we could compare RNP and gRNA nucleofection in stimulated *vs* naive CD3$^+$ T cells from wild type (WT) and Cas9$^+$ mice, respectively (**Figure 1A**).

We prepared gRNA by combining chemically modified tracrRNA and crRNA (26). We used 50 and 150 pmol *Cd90* gRNA_1 (**Table S1**) to electroporate naïve mouse CD3$^+$ T cells using Lonza Nucleofector IIb program X001. Seven days after nucleofection, flow cytometry analyses revealed around 60 % loss of surface protein expression and similar viability of around 25 % with complete RPMI media (**Figures 1B, C**). To investigate whether gRNA-only is as efficient as RNP-mediated knockout in naive mouse T cells collected from L2G85.CD90.1 mice (due to the lack of Cas9 expression referred to as WT), we prepared RNPs using 10 μg recombinant Cas9 protein and 150 pmol gRNA (3). RNP electroporation showed similar knockout efficiency in IL-7 pretreated naïve mouse T cells compared to gRNA electroporation (**Figure 1B**). Thus, we fixed our protocol for 150 pmol gRNA and RPMI, but verified this in an extended approach comparing RNP and gRNA-only nucleofection of unstimulated and by anti-CD3, anti-CD28 and IL-2 pre-stimulated CD3$^+$ T cells from WT and Cas9$^+$ mice for 72 hours. Viability was significantly enhanced in activated T cells (60 %; **Figure 1D**) as compared to naïve T cells (below 30%; **Figure 1C**). Nevertheless, no significant difference in knockout efficiency occurred between gRNA and RNP electroporation in neither unstimulated nor pre-stimulated T cells (**Figure 1E**). Collectively, we identified optimized conditions and demonstrated that gRNA delivery in Cas9$^+$ naïve and pre-stimulated CD3$^+$ T cells is as efficient as RNP nucleofection in WT cells.

## Two Genes Can Be Knocked Out Simultaneously in Stimulated Primary Mouse Cas9$^+$CD3$^+$ T Cells

We extended targeting to other genes encoding surface molecules, i.e. *Cd4* in isolated CD3$^+$CD4$^+$ T cells and *Cd8* in isolated CD3$^+$CD8$^+$ T cells (**Figure S1A**). Since efficacy and

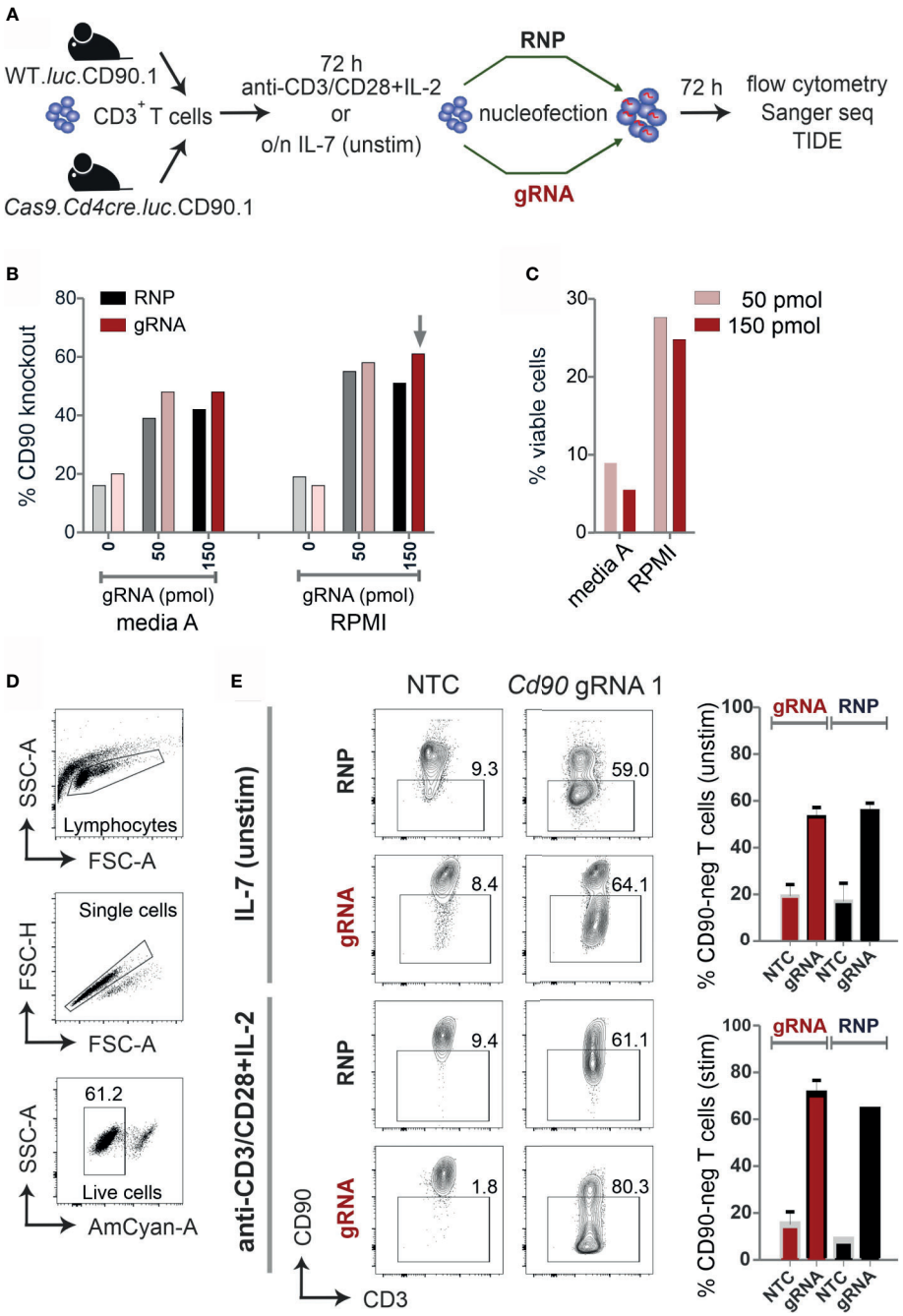


**FIGURE 1** | CRISPR/Cas9-mediated knockout is equally efficient with RNP nucleofection in WT or gRNA-only nucleofection in Cas9$^+$CD3$^+$ primary mouse T cells. **(A)** Flow chart of the CRISPR/Cas9 methods. **(B–E)** All analyses were performed by surface antibody staining and flow cytometry. **(B)** gRNA concentration optimization, comparison between Amaxa and RPMI media in resting CD3$^+$ T cells. (n=1) **(C)** Viability of resting CD3$^+$ T cells using gRNA nucleofection and different media. **(D)** Gating strategy and viability of pre-stimulated CD3$^+$ T cells using gRNA nucleofection and RPMI. **(E)** Comparison of CD90 KO efficiency between RNP nucleofection in WT T cells and gRNA nucleofection in Cas9$^+$ T cells, either resting (IL-7) or pre-stimulated CD3$^+$ T cells, 3 days after nucleofection using IIb/X001 condition. Data are presented as mean ± SEM and representative of three independent experiments.

viability had been better with pre-stimulated T cells, we electroporated a combination of three gRNAs per gene this time using the 4D Nucleofector, CM137 program. After 72 h, CD90 and both co-receptors were lost with a consistent efficiency of above 90 %, all at once demonstrating that both T-cell subsets can be targeted equally well. Furthermore, different genes could be aimed at by a combination of three gRNAs per gene (**Table S1**) in the same cell, as proven by the concurrent loss

of CD90 and CD8 in $CD3^+CD8^+$ T cells (**Figure S1B**). Overall, in both $CD4^+$ and $CD8^+$ T cells, only a slight decrease of the mean knockout efficiency per gene was observed when one additional gene was targeted simultaneously (**Figure S1C**).

## The Expression of Transcription Factor Nfatc1 Is Lost Upon gRNA-Only Nucleofection in Stimulated Murine $Cas9^+CD3^+$ T Cells

All NFAT proteins share a conserved core region composed of a DNA-binding 'Rel-similarity domain' and a less conserved N-terminal regulatory domain (**Figure 2A**, **Figure S2A**). Distinct NFAT family members and their isoforms have both redundant and specific functions, the latter most obvious for NFATc1 (19, 27, 28).

We had shown that co-transplantation of NFAT-deficient T cells as opposed to WT T cells ameliorates GvHD after HCT in a major mismatch model (14). At that time $CD3^+$ T cells were gained from *Nfatc1*$^{fl/fl}$.*Cd4cre* or *Nfatc2*$^{-/-}$ mice. For translational application, CRISPR/Cas9-mediated gene editing seemed feasible, but needed to be examined.

We designed several gRNAs for *Nfatc1* by the online tool DESKGEN (**Table S1**; **Figure 2B**). Comparing the efficiency of all individual gRNAs by single nucleofection determined *Nfatc1* gRNA_4, which binds to exon 4, as superior to all others (**Figure 2C**). The combination with exon 3-targeting gRNA_9 could enhance the degree of protein loss.

Direct evaluation of the mutational burden on DNA level - achieved by either cloning and sequencing or TIDE - revealed a similar high degree after gRNA-only_4 nucleofection (**Figures 2D, E**). In around 80 % of the cells, a single G nucleotide was deleted, expected to cause a frame shift (**Figure 2D**). About 7 % cells showed a deletion of six nucleotides as revealed by cloning PCR products of the target region in TA vector and Sanger sequencing. Although we could not detect any insertions using Sanger sequencing, TIDE data showed insertion of 2 to 10 nucleotides in very low frequencies of cells (**Figure 2E**). Of note, although during NHEJ most of the indels are in the length of a few nucleotides, it is still possible to have longer ones, not detectable by neither Sanger sequencing nor TIDE.

Interestingly, a reduction could be seen on mRNA level for both exon 1 and exon 2-containing isoforms even when the primers were chosen to bind 5' of the seeding sequence of the employed gRNAs (**Figure 2F**). Loss of mRNA expression improved upon the combination of two or three gRNAs and did not alter when *Nfatc2* was additionally targeted (**Figure 2G**).

NFATc2, like NFATc1, comes in several isoforms, in which at least exon 5, 6, 7 are expressed in all. Resembling the strategy for *Nfatc1*, we had designed several *Nfatc2*-specific gRNAs, of which three gave the best results, i.e. two binding in exon 3 encoding most of the regulatory domain and one in exon 6 encoding part of the RSD (**Table S1**, **Figure S2B**). Here again, the knockout effect got stronger by combining *Nfatc2*-specific gRNAs specific for different exons (**Figure S2C**). Mutations caused by one gRNA were less efficient as in *Nfatc1*, but more complex (**Figures S2D, E**).

To evaluate the effect on NFAT-transactivated genes, we checked the expression of the cytokines IFN-γ, IL-2 and TNF-α as well as the surface molecule CD40L by flow cytometry. Although T cells had been stimulated for three days beforehand, which instigates instant NFAT activation, aiming at either NFATc1 or NFATc2 could still reduce target gene expression (**Figure 2H**). The combination of three *Nfatc1*-specific gRNAs with three *Nfatc2*-specific gRNAs elicited an augmented effect on NFAT response genes as compared to targeting NFATc1 or NFATc2 alone (**Figure 2H**). Taken together, we achieved above 85 % knockout efficiency of the transcription factor NFAT by applying gRNA-only nucleofection, which we confirmed on DNA, RNA and protein level. This had functional consequences for the manipulated T cells.

## NFAT-Reduced Pre-Stimulated Murine $Cas9^+CD3^+$ T Cells Expanded Poorly During HCT

To explore whether NFAT ablation by CRISPR/Cas9 reduces the allo-reactivity like before (14), $Cas9^+CD3^+luc^+.CD90.1^+.H\text{-}2^b$ T cells were nucleofected by three gRNAs targeting either *Nfatc1* or *Nfatc2* (**Figure 3A**). To achieve an enhanced degree of knockout (**Figure 1E**), we pre-stimulated the T cells before nucleofection. Subsequently, they were co-transferred with $CD90.2^+.H\text{-}2^b$ bone marrow (BM) from *Rag1*$^{-/-}$ mice into lethally irradiated $CD90.2^+.H\text{-}2^d$ BALB/c mice. All mice, which received T cells, lost weight continuously over six days, but less with a prior NFATc1 knockout and significantly less upon NFATc2 knockout (**Figure 3B**). Bioluminescence imaging (BLI) of the living mice over time revealed less proliferation and expansion of T cells, which had been NFAT-ablated *vs* transfected by non-targeting crRNA (NTC) (**Figure 3C**). This could be further corroborated *ex vivo* by BLI of all organs on day 6 (**Figure 3D**). Detailed comparison of lymphoid and non-lymphoid organs of mice with NTC *vs Nfatc1*-specific gRNA-nucleofected T cells documented a significant halt in the expansion of NFATc1 knockouts (**Figure 3E**). This included the gut, one of the prime target organs during GvHD. Accordingly, the gut homing receptor α4β7 integrin was less well expressed on NFATc1-ablated $CD4^+$ and $CD8^+$ T cells (**Figure 3F**). Additionally, those T cells produced fewer IFN-γ, the dominant cytokine during aGvHD (**Figure 3G**), while NFATc1 knockout Tregs had an advantage over NTC-transfected ones (**Figure 3H**). This supports our former data that Tregs are less dependent on NFAT proteins and that they function well, when one or two NFAT family members are missing (14, 15). In sum, with regard to allo-reactivity, ablating NFAT by gRNA-only in pre-stimulated $Cas9^+CD3^+$ T cells seemed to be equivalent to the loss achieved using T cells from knockout mice, as seen in our earlier work (14).

## Pre-Stimulation of T Cells Alters Their Behavior *In Vivo*

Unexpectedly, mice, which received NFATc1 or NFATc2-ablated T cells, were not protected over time and deceased like those with NTC-T cells (data not shown). One possibility was

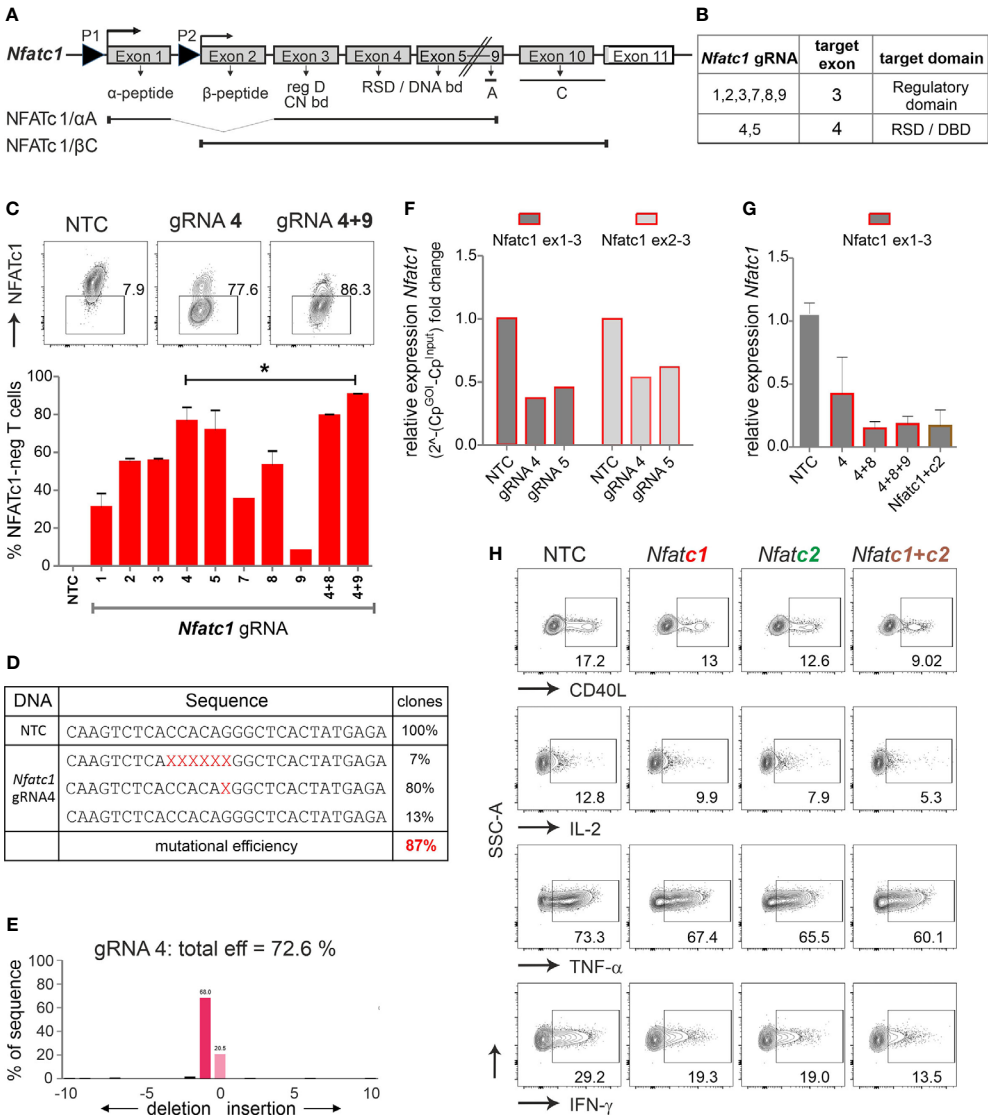


**FIGURE 2** | The combination of exon 3 and exon 4-targeting gRNAs efficiently erases *Nfatc1* in stimulated murine Cas9$^{+}$CD3$^{+}$ T cells. **(A)** Genomic structure of *Nfatc1* encoding six different isoforms due to two different promoters, of which P1 is inducible and P2 is constitutive, different splicing events and two non-depicted polyA sites. The most prominent isoforms NFATc1/αA and NFATc1/βC are indicated. The first common exon 3 encodes the regulatory domain, which includes calcineurin interaction and phosphorylation sites. Exon 4 is necessary for the expression of the Rel similarity domain, which enforces DNA binding. **(B)** Table with *Nfatc1* gRNAs and their target exons. **(C)** Variation in knockout efficiency between gRNAs specific for *Nfatc1* measured by intracellular flow cytometry for NFATc1. The efficiency increases by the use of two gRNAs per gene mean ± SEM, of three independent experiments, unpaired Student's t test *P < 0.05. **(D)** Detection of indels in sequences of clones established after *Nfatc1* gRNA_4 nucleofection detected by Sanger sequencing, data are from one experiment. **(E)** Recognition of indels using TIDE. The mutational load was also calculated by TIDE. Of note, with variance $R^{2}$ = 0.92, 8 % were recognized as noise or large indels. **(F)** mRNA isolated from cells collected 72 hours post nucleofection. qRT-PCR with primers binding to exon 1 plus 3 or exon 2 plus 3 in *Nfatc1* RNA, i.e. 5' of the gRNA_4 binding site. **(G)** qRT-PCR with primers binding to exon 1 plus 3 in *Nfatc1* cDNA after nucleofection of one, two or three *Nfatc1*-specific gRNAs, additionally with three *Nfatc1*-specific (4 + 8+9) and three *Nfatc2*-specific (1 + 2+3) gRNAs, mean + SEM, from two independent experiments. **(H)** Effect on target genes upon *Nfatc*1 and/or *Nfatc2* knockout. Flow cytometry of intracellular cytokines IL-2, TNF and IFN-γ as well as surface expression of CD40L. Data represent three independent experiments.

that the few cells without knockout outcompeted the NFAT-deficient ones. Therefore, we performed an experiment with NFATc1 and NFATc2-deficient cells gained from *Nfatc1*$^{fl/fl}$.*Cd4cre* and *Nfatc2*$^{-/-}$ mice, respectively (14). We treated them according to our model (**Figure 3A**) and compared them to NTC and gRNA-nucleofected for NFATc1 plus NFATc2. Very different from our results using these same NFAT-deficient T cells without stimulation, pre-activated CD3$^{+}$ T cells from either *Nfatc1*$^{fl/fl}$.*Cd4cre* or *Nfatc2*$^{-/-}$ caused long-term weight loss and enhanced clinical scores beginning one week after transplantation in the major mismatch model of HCT (**Figure S3A**). The NFAT single-deficient T cells were responsible for a premature death in comparison to NTC-nucleofected or NFATc1c2

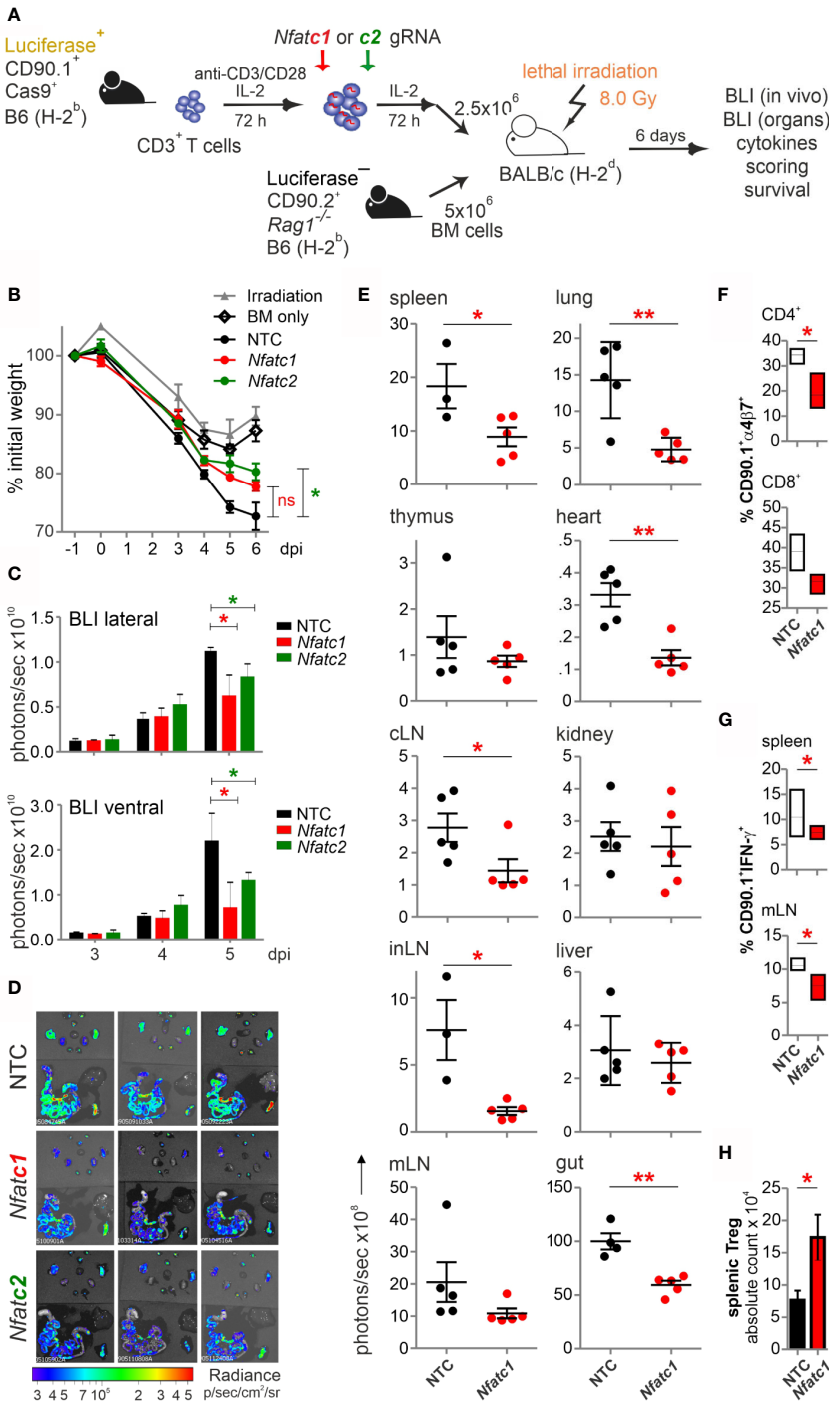


**FIGURE 3** | Knockout of NFATc1 or NFATc2 in pre-stimulated murine Cas9$^+$CD3$^+$ T cells limits signs of GvHD after co-transfer in a major mismatch model. **(A)** Experimental set up of sole NFAT-specific gRNA nucleofection and GvHD induction due to an H-2$^b$ →H-2$^d$ transfer with pre-stimulated Cas9$^+$CD3$^+$ T cells. **(B–H)** gRNAs used: *Nfatc1* gRNA 4 + 8+9, *Nfatc2* gRNA 1 + 2+3. **(B)** Weight changes of mice with NTC and NFAT-specific gRNA-nucleofected and co-transplanted Cas9$^+$CD3$^+$ T cells. Mice were evaluated every day pre and post transplantation and weight loss was calculated considering day-1 weight as 100 %. [ns, not significant] **(C)** Lateral and ventral view by BLI of living mice on day 3, 4, and 5 dpi with NTC, NFATc1 or NFATc2-ablated pre-stimulated Cas9$^+$CD3$^+$ T cells. Plotted are photons per second; mean + SD. **(D)** *Ex vivo* BLI images of lymphoid and non-lymphoid organs of the mice under **(C)** at 6 dpi; mean ± SD. **(E–H)** Analyses six days after GvHD induction with NTC and *Nfatc1* gRNA-nucleofected, pre-stimulated Cas9$^+$CD3$^+$ T cells; mean ± SD, unpaired Student's t test (*$p < 0.05$, **$p < 0.01$), representative of two independent experiments. **(E)** Quantitation of *ex vivo* BLI analyses of lymphoid and non-lymphoid organs. **(F)** Staining of integrin α4β7 together with CD90.1, CD4 and CD8 followed by flow cytometry. **(G)** Intracellular staining of IFN-γ after surface staining for CD90.1, CD4 and CD8 followed by flow cytometry. **(H)** Intracellular staining of Foxp3 after surface staining for CD90.1, CD4 and CD25 followed by flow cytometry. [dpi, days post irradiation].

double-deficient ones, created by gRNA nucleofection of $Cas9^+CD3^+$ T cells. With this, non-manipulated $Cas9^+CD3^+$ T cells overgrowing the factually nucleofected ones were unlikely to be the cause of loss of protection over time.

One remaining possibility was that the 3-day pre-stimulation period changed the (allo-) reactivity of T cells. Therefore, we adapted our protocol to pre-stimulating $Cas9^+CD3^+$ T cells for only 24 h before gRNA nucleofection and transplanting them without a major rest. In parallel, we verified the knockout on the level of *Nfatc1* and *Nfatc2* RNA (**Figure S3B**). We chose to transfer just as many T cells ($1.2x10^6$) as we had done before with naive T cells from NFAT-deficient mice (14). Now we observed a constant benefit upon NFAT ablation (**Figure S3C**). Still, shortly pre-stimulated $Cas9^+$ T cells did not behave equally when knocked out for NFATc1 *vs* NFATc2 since NFATc1 deficiency was even more effective than the DKO regarding GvHD scores. This was in contrast to the former data with naive T cells from NFAT-deficient mice. Despite that, knocking out NFATc1, NFATc2 or both by gRNA-only in shorter pre-stimulated and directly co-transplanted $Cas9^+CD3^+$ T cells protected mice from aGvHD, also obvious for skin GvHD on day 21 (**Figure S3D**).

## In Unstimulated T Cells, CRISPR Efficiency Depends on the Electroporator

Realizing the awkward performance of pre-stimulated $CD3^+$ T cells after transfer, even not fully normal when pre-stimulation time was shortened to 24 h, we had to reconsider to manipulate and transfer naive T cells. We simply tried to nucleofect by making use of a more recent version of the electroporator. The technology and electrode material is different between the instruments (Aluminium for IIb and conductive polymer for 4D) and even preset programs are not comparable between them. CD90, PD1 and NFATc1 expression was evaluated after two gRNAs per gene had been transfected into naive $Cas9^+CD3^+$ T cells and all three proteins could now competently be reduced (**Figure 4A**, **Figure S4A**). Survival of naive T cells had been another issue (**Figure 1C**). First we re-evaluated the need of pre-culturing the naive $Cas9^+CD3^+$ T cells in IL-7 (3). No influence on survival could be observed by the addition of IL-7 for overnight rest, but undoubtedly, an improved knockout efficiency in the absence of IL-7 before nucleofection for three genes tested (*Cd90* in **Figure 4B**). Next, we evaluated how nucleofected cells could be supported *in vitro* before stimulation. Different concentrations of IL-7 were given alone or in combination with IL-2 over three days after nucleofection. In the presence of IL-7, nucleofected naive $Cas9^+CD3^+$ T cells survived better (**Figure 4C**). Here, any addition to 5 ng/ml IL-7 was not superior. Naivety – measured by CD62L and CD44 expression – was kept up, while central and effector memory occurrence was even counteracted by IL-7 in $CD4^+$ and $CD8^+$ T cells (**Figure 4D**). Tregs, which are highly sensitive *in vitro*, survived in sufficient frequencies, but surprisingly did not benefit from a further addition of IL-2.

To test whether IL-2 was functional, we repeated the experiment including IL-2 alone (**Figure S4**). Compared to IL-7 or IL-7 plus IL-2, IL-2 alone negatively affected the overall survival of $CD3^+$ T cells, but could – in a known feedback loop *via* STAT5 activation – upregulate the high affinity receptor of IL-2, i.e. CD25 (**Figure S4B**). The percentage of Tregs was indeed supported by IL-2, although absolute Treg numbers did not differ between IL-2, IL-7 or double treatment (**Figure S4C**). Since IL-2 enforces central memory in $CD4^+$ and effector memory in $CD8^+$ T cells (**Figure S4D**), such treatment is not advisable if one wants naive T cells for analyses or transfer.

For *in vivo* experiments, many cells might be needed, but doubling the number of naive $Cas9^+CD3^+$ T cells per cuvette during nucleofection decreased the knockout efficiency, which was noticeable for CD90 and NFATc1 (**Figure S4E**). Lastly, we tested if gRNA-only-nucleofected naive $Cas9^+CD3^+$ T cells need to be rested *in vitro* to achieve the loss of target gene expression during transfer mouse models. After three days of *in vitro* stimulation, the 2-day period of rest appeared to enhance the number of gene-ablated cells (**Figure 4E**). On the other hand, cells, which were immediately transferred after nucleofection, exhibited the same high degree of 80 % knockout, when regained after six days as when they had been rested in IL-7 post nucleofection for two days prior to transplantation (**Figure 4E**). Since we nucleofected the mixture of resting $CD3^+$ T cells, it was necessary to document whether the minor, but important subpopulation of Tregs got targeted together with all Tcon. Indeed, the knockout efficiency was high in Tregs as well, when measured directly transplanted and six days after GvHD induction (**Figure 4F**). Thus, all $Cas9^+CD3^+$ T cells can be efficiently gene-targeted by gRNA-only without pre-stimulation if the right nucleofector is used, they are supported directly by the *in vivo*-situation or by some IL-7 after nucleofection and before activation *in vitro*.

## The Procedure of Nucleofection Only Minimally Affects the Metabolism of $Cas9^+CD3^+$ T Cells

From their naïve to effector function, T cells undergo metabolic reprogramming and shift from oxidative phosphorylation (OXPHOS) towards aerobic glycolysis. The co-secretion of protons and lactate during aerobic glycolysis results in the acidification of the media, which can be measured as the extracellular acidification rate (ECAR) in the Glycolysis Stress Test (GST). The Mitochondrial Stress Test (MST) is based on changes of the oxygen consumption rate (OCR) that is indicative for OXPHOS.

In order to determine whether nucleofection of naive $Cas9^+CD3^+$ T cells influences their metabolic plasticity during activation, we performed metabolic flux analyses in cells, which underwent nucleofection with or without NTC in comparison to stimulated cells. As anticipated, without stimulation, ECAR and OCR activity was hardly detectable (data not shown) in T cells, while overnight (15 h) stimulation revealed substantial glycolytic and OXPHOS activity. Nucleofection, irrespective of incorporated RNA, affected both glycolysis and OXPHOS. The strongest effects were observed for maximal glycolytic capacity and glycolytic reserve, while the basal OCR/ECAR ratio was not skewed suggesting a balanced effect on the basal bioenergetic phenotype (**Figure 5A**). Strikingly, continuously (for 72 h)

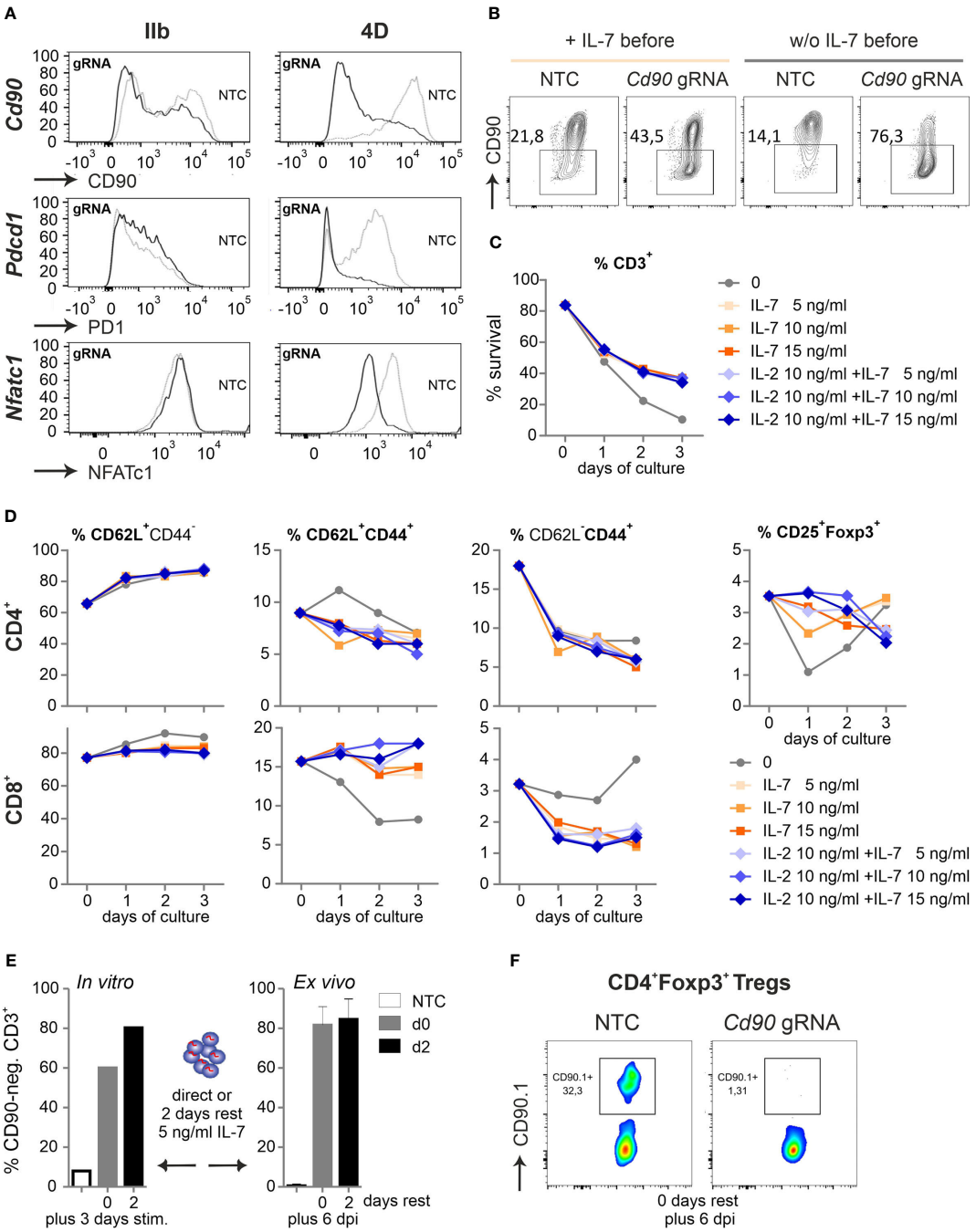


**FIGURE 4** | Naive Cas9$^+$CD3$^+$ T cells can be efficiently gene-ablated. **(A)** Comparison of two nucleofector versions for knocking out CD90 (gRNA 2 + 3), PD1 (gRNA 1 + 2) and NFATc1 (gRNA 4 + 8+9) by gRNA-only in resting Cas9$^+$CD3$^+$ T cells analyzed by flow cytometry after 3 d rest with IL-7 and 3 d of stimulation. **(B)** CD90 KO (gRNA 2 + 3) with and without o/n IL-7 pre-incubation, followed by nucleofection, 3 d rest with IL-7 and 3 d of stimulation. **(C, D)** Flow cytometric analyses over three days of resting Cas9$^+$CD3$^+$ T cells treated with 5, 10 and 15 ng/ml IL-7 in the absence or presence of 10 ng/ml IL-2 after NTC nucleofection. **(C)** Percentage of living CD3$^+$ T cells analyzed by Zombie live-dead staining (mean ± SD). **(D)** Frequency of CD62L$^+$CD44$^-$ naive, CD62L$^+$CD44$^+$ central memory and CD62L$^-$ CD44$^+$ effector memory CD4$^+$ and CD8$^+$ T cells as well as CD4$^+$CD25$^+$Foxp3$^+$ Tregs determined by surface and intracellular staining followed by flow cytometry. **(E)** CD90.1 staining and flow cytometry analysis of CD3$^+$ T cells not rested or for 2 d with IL-7 post gRNA nucleofection (CD90 gRNA 1 + 2+3) followed by 3 d *in vitro* stimulation, n = 1; or transplanted and analyzed *ex vivo* 6 dpi; mean + SD. **(F)** CD90.1 expression on CD4$^+$Foxp3$^+$ T cells *ex vivo* 6 dpi.

stimulated T cells did not display any differences in terms of their metabolic profile (**Figure 5B**). The latter observation indicates that nucleofection of naive Cas9$^+$CD3$^+$ T cells might stress them (metabolically) only transiently and that upon a short recovery period T cells preserve their full capacity to meet their basic bioenergetic demands, but also to adapt to increased demands through upregulation of aerobic glycolysis or OXPHOS.

## Naive NFAT-Targeted Cas9$^+$CD3$^+$ T Cells Do Not Cause Severe GvHD

Pre-stimulated and nucleofected Cas9$^+$CD3$^+$ T cells were able to cause GvHD in a major mismatch model, during which NFAT deficiency reduced T-cell expansion and proliferation (**Figure 3**). However, the clinical score was untypically low and NFAT single-ablated T cells could not protect over time (**Figure S3**). Now we co-transplanted naive Cas9$^+$CD3$^+$ T cells with BM cells (**Figure 6A**). The clinical scores doubled, demonstrating the undisturbed power of naive T cells (**Figure 6B**). Knockout of NFATc1, NFATc2 or both reduced the clinical scores. Accordingly, NFAT-ablated cells - measured by *in vivo* BLI – expanded significantly less (**Figures 6C, D**).

Next, we analyzed whether it would be advisable to rest naive, NFAT-ablated Cas9$^+$CD3$^+$ T cells in IL-7 before transfer. We transferred the same number of BM cells, but a substantially downgraded number of T cells, still causing a clinical score above 6 (**Figure 6E**). Two days of rest enabled the T cells to transmit

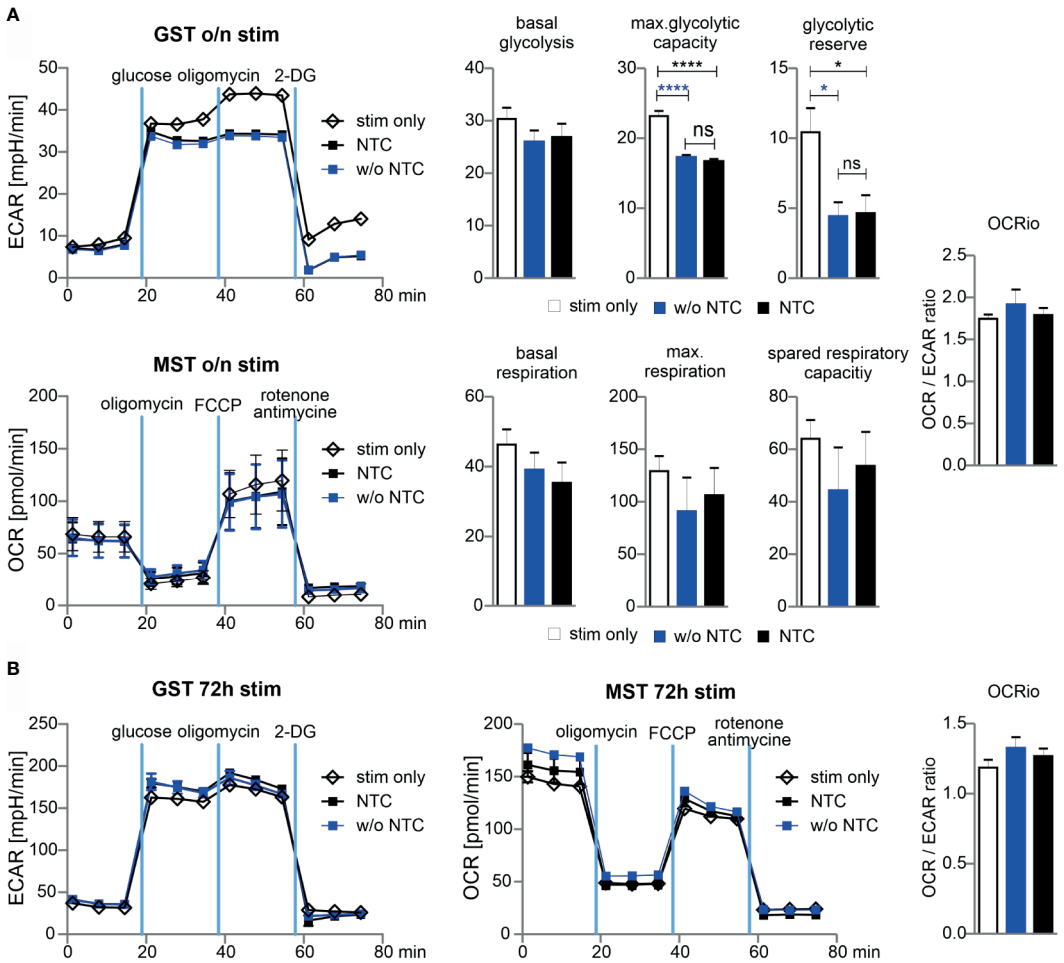


**FIGURE 5** | Glycolytic reserve has been compromised upon electroporation of T cells. **(A)** Naive mouse Cas9$^+$CD3$^+$ T cells were nucleofected followed by overnight stimulation with anti-CD3/CD28 and IL-2. Metabolic status was analyzed performing a GST and MST on a the XFe96 Seahorse metabolic flux analyzer. ECAR was measured at baseline, in response to glucose to calculate basal glycolysis, after oligomycin injection for max. glycolytic capacity and reserve, and after 2-DG injection for non-glycolytic acidification. Basal glycolysis = glucose - baseline; glycolytic capacity = oligomycin - baseline; glycolytic reserve = capacity - glycolysis. Oxygen consumption rate (OCR) was measured under basal condition followed by sequential injection of oligomycin, FCCP, and rotenone together with antimycin A to shut down mitochondrial respiration (values represent non-mitochondrial respiration). Basal respiration = baseline - non-mitochondrial respiration; maximal respiration = FCCP - non-mitochondrial respiration; spare respiratory capacity = maximal respiration - basal respiration. Student's two-tailed *t*-test (*p < 0.05, ****p < 0.001); mean ± SD, data represents two independent experiments. [ns, not significant] **(B)** Naive mouse Cas9$^+$CD3$^+$ T cells were nucleofected followed by 72 hours stimulation with CD3/CD28 and IL-2. Metabolic status was determined as for **(A)**. Student's two-tailed *t*-test (*p < 0.05, ****p < 0.001); mean ± SD; of two independent experiments.

the high GvHD score earlier, but their potential for induction of clinical scores and weight loss were alike irrespective of rest or immediate transfer after nucleofection. Knockout of NFATc1 by gRNA-only in naive $Cas9^+CD3^+$ T cells limited GvHD symptoms significantly in both settings (**Figure 6E**). We conclude that gRNA-only nucleofection of unstimulated $Cas9^+CD3^+$ T cells not only leads to efficient gene editing, but preserves their functional abilities upon allo-HCT.

## Transfer of NFAT-Ablated Naive T Cells Protect Mice From GvHD Over Time

To evaluate if the direct transfer of NFAT-deficient T cells created by gRNA-only nucleofection in naive $Cas9^+CD3^+$ T cells impinged long-term protection from severe GvHD, we once again knocked out NFATc1. NFATc1 ablation was verified in the total $CD3^+$ T cell population and individually in $CD4^+$ and $CD8^+$ Tcon as well as $CD4^+Foxp3^+$ Tregs, each time in comparison to NTC nucleofection, by intracellular flow cytometry (**Figure 7A**). When we rested some of those cells in IL-7 for two days, stimulated them with anti-CD3/28+IL-2 and restimulated by PMA/Ionomycin for 5 h *in vitro*, IL-2 and IFN-γ expression was compromised due to NFATc1 deficiency (**Figure 7B**). *In vivo*, different from the transfer with pre-stimulated T cells, clinical scores as well as weight loss remained less severe over time in comparison to the transfer of naive WT $Cas9^+CD3^+$ T cells (**Figure 7C**). Accordingly, all mice, which received NTC-nucleofected $Cas9^+CD3^+$ T cells, had died by 35 days, while half of all mice getting NFATc1 knockout T cells were still alive after 90 days. Similarly, NFATc2 ablation in naive $Cas9^+CD3^+$ T cells and their subsequent transfer limited the degree of GvHD stably over time (**Figure 7D**). With this, naive T cells behaved the same in our major mismatch model irrespective whether they were gathered from NFAT-deficient mice (14) or whether they were knocked out *in vitro* by gRNA-only nucleofection of $Cas9^+CD3^+$ T cells.

## Ablation of the NFAT Target Gene *Irf4* in Donor T Cells Ameliorates GvHD

If T cells are not stimulated before allo-HCT, NFAT target genes are not yet trans-activated. This might be one functional difference between transplanted naive and activated T cells. Besides effector molecules like CD40L or cytokines, NFAT induces transcription factors, thereby influencing gene expression in an extensive manner. As we found NFAT to upregulate and cooperate with IRF4 (29, 30), we determined whether IRF4 is a direct target gene. Both NFATc1 and NFATc2 are bound to the immediate upstream region of *Irf4* in ChIPseq experiments of $CD8^+$ T cells (**Figure S5A**) (31, 32). Accordingly, constitutive active NFATc2 transactivated the *Irf4* promoter in a reporter assay (**Figure S5B**), while activation of T cells from *Nfatc1*$^{caaA}$.dLckcre mice, which express constitutive active NFATc1/αA in post-thymic T cells, had a strong positive impact on IRF4 protein levels (**Figure S5C**).

This prompted us to test our established method of CRISPR/Cas9 editing by gRNA-only nucleofection with this NFAT target gene for allo-HCT. gRNAs for exon 1 and exon 6 were tested in different combinations. The combination of three exon-1-specific gRNAs for nucleofection of naive $Cas9^+CD3^+CD90.1^+$ T cells achieved 80 % IRF4-negative T cells after 2 days rest with IL-7 and 3 days of stimulation *in vitro* (**Figure 8A**). Editing of *Irf4* and direct transfer in conjunction with allo-HCT did not prevent weight loss, but reduced the clinical score significantly (**Figures 8B, C**). Proliferation and expansion of transplanted T cells was extensively impaired (**Figure S5D**, **Figure 8D**). This could also be observed *ex vivo* in individual organs (**Figures 8E, F**). Accordingly, the absolute number of *Irf4*$^{-/-}$ $CD90.1^+$ T cells – including that of tTregs – was less compared to NTC-nucleofected T cells (**Figure 8G**). However, the frequency of *Irf4*$^{-/-}$ Tregs was preserved within the transplanted T-cell fraction (**Figure 8G**). This might be due to relatively more IL-2 and TNF-expressing $CD4^+$ and $CD8^+$ splenic T cells (**Figure 8H**, **Figure S5E**), which support Tregs *via* CD25, i.e. the high-affinity IL-2R, and TNFR2. On the other hand, we observed an enhanced Th1 phenotype, i.e. IFN-γ and again TNF production, caused by IRF4 ablation in $CD4^+$ and $CD8^+$ T cells (**Figure 8H**, **Figure S5E**). Nevertheless, the absolute numbers of cytokine-expressing as well as granzyme B and perforin-positive $CD4^+$ and $CD8^+$ *Irf4*$^{-/-}$ T cells were contracted significantly in comparison to NTC-nucleofected T cells (**Figure 8H**, **Figures S5F, G**). In sum, despite the shift towards an unfavorable Th1 differentiation, deletion of the NFAT target gene IRF4 in co-transplanted naive T cells during allo-HCT protected from severe GvHD.

# DISCUSSION

We present an effective CRISPR/Cas9-based method to edit genomes in primary murine T cells. If Cas9 transgenic mice are available, gRNA-only nucleofection is sufficient in pre-activated and even in naive $Cas9^+CD3^+$ T cells to achieve at least 80 % knockout. More than one gRNA per gene increased the degree of knockout, especially when targeting different exons encoding different protein domains. By nucleofection of multiple gRNAs, it is possible to ablate several genes concurrently with almost unchanged effectiveness per gene. Importantly, nucleofected resting $Cas9^+CD3^+$ T cells could be transferred to mice without any further treatment or rest, acquired their knockout *in vivo*, but otherwise behaved like transplanted naive $CD3^+$ T cells. Further requirement fulfilled was that nucleofection only transiently affected the metabolism of $Cas9^+CD3^+$ T cells.

Primary mouse T cells are usually not easily genome-edited by non-viral methods, wherefore transgenes or shRNAs are mostly transferred retro- or lentivirally into pre-activated cells, often with a subsequent selection by antibiotics or flow cytometric sorting. Researchers have created Cas9 transgenic mice (8, 10). To manipulate their T cells, however, gRNAs are still introduced per retroviral transduction (7, 8). Apart from the fact that this is more tedious and time consuming than electroporation, retroviral backbones could cause immunogenicity and toxicity (33). Of note, T cells have to be activated before retroviral infection. Thus, overall transfection of transgenic $Cas9^+$ cells by electroporation appears advantageous.

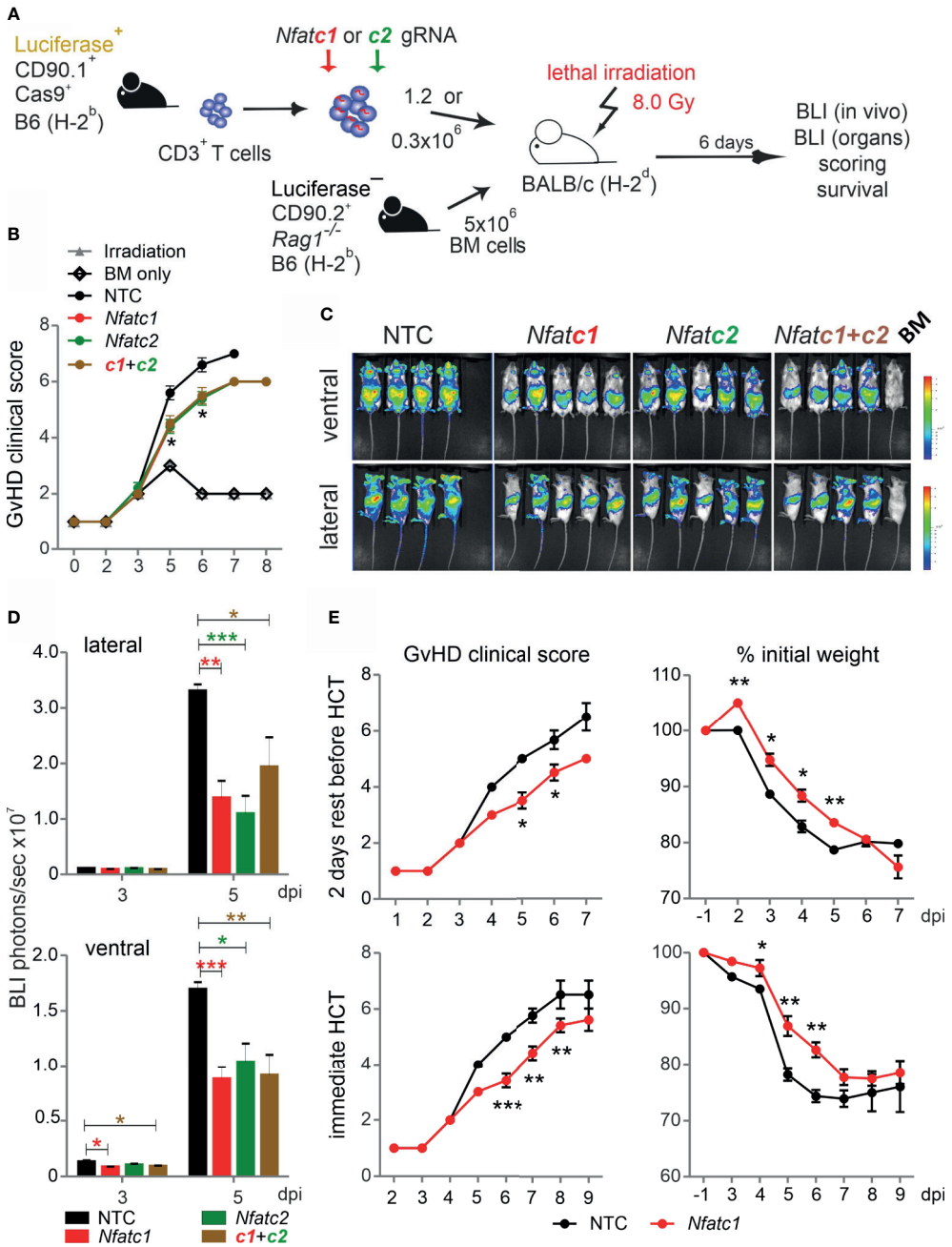


**FIGURE 6** | *Nfatc1*$^{-/-}$ 'CRISPR'ed unstimulated murine Cas9$^{+}$CD3$^{+}$ T cells ameliorate aGvHD. **(A)** Experimental set up of sole NFAT-specific gRNA nucleofection and GvHD induction due to an H-2$^{b}$ → H-2$^{d}$ transfer with naive Cas9$^{+}$CD3$^{+}$ T cells. **(B–E)** gRNA used: *Nfatc1* gRNA 4 + 8+9, *Nfatc2* gRNA 1 + 2+3. **(B–D)** Naive Cas9$^{+}$CD3$^{+}$ T cells were nucleofected with NTC, *Nfatc1*, *Nfatc2*, and *Nfatc1* plus *Nfatc2* targeting gRNAs and 1.2x10$^{6}$ cells transplanted immediately thereafter. **(B)** Clinical scores of GvHD-induced mice determined daily for 8 days. **(C)** Ventral and lateral in *vivo* BLI at 5 dpi. Data represent two independent experiments. **(D)** Quantitation and statistical analyses of BLI of living mice in lateral and ventral view on 3 and 5 dpi. Plotted are photons per second. Student's two-tailed *t*-test (*p < 0.05, **p < 0.005, ***p < 0.001); mean +SD. **(E)** Naive Cas9$^{+}$CD3$^{+}$ T cells were nucleofected by NTC or *Nfatc1* targeting gRNAs and 0.3x10$^{6}$ cells transplanted after 2 days of rest in comparison to immediately. Clinical scores and weight loss were assessed daily over the indicated period. Student's two-tailed *t*-test (*p < 0.05, **p < 0.005, ***p < 0.001); Mean ± SD.

Nonetheless, electroporation entails other challenges. Electroporation of cells can be irreversible, when it disrupts the plasma membrane, causing loss of cell homeostasis and leading to cell death. On top, applied electric fields, even if they are transient, can reach the mitochondrion, which harbors the electron transport chain and can release cytochrome C, which would affect the metabolic capacities and again induce cell death (34). Thus, conditions have to be acquired, which allow the cells

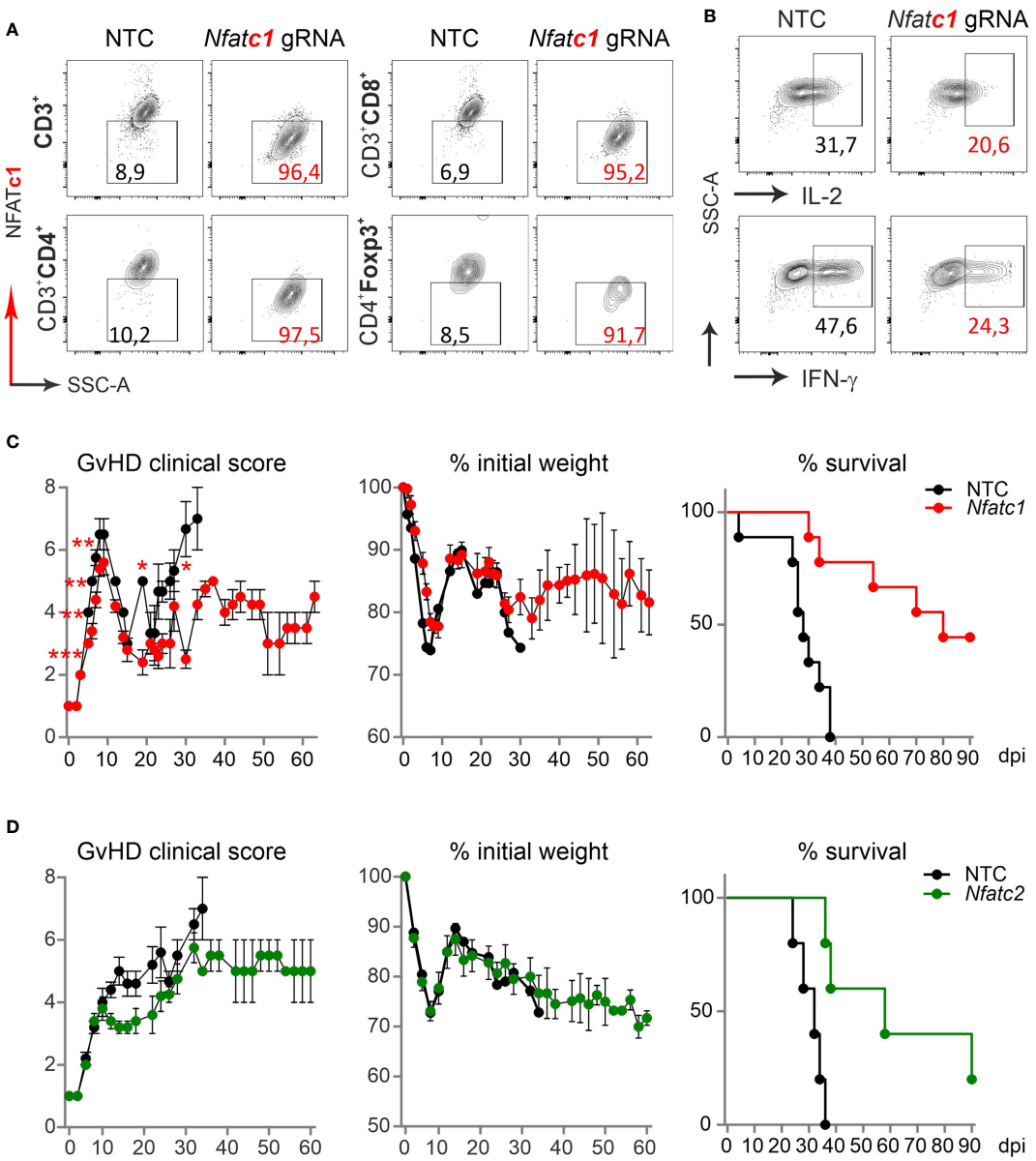


**FIGURE 7** | *Nfatc1*$^{-/-}$ and *Nfatc2*$^{-/-}$ 'CRISPR'ed unstimulated murine Cas9$^{+}$CD3$^{+}$ T cells protect over time and prolong survival. **(A, B)** Naïve Cas9$^{+}$CD3$^{+}$ T cells were nucleofected by NTC or *Nfatc1*-targeting gRNAs and knockout efficiency was assessed by surface and intracellular flow cytometry after 2 days of IL-7 rest and 3 days of stimulation in *vitro*. **(A)** Detection of NFATc1 in CD4$^{+}$ and CD8$^{+}$ as well as CD4$^{+}$Foxp3$^{+}$ T cells. **(B)** Intracellular IL-2 and IFN-γ staining in *Nfatc1* gRNA-nucleofected naïve Cas9$^{+}$CD3$^{+}$ T cells. **(C, D)** Naive Cas9$^{+}$CD3$^{+}$ T cells were nucleofected by NTC or NFAT-specific gRNAs (*Nfatc1* gRNA 4 + 8+9, *Nfatc2* gRNA 1 + 2+3) and 0.3x10$^{6}$ cells transplanted directly thereafter (H-2$^{b}$ → H-2$^{d}$ transfer). Clinical scores and weight loss were determined over 60 days, whereas survival over 90 days. Student's two-tailed *t*-test (*p < 0.05, **p < 0.005, ***p < 0.001); mean ± SEM, n≥5. Data represent two independent experiments.

to fully recover from the transient perturbation. In fact, we did not observe any stress on mitochondria, in line with quiescence and survival of the manipulated T cells. Metabolic competence including a shift from OXPHOS towards aerobic glycolysis upon stimulation is a prerequisite for proper T cell function (35). In addition, although Tregs are known to rather utilize OXPHOS to exert their suppressive activity, they also rely on glycolysis for proliferation and migration (36). Monitoring T-cell bioenergetics after *in vitro* stimulation revealed a preserved full metabolic capacity for all T-cell subtypes three days after nucleofection. Induced stress on preferentially glycolytic reserve capacities was apparent only transiently after the intervention. Overall, we found conditions - with the right nucleofector and program - which ensure good survival rates of resting Tcon as well as Tregs allowing them to respond with unperturbed proliferation and metabolic reprogramming.

It surely makes a difference whether T cells lose a certain gene before they are activated or thereafter. For example, NFAT

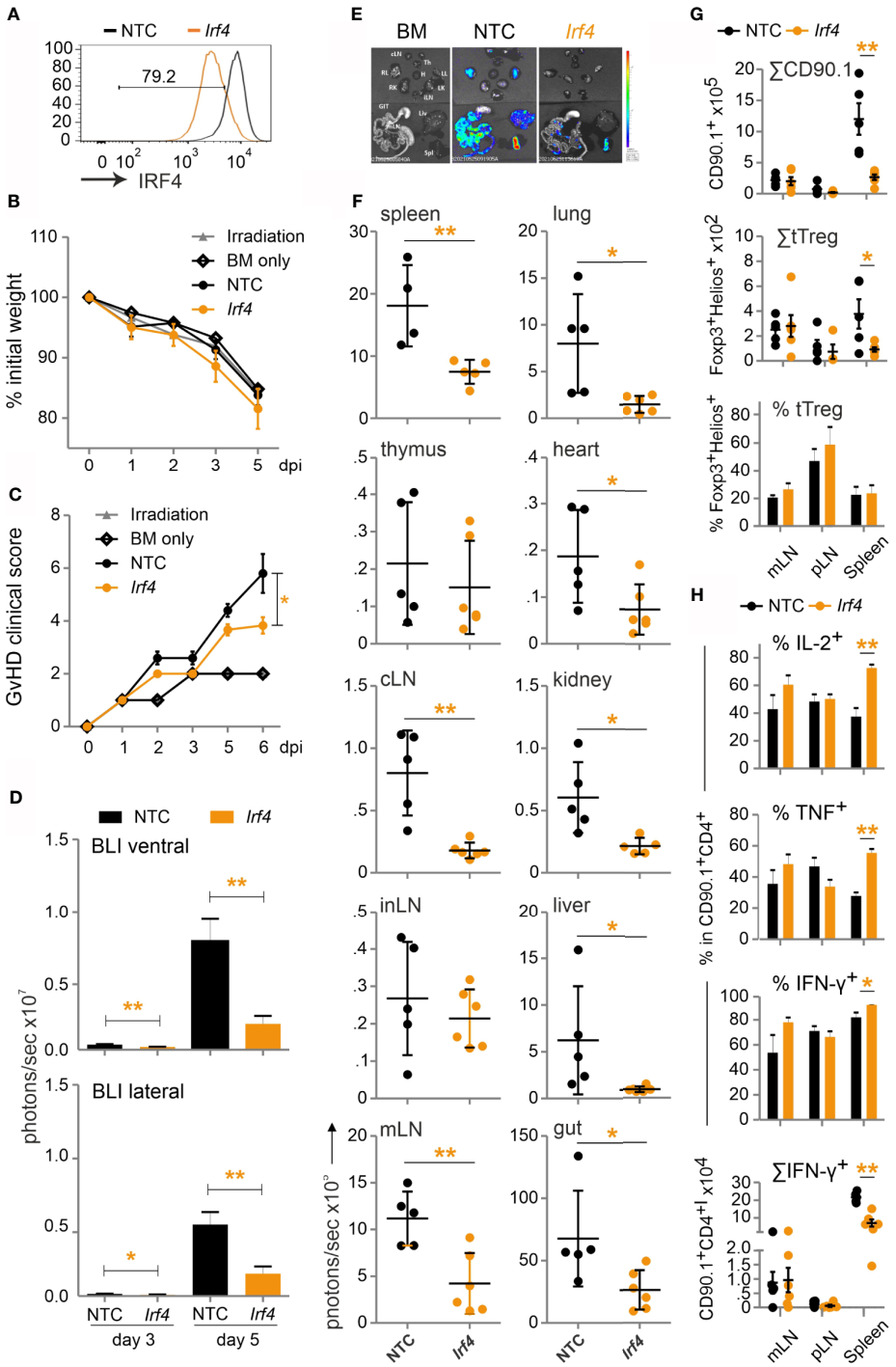


**FIGURE 8** | *Irf4*$^{-/-}$ CRISPR'ed unstimulated murine Cas9$^{+}$CD3$^{+}$ T cells ameliorate GvHD despite enhanced Th1 differentiation. **(A–H)** Naïve Cas9$^{+}$CD3$^{+}$ T cells were nucleofected with either NTC or combination of three gRNAs targeting *Irf4*. **(A)** IRF4 knockout efficiency analyzed in Cas9$^{+}$CD3$^{+}$ T cells by intracellular staining and flow cytometry post 2 days rest with IL-7 and 3 days of stimulation. **(B–H)** GvHD induction due to an H-2b →H-2d transfer with naïve Cas9$^{+}$CD3$^{+}$ T cells. Mann Whitney test (*$p < 0.05$, **$p < 0.005$). Data represent mean ± SD from one experiment with n≥5 mice per group. **(B)** Weight was measured post transplantation up to dpi 5 and percentage of weight loss was calculated considering d0-weight as 100%. **(C)** Clinical score of GvHD-induced mice determined daily for 6 days. **(D)** Quantitation of ventral and lateral *in vivo* BLI at 3 and 5 dpi. **(E)** *Ex vivo* BLI images of lymphoid and non-lymphoid organs at 6 dpi. **(F)** Quantitation of *ex vivo* BLI analyses of lymphoid and non-lymphoid organs at 6 dpi. **(G)** Absolute count of CD90.1$^{+}$ donor T cells and CD90.1$^{+}$CD4$^{+}$CD25$^{+}$Foxp3$^{+}$ donor T cells by flow cytometry. Percentage of Helios$^{+}$ tTreg within CD90.1$^{+}$CD4$^{+}$CD25$^{+}$Foxp3$^{+}$ donor T cells by intracellular flow cytometry. **(H)** Frequency of IL-2$^{+}$, TNF$^{+}$ and IFN-γ$^{+}$ within CD90.1$^{+}$CD4$^{+}$ donor T cells determined by intracellular staining and flow cytometry as well as absolute count of CD90.1$^{+}$CD4$^{+}$ IFN-γ$^{+}$ donor T cells.

proteins transmit TCR signals, i.e. antigen recognition, which leads to a plethora of transactivated genes (16, 37, 38). First identified was the positive regulation of cytokine expression like that of IL-2 and IFN-γ. Not only IFN-γ, but already IL-2 influences immune cell differentiation (39). We recently showed that an enhanced amount of IL-2 – due to the dominance of NFATc1/αA – at the beginning of activation shifts the immune response to a more tolerogenic one, although upregulation of IL-2 is transient (19). This might be one reason, why only naive T cells, but not effector cells play a major role in acute GvHD in mice and men (40–42). At least our data gathered after the transfer of pre-activated/effector T cells in a major mismatch model confirmed this notion - and made it necessary to search for the right condition to manipulate naive T cells. Such data about the alloreactivity of naive *vs* pre-stimulated T cells stirred clinical studies and naive T cells were depleted by anti-CD45RA from the HCT grafts (43). It failed, possibly because $CD45RA^+$ Tregs were excluded as well, while Tregs are needed to limit acute GvHD (44). This emphasizes the need of murine transfer models, in which more than one subtype of immune cells is studied. With respect to gene editing by CRISPR/Cas9 and T-cell transfer models, it highlights attempts like ours, in which $CD3^+$ T cells instead of one T-cell subtype is edited and transplanted for GvHD. In this context, it is noteworthy that $CD4^+$ and $CD8^+$ Tcon as well as $CD4^+CD25^+Foxp3^+$ Tregs were all efficiently gene-ablated in the mixture of $Cas9^+CD3^+$ T cells.

Since all T-cell subtypes are all edited equally well, this allows the approach to be used in wide-ranging scenarios. For hard to isolate subtypes or subtypes which differentiate *in vivo* after transfer, Cas9 transgenic mice can be bred to different Cre lines (for example to *Il21cre* for follicular T-helper ($T_{FH}$) cells). Total $CD3^+$ T cells will be nucleofected with gRNA and transplanted in recipient mice. Gene editing, however, will occur exclusively upon subtype-specific Cre expression. This definitely is an important application, which is not possible using the RNP method.

Therefore, $Cas9^+$ mice, especially when they are already inbred as B6.*Cas9.Cd4cre.luc*.CD90.1 (or other Cre lines), are a suitable tool for comfortable subsequent studies. Although we avoided a repetition of GvL experiments with NFAT single-ablated T cells (14), successive experiments involving other genes or varying the GvHD model (acute GvHD due to minor mismatch or chronic GvHD) could easily include this aspect. In our context, we might want to knockout further NFAT target genes – additionally and in parallel – to test if the 'NFAT phenotype' is due to a certain gene's altered expression. The limited study with the ablated NFAT target gene *Irf4* demonstrated already that absence of IRF4, which is dominantly required for Th2, Th17 and $T_{FH}$ cell differentiation (45), provokes a disadvantageous Th1 phenotype under GvHD-inducing conditions. This is in contrast to allo-HCT with NFAT-deficient T cells, which can also implement specific T-helper characteristics (46), but restricted the overall cytokine expression irrespective of the individual NFAT family member ablated [this study and (14)]. Both, NFAT proteins and IRF4, enable proliferation including metabolic reprogramming of naive T cells by a shared pathway (29, 30, 47), a fact that limited T-cell expansion and ameliorated GvHD upon loss of NFATc1, NFATc2 or IRF4. Whether IRF4-deficient Tregs are equally able to preserve the GvL effect like NFAT single-deficient Tregs has to be tested next.

$Cas9^+$ mice and our protocols are suitable for translational studies. Sparked by our observation that ablation of a single NFAT member in co-transplanted T cells protects like clinical calcineurin inhibition (14), we want to proceed towards translation into the clinic. Here we found that we have to take enormous care in modifying only resting human T cells, as NFAT single-deficient effector T-cells would harm the allo-HCT-receiving patient even more than non-manipulated effector T cells would.

Overall, we introduce a method to gene edit murine primary T cells by CRISPR/Cas9, in efficiency comparable to RNP transduction (3), but faster and to our opinion even easier as documented to be capable in a mixture of $CD3^+$ T cells, which perform *in vivo* like naive $CD3^+$ T cells derived from gene-deficient mice.

## 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 author.

## ETHICS STATEMENT

The animal study was reviewed and approved by Government of Lower Frankonia (Regierung von Unterfranken/55.2.2-2532-2-592).

## AUTHOR CONTRIBUTIONS

SM designed and performed research as well as analyzed and discussed the data and took part in writing the manuscript. IJ, DS, and LB did experiments and analyzed data. NH and RS supported experiments. AB and AR offered resources or provided financial support and discussed the data. DM designed research and discussed data. FB-S conceptualized the research goals, acquired major funding, designed research, analyzed and discussed the data, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.683631/full#supplementary-material

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REVIEW
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# Recent Metabolic Advances for Preventing and Treating Acute and Chronic Graft Versus Host Disease

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The therapeutic efficacy of allogeneic hematopoietic stem cell transplantation (allo-HSCT) is limited by the development of graft-versus-host disease (GVHD). In GVHD, rigorous pre-conditioning regimen resets the immune landscape and inflammatory milieu causing immune dysregulation, characterized by an expansion of alloreactive cells and a reduction in immune regulatory cells. In acute GVHD (aGVHD), the release of damage- and pathogen- associated molecular patterns from damaged tissue caused by the conditioning regimen sets the stage for T cell priming, activation and expansion further exacerbating tissue injury and organ damage, particularly in the gastrointestinal tract. Studies have shown that donor T cells utilize multiple energetic and biosynthetic pathways to mediate GVHD that can be distinct from the pathways used by regulatory T cells for their suppressive function. In chronic GVHD (cGVHD), donor T cells may differentiate into IL-21 producing T follicular helper cells or tissue resident T helper cells that cooperate with germinal center B cells or memory B cells, respectively, to produce allo- and auto-reactive antibodies with subsequent tissue fibrosis. Alternatively, donor T cells can become IFN- γ/IL-17 cytokine expressing T cells that mediate sclerodermatous skin injury. Patients refractory to the first line standard regimens for GVHD treatment have a poor prognosis indicating an urgent need for new therapies to restore the balance between effector and regulatory immune cells while preserving the beneficial graft-versus-tumor effect. Emerging data points toward a role for metabolism in regulating these allo- and auto-immune responses. Here, we will discuss the preclinical and clinical data available on the distinct metabolic demands of acute and chronic GVHD and recent efforts in identifying therapeutic targets using metabolomics. Another dimension of this review will examine the changing microbiome after allo-HSCT and the role of microbial metabolites such as short chain fatty acids and long chain fatty acids on regulating immune responses. Lastly, we will examine the metabolic implications of coinhibitory pathway blockade and cellular therapies in allo-HSCT. In conclusion, greater understanding of metabolic pathways

involved in immune cell dysregulation during allo-HSCT may pave the way to provide novel therapies to prevent and treat GVHD.

**Keywords: metabolism, graft-versus-host disease, intestinal epithelial cells, alloreactive T-cells, graft-versus-tumor**

## INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is an effective and widely used cellular therapy for various malignant and benign hematological disorders. The beneficial effect of allo-HSCT is dependent on donor T cells which promote bone marrow engraftment and mediate the graft-versus-tumor (GVT) effect against residual cancer cells that survive conditioning regimen. However, the downside of donor T cell alloreactivity can be graft-versus-host disease (GVHD) which involves the attack of histocompatibility-disparate healthy recipient tissues and is the most common cause of non-relapse morbidity and mortality after allo-HSCT (1, 2).

Acute GVHD (aGVHD) generation requires a multi-step process (**Figure 1**). Proinflammatory early events begin with a conditioning regimen-mediated tissue injury that causes the release of inflammatory triggers, such as damage associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs). Inflammation rapidly recruits host cells of the innate and adaptive immune system that contribute to tissue injury and activation of antigen-presenting cells (APCs) of donor and host origin that prime the adaptive immune response. Following activation, alloreactive donor T cells expand, and differentiate into effector cells that secrete proinflammatory cytokines (2, 3). Effector cells are recruited to target organs where cytokines (i.e., IFN-γ) can cause tissue damage such as intestinal stem cell injury in the gastrointestinal tract and cytotoxic molecules (i.e., perforin) amplify end organ damage (2, 3).

Similar to aGVHD, chronic GVHD (cGVHD) is initiated by conditioning regimen injury and subsequent inflammation. Alloreactive T- and B- cells are activated by host APCs and T cells. T cells are polarized to Th1, Th2, or Th17 cells. Auto- and allo-reactive T cells escape deletion due to thymic injury and deficient generation of thymus-derived regulatory T cells (Tregs) leads to low numbers of peripheral Tregs, T follicular regulatory (Tfr) cells and immune dysregulation (**Figure 2**). Under these conditions, activated T cells can differentiate into T follicular helper (Tfh) or pre-Tfh cells that secrete IL-21 or IL-17, and signal B cells to produce auto- and allo- antibodies that are deposited in cGVHD target organs and contribute to further tissue injury and chemokines release (4). Fc receptors on recruited monocytes and macrophages are ligated by deposited immunoglobulin, stimulating fibroblasts to secrete extracellular matrix components favoring fibrosis that cause an obstructive lung disease known as bronchiolitis obliterans (1).

Despite differences in pathophysiology between aGVHD, characterized by a cellular tissue destructive process, and cGVHD characterized by T cell: B cell cooperativity, antibody deposition and fibrosis, corticosteroids are the first line therapy for both GVHD types. Steroids are broadly immunosuppressive, have considerable side-effects and increase susceptibility to opportunistic infections. Steroid refractory patients have a poor prognosis (2) highlighting the need to continue to pursue novel therapies to control GVHD, retain GVT response and avoid broad immune suppression. Recently strategies targeting metabolic pathways in immune cell populations have been garnering attention given the specialized substrate and energy requirements of immune cell types. The high proliferative, differentiation and migratory needs of GVHD-causing T- and B- cells; and of cells involved in tissue repair depend upon sufficient substrate availability that can be used by metabolic pathways for robust ATP production, biosynthesis, and reduction-oxidation (redox) reactions. Since these processes occur in different environments by distinct cell types, there is a therapeutic opportunity to inhibit GVHD pathogenesis while favoring cell repair mechanisms.

## METABOLIC PATHWAYS KNOWN TO CONTRIBUTE TO GVHD

Studies have linked glycolysis, fatty acid synthesis (FAS), and glutaminolysis to GVHD. During glycolysis, which occurs in the cytoplasm, glucose is broken down to generate two molecules of pyruvate and ATP (5). Pyruvate can enter the tricarboxylic acid (TCA) cycle to fuel ATP production or to support TCA cycle anaplerosis. The first step of the TCA cycle is the formation of citrate from acetyl-CoA and oxaloacetate. A complete turn of the TCA cycle yields GTP, $CO_2$ and reduced forms of nicotinamide adenine dinucleotide (NAD) + hydrogen (NADH) and flavin adenine dinucleotide ($FADH_2$). These each can be used as a cofactor for use by multiple enzymes or to shuttle electrons into the mitochondrial electron transport chain (ETC) Complex I and Complex II. The final electron acceptor is $O_2$; high amounts of ATP are produced in a process known as oxidative phosphorylation (OXPHOS) (5).

Highly proliferative cells can preferentially rely on ATP from cytosolic glycolysis instead of mitochondrial TCA cycle through a process called aerobic glycolysis. Also known as the Warburg effect (6), pyruvate is converted into lactate by lactate dehydrogenase (LDH), replenishing NAD+ that is required for glycolysis. Alternatively, pyruvate is decarboxylated by pyruvate dehydrogenase complex (PDH) to form acetyl-CoA that enters the TCA cycle eventually feeding into the mitochondrial ETC. Thus, PDH regulates the metabolic finetuning between glycolysis and FAO through regulation of acetyl-CoA.

Glycolysis also produces intermediates for the downstream synthesis of nucleotides and amino acids *via* the pentose phosphate pathway (PPP) and nicotinamide adenine dinucleotide phosphate + hydrogen (NADPH) for protection against oxidative

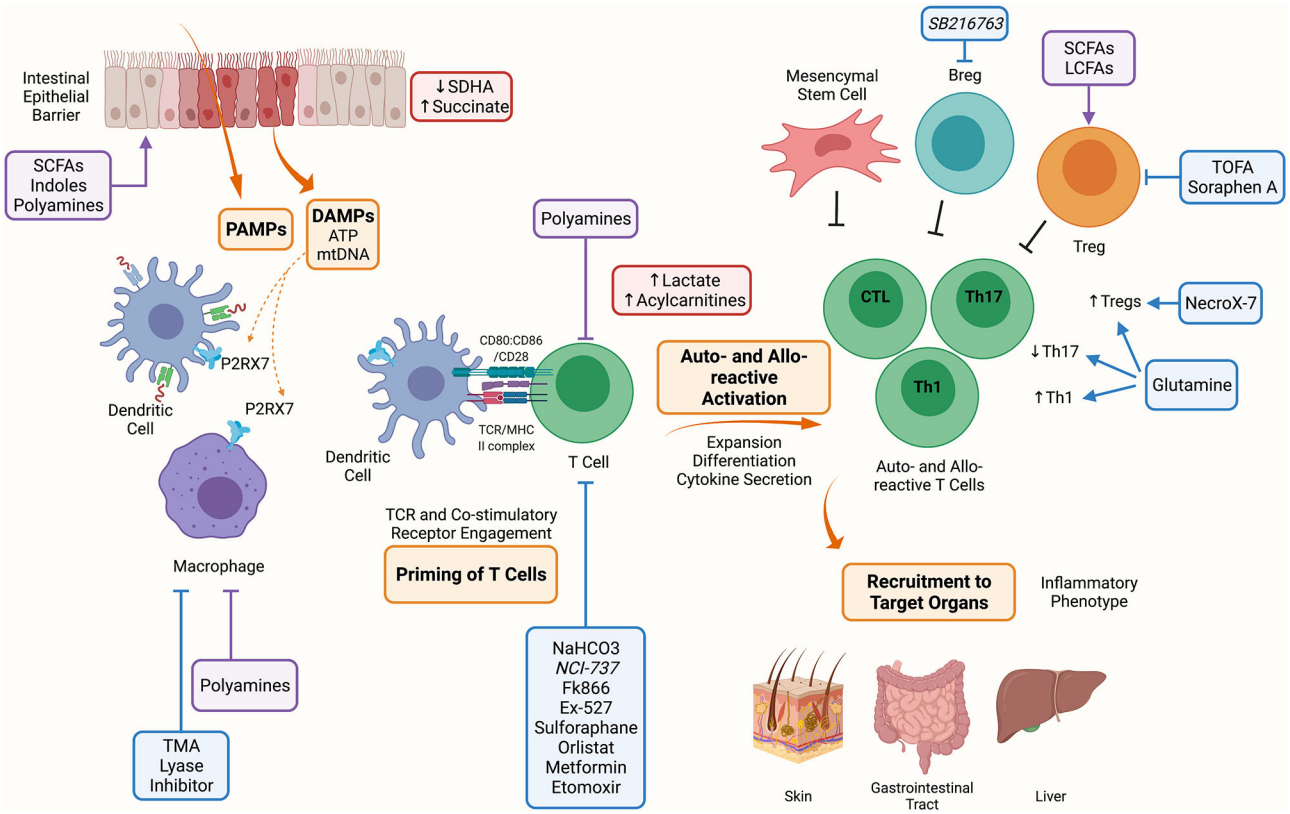


**FIGURE 1** | Metabolic Targets of Acute Graft Versus Host Disease. The conditioning regimen mediates tissue damage releasing PAMPs and DAMPs, these prime APCs to activate T cells. Alloreactive T cells expand and differentiate into inflammatory Th1, Th17 and CTLs. Alloreactive T cells contribute to the inflammatory milieu through secretion of IL-2, IFN-γ and TNF-a. These activated alloreactive donor T cells can be recruited to target organs including the skin, GI tract and liver to mediate the inflammatory phenotype characteristic of aGVHD. Indicated in blue are metabolic targets for the treatment of aGVHD. Trimethylamine (TMA) lyase inhibitor prevents the polarization of macrophages into M1 cells. Inhibitors against the nicotinamide phosphoribosyl transferase pathway (Fk866), lactate dehydrogenase (NCI-737), sirtuin-1 (Ex-527), lysosomal lipase (orlistat), fatty acid oxidation (etomoxir); along with activators of 5'-AMP-activated protein kinase (AMPK) (metformin) and nuclear factor 2 (sulforaphane) may or have been shown to target allo-reactive T cell function in aGVHD. $NaHCO_3$ has been shown to abrogate lactate accumulation in allo-reactive T cells. Inhibitors of fatty acid synthesis (FAS) (5-(Tetradecyloxy)-2-furoic acid (TOFA), Soraphen A), reactive oxygen species (NecroX-7), and glutamine administration improve the suppressive function and increase Tregs aiding to alleviate aGVHD. Lastly, GSK3β inhibitor (SB216763) has been shown to enhance regulatory B cells (Bregs) differentiation and suppressive function and may be a therapeutic target in aGVHD. Indicated in red are metabolites that have been shown to accumulate during aGVHD, these include succinate in IECs due to reduced SDHA activity, and lactate and acylcarnitines in allo-reactive T cells. Indicated in purple are microbial metabolites that act on cells during aGVHD, these include short chain fatty acids (SCFAs), long chain fatty acids (LCFAs), indoles and polyamines which can act on immune (macrophages, T cells, Tregs) and non-immune (IECs) cells. Created with BioRender.com.

stress (5). Metabolic reprogramming of T cells and other immune cell types to use of metabolic pathways that optimally exploit available substrates may prove advantageous by rapidly generating ATP in a substrate limited environment or promoting glycolytic intermediate flux into biosynthetic pathways (7). TCA cycle intermediates also can serve as connection points to multiple metabolic processes. For example, TCA intermediates, including citrate, itaconate, succinate, fumarate, and L-malate, can accumulate and regulate pro- and anti-inflammatory gene expression in immune cells (8). In the setting of cellular stress TCA intermediates can be released from the mitochondria and act as regulators of the immune system (8). Such intermediates promote reactive oxygen species (ROS) production that can signal inflammatory responses and mediate post-translational modification of metabolic pathway enzymes. The export of citrate and succinate from the mitochondria is linked to the production of pro-inflammatory mediators in macrophages including ROS, nitric oxide, and prostaglandin E2 (8, 9). Alpha ketoglutarate can regulate NF-kB signaling mediating pro-inflammatory responses (10) and mutations in the alpha ketoglutarate generating enzyme, isocitrate dehydrogenase (IDH), are associated with diseases of chronic inflammation (11). Conversely, itaconate, alpha ketoglutarate and fumarate enhance immunosuppression (8). Overall, TCA intermediates can have pro and/or anti-inflammatory effects.

Fatty acid oxidation (FAO) is linked to the FA beta-oxidation that occurs in the mitochondrial matrix, wherein lipids are metabolized to produce acetyl-CoA and electron carriers. The formation of malonyl-CoA from acetyl-CoA by acetyl-CoA carboxylase (ACC) early in FAS, inhibits carnitine palmitoyltransferase (CPT1a), the rate limiting step of FAO,

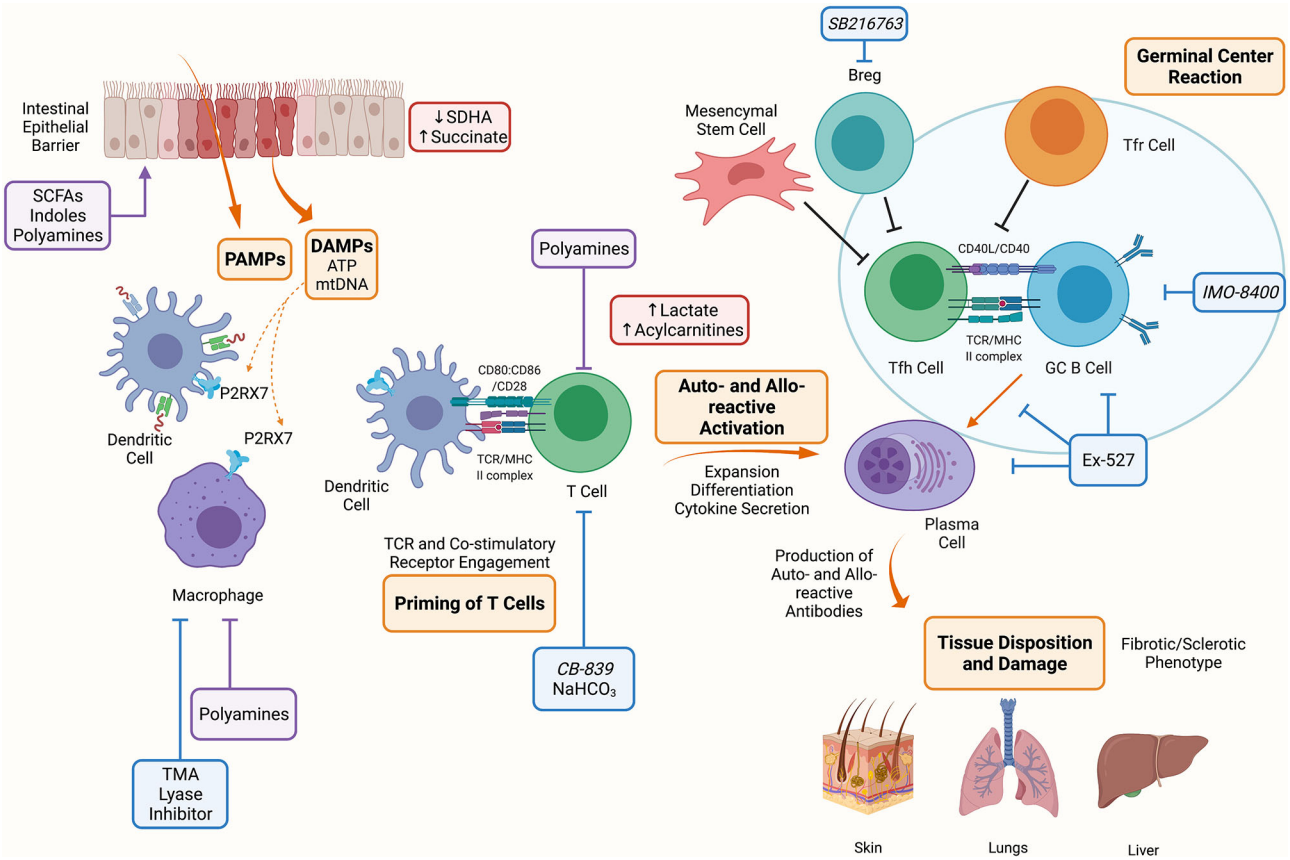


**FIGURE 2** | Metabolic Targets of Chronic Graft Versus Host Disease. The conditioning regimen mediates tissue damage releasing PAMPs and DAMPs, these prime APCs to activate T cells. Alloreactive T cells expand and differentiate into Tfh cells which provide costimulatory signals to B cells resulting in the formation of the germinal center and production of auto- and allo-antibodies. These antibodies deposit onto target organs such as skin, lungs and liver mediating the fibrotic and sclerotic phenotype characteristic of cGVHD. Indicated in blue are metabolic targets for the treatment of cGVHD. Trimethylamine (TMA) lyase inhibitor prevents the polarization of macrophages into M1 cells. $NaHCO_3$ has been shown to abrogate lactate accumulation in allo-reactive T cells. CB-839 is a glutaminase inhibitor that may be effective in cGVHD since GLS deficient T cells are unable to establish disease. Ex-527 is an inhibitor against Sirtuin-1 that reduced Tfh cell, B cell and plasma cell differentiation and IMO-8400 is a TLR 7/8/9 inhibitor that may be effective against B cells in cGVHD. Lastly, SBGSK3β inhibitor (SB216763) has been shown to enhance regulatory B cells (Bregs) differentiation and suppressive function and may be a therapeutic target in cGVHD. Indicated in red are metabolites that have been shown to accumulate during aGVHD, these include succinate in IECs due to reduced SDHA activity, and lactate and acylcarnitines in allo-reactive T cells. Indicated in purple are microbial metabolites that act on or influence the function of cells during cGVHD, these include short chain fatty acids (SCFAs), long chain fatty acids (LCFAs), indoles and polyamines which can act on immune (macrophages, T cells) and non-immune (IECs) cells. Created with BioRender.com.

strictly controlling the processes of FAS and FAO (12). The intricate balance of these metabolic pathways serves to support the effector function of rapidly proliferating, expanding and differentiating immune cells (**Figure 3**).

## MODULATING DONOR T CELL METABOLISM TO CONTROL GVHD AND PRESERVE GVT

### Glycolysis and Glutaminolysis

T cell function, including the ability of T cells to eliminate tumor cells, depends on their mitochondrial fitness (13). A recent study in allo-HSCT divulged the critical role of glycolytic activity of T cells in mediating GVT responses (14). Tumors evade immune surveillance by downregulating antigen presentation, secreting soluble cytokines, recruiting Tregs to the tumor milieu, and promoting factors that support immune tolerance and immune evasion (15). Leukemic cells can also evade T cell lysis by creating a distinct environment containing immune suppressive metabolites including lactic acid. T cells isolated from the same allo-HSCT patients exhibited distinct metabolic phenotypes based on the status of tumor relapse. T cells harvested during tumor relapse exhibited reduced glycolytic activity and OXPHOS compared to those harvested during remission. These findings correlated with increased serum lactic acid levels in tumor-relapsing patients. Mechanistically, production of lactic acid by tumor cells impaired T cell metabolic fitness, proliferation, and cytokine production and thus reduced GVT responses.

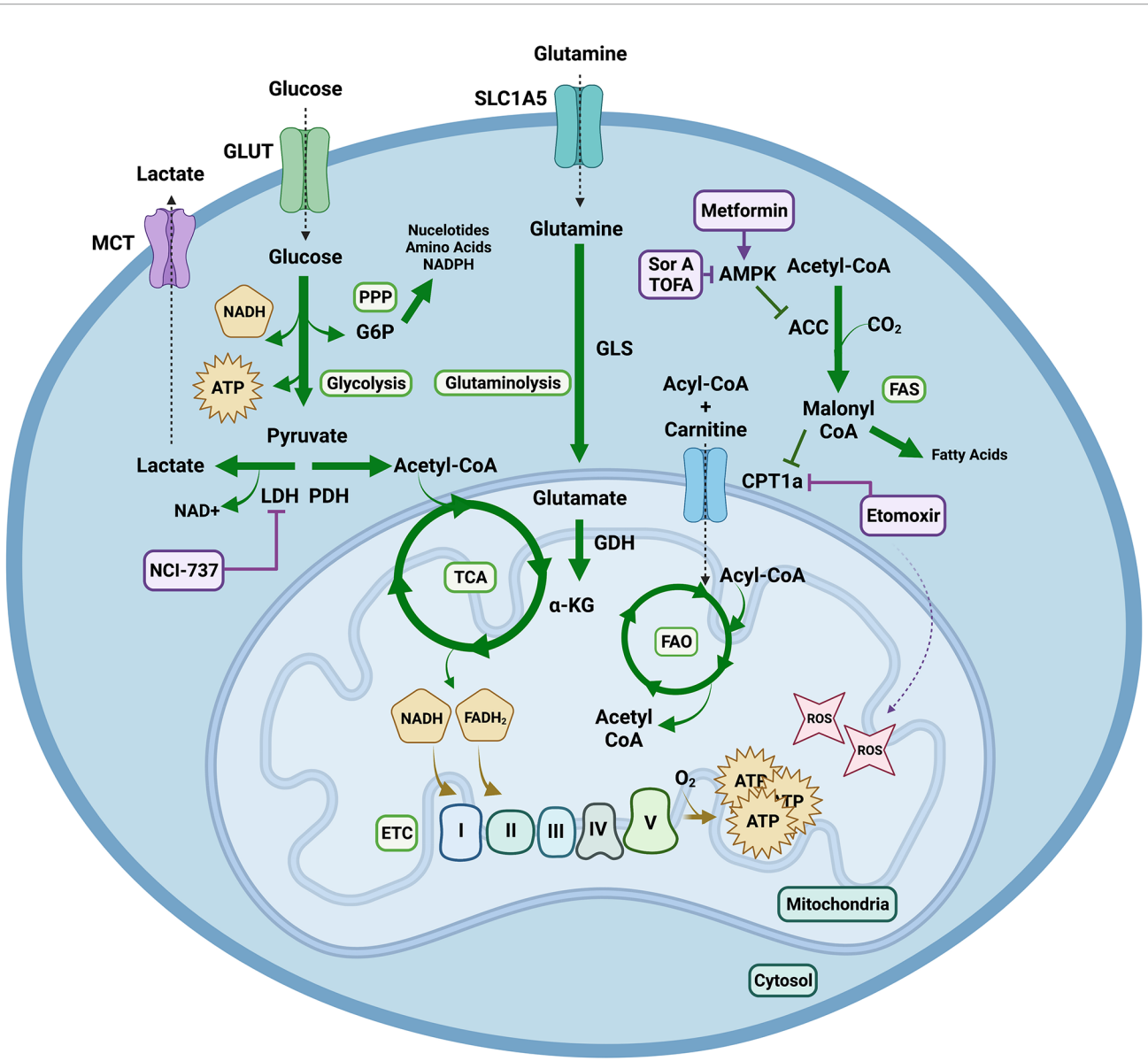


**FIGURE 3** | Metabolic Pathways Contributing to Graft Versus Host Disease. Extracellular glucose is transported into the cell and utilized to generate two molecules of pyruvate and ATP in glycolysis. Pyruvate has multiple fates. It can be converted into lactate *via* lactate dehydrogenase (LDH) replenishing nicotinamide adenine dinucleotide (NAD) + for glycolysis. This can be exploited by highly proliferative cells to continuously produce ATP *via* glycolysis, even in the presence of $O_2$, in a process known as aerobic glycolysis or the Warburg effect. Lactate can be exported from the cell through monocarboxylic transporters (MCT). Alternatively, pyruvate can be decarboxylated into acetyl-CoA *via* pyruvate dehydrogenase (PDH) to enter the tricarboxylic acid (TCA) cycle. One turn of the TCA cycle yields GTP, $CO_2$ and reduced forms of nicotinamide adenine dinucleotide hydrogen (NADH) and flavin adenine dinucleotide ($FADH_2$); which can shuttle electrons into the mitochondrial electron transport chain (ETC) where $O_2$ serves as the final electron acceptor to generate high amounts of ATP in a process known as oxidative phosphorylation (OXPHOS). OXPHOS can mediate production of reactive oxygen species (ROS) such as superoxide. During glutaminolysis glutamine is hydrolyzed into glutamate, which can be converted by glutamate dehydrogenase (GDH) into alpha ketoglutarate (α-KG) in order to enter the TCA cycle. Additionally, fatty acids (FA) undergo beta oxidation in the mitochondrial matrix to produce acetyl-CoA and electron carriers. The formation of malonyl-CoA from acetyl-CoA by acetyl-CoA carboxylase (ACC) early in fatty acid synthesis (FAS), inhibits carnitine palmitoyltransferase (CPT1a), the rate limiting step of fatty acid oxidation (FAO), strictly controlling the processes of FAS and FAO. Lastly, the nuclear energy sensor 5'-AMP-activated protein kinase (AMPK) can inhibit ACC releasing inhibition of CPT1a thus indirectly promoting FAO. Pharmacological agents targeting these metabolic pathways include NCI-737 inhibiting LDHA, metformin activating AMPK, soraphen A and 5-(Tetradecyloxy)-2-furoic acid (TOFA) inhibiting ACC and etomoxir inhibiting CPT1a. Notably concentrations of etomoxir above 5uM have been shown to induce ROS production. Created with BioRender.com.

At physiologic pH, lactic acid dissociates into H+ and $La^-$. Using $^{13}C$ lactate and LC/MS approaches, the authors showed that lactate is consumed by proliferating T cells in an MCT-1-dependent manner and integrated into their TCA cycle. Although speculative, lactate consumed in this manner may impede T cell function by end-point inhibition of glycolysis or an increase in reductive stress by skewing NADH/NAD+ ratios towards excess NADH accumulation. Interestingly their data

suggests that sustained exposure to elevated lactate above a critical threshold of 10mM (which didn't impede T cell function) whereas levels >15mM) impaired T cell function. Administration of sodium bicarbonate ($NaHCO_3$) to counteract acidosis, restored GVT responses in mouse tumor models by rescuing T cell metabolic fitness and function. Interestingly, a short-term treatment of $NaHCO_3$ in allo-HSCT patients increased their T cells' respiratory capacity and effector cytokine production. These findings provide evidence that the metabolic reprogramming of donor T cells may be exploited to enhance their GVT activity in patients with tumor relapse.

By employing novel, noninvasive hyperpolarized $^{13}C$-pyruvate magnetic resonance imaging (MRI) Assmann et al. (16) were able to diagnose GVHD prior to the onset of clinical manifestations in a mouse model of cGVHD. Imaging analysis at an early time-point identified that allo-HSCT mice had a higher conversion of pyruvate into lactate in the liver than those of syngeneic controls. However, no difference was observed at later timepoints which may be due to the change in metabolic shifts and reduced T cell activity. Further analysis using transcriptomic, metabolite and *ex vivo* metabolic activity assays demonstrated that pathogenic donor CD4+ T cells were highly glycolytic. Single cell sequencing of circulating CD4+ T cells isolated from two allo-HSCT patients revealed similar metabolic changes due to the increased transcription of glycolytic enzymes even before the onset of aGVHD clinical signs (16). Overall, this novel technique is informative in assessing the onset of GVHD and pointing to reducing glycolysis and "starving" GVHD pathogenic cells. Direct targeting of glycolysis can be achieved by administering an inhibitor such as NCI-737 to lactate dehydrogenase A (LDHA), the main enzyme responsible for the Warburg effect that has shown promise as an anti-cancer agent in preclinical trials (17). Metformin is an FDA approved biguanide with a low toxicity profile and is in widespread use as a treatment for type 2 diabetes and metabolic syndrome. Metformin lowers glucose by increasing insulin sensitivity, decreasing absorption, and blocking gluconeogenesis. Metformin effects on 5'-AMP-activated protein kinase (AMPK) are discussed in 3.3 below.

Activated effector T cells also need to increase their glutamine uptake to allow for adequate ATP production by glutaminolysis (18). Glutamine is a conditionally essential amino acid in proliferating cells and is hydrolyzed by glutaminase (GLS) to produce glutamate. Glutamate can be converted by glutamate dehydrogenase to alpha ketoglutarate to enter the TCA cycle or when combined with cysteine and glycine to form glutathione, an antioxidant that protects cells from detrimental redox reactions. Our recent study demonstrated that GLS deficiency impaired Th17 and promoted Th1 cell differentiation that was associated with altered gene expression and chromatin accessibility. GLS deficient T cells were unable to drive Th17 mediated inflammatory diseases (18). Relevant to GVHD, other reports have implicated Th17 cells in murine aGVHD pathogenesis (18), and a Th17-prone population in pre-symptomatic aGVHD patients (19). In aGVHD patients, a CD146+CCR5+ Th17-prone cell population correlated with disease (19). Glutamine administration in a mouse model of aGVHD inhibited tissue injury in target organs, increased FoxP3+CD4+CD25+ Tregs on Day 7, decreased serum TNF-α on Days 7, 14, and 21 after murine allo-HSCT and prolonged survival (20). Reduced plasma amino acid levels including glutamine have been characterized in patients after allo-HSCT and associated with systemic inflammation (21). Indeed in a retrospective study patients who receive glutamine supplementation had less clinically documented infection and 100-day mortality (22). In a murine multi-organ system model of cGVHD with bronchiolitis obliterans, GLS knockout (KO) T cells were unable to cause disease (18). Th17 cells support germinal center reactions, a hallmark of disease in this cGVHD model and pharmacological inhibitors (RORγt small molecules or anti-IL17 neutralizing antibody) against an activated Th17-prone T cell subset mitigated disease. Anti-IL-17 antibodies have been FDA approved. A small molecule GLS inhibitor, CB-839, is in advanced clinical testing as an anti-cancer agent (23, 24) and could be repurposed to treat cGVHD. In contrast, local (oral) glutamine delivery has been shown to reduce treatment-related mucositis in patients with cancer, presumably through its antioxidant effects (25).

## Reactive Oxygen Species

NAD is a key coenzyme involved in metabolic pathways including glycolysis, TCA cycle, OXPHOS, FAO and one carbon metabolism (serine biosynthesis) that must be continuously replenished (26). Under inflammatory conditions and cellular stress, the NAD synthesizing enzyme nicotinamide phosphoribosyl transferase (NAMPT), the rate-limiting enzyme in the NAD salvage pathway, was upregulated enabling increases in intracellular NAD levels. NAMPT regulates the activity of various NAD dependent enzymes such as poly ADP-ribose polymerases, CD38, CD73, and sirtuins (26). The levels of NAMPT were found to be elevated in acute GI-GVHD patients (27). Specifically, NAMPT expression was most pronounced in colonic CD3+ T cells of both mice and aGVHD patients (27). A small molecule NAMPT inhibitor, Fk866, enhanced cell cycle arrest at the G1 phase and increases p53 acetylation. *In vivo* administration of Fk866 ameliorated murine aGVHD by selectively inducing apoptosis of T effector cells while sparing Tregs and memory T cells important for pathogen and tumor clearance. Further, Fk866 downregulated gene expression of IFN-γ and TNF-α in T conventional cells, inhibited Th17 differentiation and promoted Treg Foxp3 expression and lineage stability. Consistent with the murine model results, Fk866 inhibited the proliferation of human T cells from healthy and GVHD patients and promoted both *in vitro* induced Treg (iTreg) and *in vivo* Treg generation. Since Fk866 maintained GVT activity against leukemia, immunometabolism strategies that inhibit NAMPT would have advantage over more global immunosuppressants.

Sirtuin-1 (Sirt-1) is a member of the class III family of histone deacetylases (HDACs) and has been shown to modulate cellular metabolism by acting as a cellular sensor (28, 29). Sirt-1 is active in both the nucleus and the cytoplasm, and its targets are key

regulators of various metabolic pathways. Sirt-1 inhibits T cell activation and the differentiation of Th1 and Th17 cells (30, 31). A recent study reported that Sirt-1 deficient T cells have impaired potential to produce IFN-γ and induce murine aGVHD (31). Deficiency of Sirt-1 promoted Treg differentiation and stability in aGVHD recipients. The effect of Sirt-1 inhibition was extended beyond the aGVHD models as the small molecule Sirt-1 inhibitor, Ex-527, prevented and reversed cGVHD. Mechanistic studies revealed that Sirt-1 deficiency reduced Tfh cell differentiation and reduced B cell activation and plasma cell differentiation. Attenuation of cGVHD with preservation of GVT was also observed in cGVHD mice treated *in vivo* with Ex-527 (31) that is in phase II clinical trials (32). Given its desirable properties discussed above, Ex-527 may be a candidate for clinical trials in GVHD.

The initiating events for acute and chronic GVHD start with the release of DAMPs from the conditioning regimen (1, 2). The DAMP ATP is released during necrosis which can activate the purinergic P2X7 receptor on APCs leading to subsequent activation of allo-reactive T cells (33). Compared to control mice, immune deficient NSG mice injected with human peripheral blood mononuclear cells to induce GVHD had increased murine P2RX7 in the duodenum, ileum, and skin (33). In addition to APC activation, P2X7R is required for the establishment, maintenance, and functionality of central and tissue resident memory T cells (34). Mechanistically P2X7R has been shown to promote mitochondrial homeostasis and metabolic function (34). During tissue damage, free DNA is also released, and high levels of free plasma mitochondrial DNA (mtDNA) have been associated with the onset of cGVHD (35). Plasma cell free mtDNA, measured from 39 adult patients post allo-HSCT with and without cGVHD (35), was found at significantly higher levels in cGVHD patients. These data correlated with B cell responsiveness to a TLR9 agonist, as shown by CD86 upregulation and known cGVHD biomarkers such as CXCL10, ICAM-1, CXCL9, sCD25 and sBAFF (35). Previous clinical trials have tested a TLR7,8, and 9 antagonist (IMO-8400) (36), offering the possibility that IMO-8400 or other TLR7,8, and 9 inhibitors could be repositioned for GVHD purposes.

Pre-conditioning regimens can exacerbate GVHD by increasing ROS and free radicals as well as reducing antioxidants. Upon allo-stimulation, donor T cells exhibited increased ROS production (37). Mitochondria derived ROS has been shown to be essential for T cell activation and proliferation (38, 39). Hence, multiple studies have attempted to prevent GVHD by reducing oxidative stress and maintaining redox balance in allo-HSCT preclinical models. Mitochondrial HDACs such as Sirt-3, the major mitochondrial sirtuin, control ROS production by promoting antioxidant scavenging mechanisms (40). Unexpectedly, loss of Sirt-3 in donor T cells attenuated aGVHD and retained the GVT response (41) rather than aggravating aGVHD. Mechanistically, Sirt-3 deficiency led to reduced ROS production in both non-specific TCR and allo-stimulated T cells, which may be indirectly due to impaired donor T cell activation. Interestingly, NAD+ and its cofactor nicotinamide compete with each other (42); nicotinamide has been shown to have clinical efficacy in cancer trials (43).

The redox master regulator nuclear factor (erythroid derived 2) factor 2 (Nrf2 or NFE2L2) is a transcription factor that maintains metabolic homeostasis by promoting antioxidant responses and anti-inflammatory responses. Experimental evidence showed that Nrf2 maintains immune tolerance and mitigates inflammation. While Nrf2 deficiency accelerated autoimmune diseases (44, 45), Nrf2 activation attenuated auto-inflammatory responses (46). However, studies of Nrf2 in murine allo-HSCT models have yielded conflicting results as Nrf2-deficient donor T cells induced less aGVHD morbidity and mortality. In contrast, sulforaphane an aliphatic isothiocyanate that activates Nrf2, ameliorated aGVHD (47, 48). In both models, GVT responses were preserved (48, 49) and the frequency of Tregs was increased. A recent study compared the expression pattern of Nrf2 on CD3+ T cells between allo-HSCT patients and healthy controls. Elevated expression of Nrf2 on both CD4+ and CD8+ T cells was observed in allo-HSCT patients, especially at time periods of cellular stress early post transplantation, then steadily declined over time and high Nrf2 expression in CD8+ T cells was associated with reduced cGVHD (50). Other studies also have explored the strategy of scavenging ROS to counteract oxidative stress by either administering NecroX-7, a necrosis inhibitor with an antioxidant mechanism, or by overexpressing antioxidant enzyme thioredoxin (51). Administration of NecroX-7 significantly improved aGVHD recipient survival which was correlated with the reduced levels of ROS and increased frequency of Tregs (51, 52). Donor allogeneic T cells overexpressing thioredoxin 1 had an impaired potential to induce aGVHD due to less ROS accumulation (52). This preclinical finding is promising as human recombinant Trx1treatment attenuated GVHD in both murine MHC mismatched and xenograft models, importantly without losing GVT responses (52).

## Lipid Metabolism

Activated alloreactive T cells in GVHD recipients have increased energy requirements to accommodate their expansile and effector functions (53). OXPHOS, shown to be utilized by T cells activated during GVHD, is the most efficient source of ATP. Multiple substrates can be used for OXPHOS. Upregulated FA transport, FAO enzymes, rates of FAO and transcriptional coactivators (53) during GVHD support the use of FA as the principal fuel source for alloreactive T cells (37). Pharmacological blockade of FAO by *in vivo* administration of etomoxir, a competitive inhibitor of CPT1a, resulted in decreased survival of donor alloreactive T cells without affecting T cells during normal immune reconstitution (53). Whereas BM cells from mice reconstituted by BM cells without T cells had increased aerobic glycolysis, alloreactive T cells in GVHD mice increased aerobic glycolysis and OXPHOS as well as accumulated acylcarnitines, indicating high FAO rates (37). These data suggest that inhibitors of OXPHOS or FAO may reduce GVHD without compromising hematopoietic reconstitution.

Lysosomal lipase (LAL), an intracellular lipase, hydrolyzes cholesteryl esters and triglycerides to produce free FAs and cholesterol. LAL is required for T cell development,

maturation, activation, and function (54, 55). Notably, LAL deficiency in CD4+ T cells impaired pathogenic Th1 differentiation and increased Treg generation (54, 55). Loss of LAL compromised metabolic homeostasis and immune function (56). Yu and colleagues used both genetic and pharmacological approaches to inhibit LAL in an aGVHD model. Donor T cell LAL deficiency were defective in aGVHD induction as a result of lower survival, migration potential and metabolic function (57). LAL-deficient CD4+ donor T cells exhibited decreased CPT1a expression and higher oxidative stress levels, consequentially increased lipid content. Although pharmacological inhibition of LAL using orlistat diminished aGVHD, GVT responses were maintained (57).

The energy sensor AMPK promotes FAO and mitochondrial biogenesis (12). Metformin activates heterotrimeric AMPK (58). *In vivo* metformin administration increased the ratio of Treg/Th17, enhanced autophagy and reduced mTOR/STAT3 signaling (59) resulting in the amelioration of murine aGVHD severity (59). In contrast, a recent study reported that the loss of AMPK in donor T cells attenuated aGVHD (60), suggesting a pAMPK independent mechanism of GVHD amelioration by metformin (60). Allogeneic murine and human T cells upregulated pAMPK during early aGVHD and xenogeneic GVHD respectively. Donor T cell AMPK$\alpha$1/$\alpha$2 deletion (AMPK KO) attenuated aGVHD in each of two distinct murine models without compromising GVT responses. Reduced aGVHD was due to decreased AMPK-KO donor T cells. Surprisingly, no difference was observed in the canonical AMPK-related pathways of FAO, autophagy, or mTOR signaling between donor AMPK-KO and wildtype (WT) T cells. Future studies are warranted to consider metformin in preventing and treating GVHD.

# MODULATING INTESTINAL METABOLISM

## Intestinal Epithelial Cells

Alterations in the intestinal microbiota has been implicated in multiple diseases including GVHD (61). In aGVHD and cGVHD intestinal damage and microbial dysbiosis are central to pathogenesis (1, 2). Indeed, intestinal epithelial cell (IEC) damage has been shown to contribute to alloimmune and autoimmune diseases such as inflammatory bowel disease (IBD) and GVHD (62, 63). IECs form mucosal and chemical barriers including antimicrobial peptides to protect the host from invading pathogens (64). A recent study investigated the metabolic changes of IECs in aGVHD mice (62). Oxygen consumption rates (OCR), an indicator of OXPHOS, in allogeneic IECs (allo-IECs) were significantly lower from syngeneic IECs (syn-IECs) controls. Mitochondrial TCA cycle metabolite composition in IECs obtained from GVHD mice revealed high levels of succinate with low levels of succinate dehydrogenase A (SDHA), a component of mitochondria respiratory complex II. SDHA links the TCA cycle with the ETC by catalyzing the oxidation of succinate to fumarate in TCA and donating electrons to the ETC (65, 66). SDHA loss in allo-IECs was mediated by donor T cell cytotoxic molecules granzyme B and perforin. Specific deletion of SDHA in allo-IECs aggravated aGVHD related mortality. SDHA expression in intestinal biopsies was significantly lower in the colon of gastrointestinal (GI) GVHD patients than those without histologically GI GVHD. Modulating IEC metabolism to sustain or replenish or replace SDHA and favor OCR in the context of GI GVHD would represent a novel treatment strategy.

## Immunomodulatory Function of Microbial Metabolites

Allo-HSCT results in intestinal microbiota dysbiosis due to the conditioning regimen, immune attack and broad-spectrum antibiotic use (67). In the 1970s, the role of intestinal microflora in modulating GVHD was surmised from studies of germ-free mice exposed to aGVHD conditions (67). While germ-free conditions and antibiotics mitigated experimental GVHD (68, 69), clinical studies involving bacterial decontamination in allo-HSCT patients yielded mixed results (70–72). In more recent studies, aGVHD intestinal inflammation was associated with major shifts in intestinal microbiota with a loss of overall diversity, expansion of *Lactobacillales* and loss of *Clostridiales (*73). *Lactobacillales* mediated significant aGVHD protection in mice and microbiota patterns in allo-HSCT patients mirrored those in mice. A retrospective study on 857 allo-HSCT patients reported that broad-spectrum antibiotics imipenem-cilastatin and piperacillin-tazobactam use increased GVHD mortality. A similar result was recapitulated in aGVHD mice treated with imipenem-cilastatin that had compromised intestinal barrier functions and loss of protective mucus lining (74). Microbes produce various metabolites from nutrients that influence intestinal immunity by acting as a bridge between microbes and the host immune system. Accumulating evidence suggests that microbial metabolites play a key role in tissue repair and immune regulation.

## Short Chain Fatty Acids

Short chain fatty acids (SCFA), primarily acetate, propionate, and butyrate, possess immunomodulatory properties that promote peripheral Treg generation (75), suppress Th17 generation (76) and modulate macrophage function (77). Butyrate, a nutrient source and an HDAC inhibitor, promotes IEC barrier function (78). Butyrate levels were significantly lower in the intestinal tissues of allo-HSCT mice due to the reduced IEC transporter expression and receptor activation. Increasing intestinal butyrate levels in allo-HSCT mice by oral administration of butyrate or bacteria that produce butyrate ameliorated aGVHD (79) that was associated with enhanced epithelial cell junctional integrity and function (79). Among SCFA sensors, the metabolic-sensor receptor, free-fatty acid receptor 2, was found to regulate IL-22-producing innate lymphoid cells (ILC3) that support intestinal stem cell proliferation and differentiation (80). The G-protein coupled receptor GPR43 that is activated by SCFAs proved critical for anti-GVHD effects mediated by butyrate and propionate and was reduced in allo-HSCT recipients (81). *Bacteroides fragilis*

administration reduced murine acute and chronic GVHD lethality in allo-HSCT by improving gut integrity through increased levels of SCFA acetic and butyric acid (82). Consistent with mouse studies, SCFA were found to be reduced in 42 pediatric allo-HSCT patients who developed aGVHD (83). In another study involving 201 patients, a positive correlation between increased aGVHD mediated mortality and loss of butyrogenic bacteria was seen (84). Allo-HSCT patients who developed cGVHD had lower plasma concentrations of propionate and butyrate than controls (85). Although these findings support SCFA as a therapeutic to alleviate GVHD, a recent clinical study reported conflicting results as patients who had higher butyrogenic bacteria after gut GVHD were more likely to develop steroid refractory aGVHD or cGVHD (86). Butyrogens may have a protective effect against aGVHD onset but may aggravate the disease in patients with GI GVHD.

Chronic GVHD also is associated with GI dysbiosis with a loss of fecal microbiota diversity. In a case-control cohort of adult transplant patients, analysis of stool samples at various timepoints throughout allo-HSCT showed that the samples from cGVHD and control transplant patients were comparably diverse before allo-HSCT (day -30) and in the peri-engraftment period. At ~day +100, some patients continued to have dysbiotic microbial composition while others returned to a pre-transplant microbial composition with no significant differences between the cGVHD and control transplant patients (85). Shotgun metagenomic sequencing of day +100 stool samples yielded enrichment of the microbial metabolic pathways related to SCFA metabolism. Plasma concentrations of butyrate and propionate were significantly lower in cGVHD patients compared to control transplant patients (85). Since SCFAs are produced after microbial fermentation by anaerobic bacteria, a Bayesian logistic regression of stool samples revealed that the presence of the anaerobic genera *Lachnoclostridum*, *Clostridium* and *Faecalibactrium* were associated with a reduced incidence of cGVHD (85). These studies demonstrate that alterations in the gut microbiome and the production of microbial metabolites such as SCFA have implications for GVHD pathogenesis and severity. A clinical trial of potato-based resistant starch ingestion during conditioning through day +100 after allo-HSCT as a source of SCFA is in progress (ClinicalTrials.gov Identifier: NCT02763033).

## Long Chain Fatty Acids

Recent studies investigated the role of long chain fatty acids (LCFA) such as palmitic acid (PA) and stearic acid (SA) in modulating the pathogenesis of aGVHD (87, 88). Wu et al. conducted a study of serum collected from 114 allo-HSCT patients and found that the ratio of SA/PA metabolite could be an excellent biomarker in the allo-HSCT recipients to predict both aGVHD and relapse (87). Patients with lower SA/PA ratio were more likely to develop grade II–IV aGVHD than those with higher SA/PA ratios (87). To further examine the role of SA or PA in the development of GVHD, allo-HSCT mice were either fed with high PA or SA diet (88). A high PA diet neither protected nor aggravated aGVHD lethality, in contrast to a high SA diet that resulted in the enrichment of *Akkermansia* genera, specifically *A. muciniphila*, and aggravated aGVHD severity. Fecal metabolomes revealed increased SCFA acetate, butyrate, and propionate in recipients fed a high SA diet as compared controls. *A.muciniphila* or acetate administration aggravated aGVHD mortality in control fed recipients, suggesting that the gut microbiota shift and associated SCFA metabolites (mainly acetate) modulate aGVHD pathogenesis. In line with the murine findings, higher concentrations of *A. muciniphila* and acetate were found in aGVHD patients than those of non-GVHD controls (88). Further studies are required to understand the role of other LCFA and their associated metabolites in regulating GVHD lethality.

## Amino Acid and Vitamin Derived Metabolites

Indoles are either derived from plant food or microbial metabolites of dietary tryptophan. Similar to SCFA, indoles support intestinal barrier function by engaging with aryl hydrocarbon receptors and promoting IL-22+ ILC3 cell maintenance (89, 90). Colonization of the intestines of allo-HSCT mice with indole-producing bacteria reduced pathology, attenuated aGVHD and improved survival (91). Oral gavage of indole-3-carboxaldehyde (ICA), an indole derivative, ameliorated aGVHD while not abrogating donor T cell mediated GVT responses. Microbial metabolites can also promote pro-inflammatory milieu and aggravate aGVHD. Wu et al. reported that a choline rich diet or choline metabolite trimethylamine *N*-oxide (TMAO) accelerated murine aGVHD lethality by inducing M1 macrophage polarization *via* the inflammasome component NLRP3 (92). Reducing TMAO level by treating allo-HSCT mice with a trimethylamine (TMA) lyases inhibitor effectively controlled choline diet-induced aGVHD. Likewise, taurine, a metabolite of bile acid, has been shown to activate NLRP6 inflammasome signaling to promote pro-inflammatory cytokines in allo-HSCT recipient mice and thus exacerbate aGVHD (93).

Mucosal-associated invariant T (MAIT) are innate-like T cells that produce large amounts of cytokines such as IL-17A in response to bacteria and yeast through recognition of riboflavin metabolites presented by the MHC class I–like molecule MR1 (94). Hill and colleagues found that recipient MAIT cells reduced aGVHD by promoting intestinal barrier function, regulating microbial diversity, and suppressing donor alloantigen presentation and T cell expansion while driving Th1 and Th17 cells in the colon post-allo-HSCT (95). Chronic GVHD patients had a reduced number of MAIT cells compared to those without cGVHD possibly due to gut microbiota changes in cGVHD patients, including alterations of species required for the expansion of MAIT cells (96). A recent study reported that MAIT cells may be used as universal cells for cellular therapy due to their lack of alloreactivity and potency in causing xenogeneic GVHD (97).

Polyamines, cationic biogenic amines are derived from dietary arginine by both host and microbes (89). A study

involving two cohorts of 43 and 56 patients reported that polyamine metabolites N-acetyl putrescine and N-acetyl spermidine were increased in allo-HSCT patients without GVHD (98). These metabolites have been shown to inhibit T cell (99) and pro-inflammatory macrophage activation (100) with an IEC protective role (101). Future investigation of the roles of microbial metabolites in clinical settings will provide more insight into their contributions to the pathogenesis of GVHD. Overall, altering the diet or microbiome to promote the production of beneficial metabolites and reduce the level of unwanted metabolites are a viable avenue to reduce GVHD.

## METABOLIC EFFECTS OF COINHIBITORY PATHWAY BLOCKADE AND CELLULAR THERAPY IN ALLO-HSCT

The inhibitory receptor, PD-1, inhibits glycolysis and promotes lipolysis and FAO (102, 103). PD-1 pathway blockade post-allo-HSCT augmented aGVHD in mice (104, 105) and patients (106). Increased programmed death ligand 1(PD-L1) expression was seen on donor T cells in mice and patients with aGVHD (107). PD-L1 KO donor T cells had enhanced apoptosis, diminished gut homing antigens, inflammatory cytokine expression, and a blunted aGVHD capacity without GVT loss (107). In GVHD mice, PD-L1 KO donor T cells had decreased glycolysis, OXPHOS, FAO, and glutaminolysis, along with increased ROS, likely contributing to the observed lower aGVHD lethality capacity.

Preclinical and clinical allo-HSCT studies have demonstrated that adoptive cellular therapy is an attractive option to reduce GVHD *via* restoring immune tolerance (108). Major hurdles hampering the wide clinical applications of cellular therapies are the requirement for expansion of low frequency regulatory cells to large numbers with retention of functionality and avoidance of plasticity in the inflammatory milieu of GVHD. Thus, studies have attempted metabolic reprogramming of regulatory cells to harness their potency and functionality in allo-HSCT settings. Among regulatory cells in controlling GVHD, Tregs have been extensively studied due to their capacity to suppress allo-immune responses. Acute GVHD patients had a lower Treg frequency with impaired stability than allo-HSCT patients without GVHD or healthy controls (109). The loss of stability due to the inflammatory milieu in allo-HSCT settings is partially dependent on the transcription factor STAT3 (110). Phospho-STAT3 inhibited peripheral Treg generation in murine aGVHD (110). Inhibition of STAT3 phosphorylation (pSTAT3) in human Tregs enhanced the suppressive capacity and stability of iTregs (111). Notably, pSTAT3- inhibited iTregs significantly reduced xenogeneic GVHD compared to vehicle control, while sparing donor GVT responses. Inhibiting pSTAT3 in iTregs induced a shift toward glycolysis by inhibiting OXPHOS (111). Metabolic reprogramming of pSTAT3- inhibited iTregs with Coenzyme Q10 treatment enhanced their suppressive capacity by elevating basal and restoring the maximal spare capacity (111). Inhibiting protein kinase C-theta increased suppression of thymic-derived Tregs, reduced mTORC2 signaling, increased OCR and upon adoptive transfer *in vivo*, decreased aGVHD mediated GI damage (112).These results support the concept that metabolic reprogramming of Tregs can be of therapeutic value to treat GVHD.

Lipid metabolism coordinates Treg proliferation and survival (113, 114). Liver kinase B1 (LKB1), a serine/threonine kinase, regulates cell growth and lipid metabolism (115, 116). Loss of LKB1impaired Treg function and stability (116–118). Tregs from aGVHD patients expressed lower LKB1 gene and protein expression than controls (109). Extending the clinical findings to a murine allo-HSCT model, the adoptive transfer of LKB1 deficient Tregs failed to control aGVHD (109). LKB1 overexpression in human Tregs partially rescued Foxp3 expression that regulates Treg stability and function. Acetyl-CoA carboxylase 1 (ACC1) catalyzes the first step in *de novo* FAS (119). Selectively deleting ACC1 in Tregs or treating Tregs *ex vivo* with an ACC1 inhibitor enhanced *in vitro* suppressive capacity and increased oxidative and glycolytic metabolism (120). Adoptive transfer of ACC1 KO Tregs reversed established cGVHD in a multi-organ system model with bronchiolitis obliterans. These studies suggest that modulating Treg lipid metabolism by either overexpressing LKB1 or employing FAS inhibitor may be a useful strategy to treat GVHD.

Over the past two decades, attention placed on testing of the adoptive transfer of mesenchymal stem cells (MSCs) to treat GVHD (121, 122) has shown variable therapeutic efficacy (123–125) that was associated with their plasticity and metabolic fitness in response to the inflammatory milieu (126, 127). Priming cord blood derived MSCs with an *in vitro* inflammatory cytokine regimen reprogrammed MSC metabolism to exhibit increased glycolytic capacity and superior immunosuppressive capacity manifested as increased survival in allo-HSCT and xenogenic GVHD recipients (122).

Glycogen Synthase Kinase 3 (GSK3) is a serine/threonine (ser/thr) protein kinase and metabolic sensor that regulates glycogen metabolism, gene transcription, cell survival and signaling (128). One isoform, GSK3β, has been shown to promote murine and human iTreg generation (129). Treatment of B cells with GSK3β inhibitor enhanced regulatory B cells (Bregs) differentiation and suppressive function (130). Chronic GVHD patients showed a reduced frequency of Bregs than allo-HSCT patients without GVHD (130). In a xenogeneic GVHD model, adoptive transfer of *ex-vivo* purified Bregs treated with the GSK3β inhibitor improved survival and reduced target organ damage in GVHD mice (130). Collectively, these studies lay a foundation for future research in exploiting the metabolic pathways to potentiate regulatory cell function in controlling and treating GVHD.

## CONCLUSION

A growing number of preclinical allo-HSCT studies are pointing to the importance of immunometabolism in modulating alloreactive donor T cell responses to control GVHD and promote GVT. Metabolic intervention with pharmacological

agents can harness regulatory cell potency and stability impairing pathogenic alloreactive donor T cell responses. Metabolic reprogramming of *ex-vivo* immune cells by gene editing technologies could be employed to target specific cell populations in an effort to enhance adoptive cellular therapy. A challenge in the field is the lack of clinical trials focused on metabolic interventions in GVHD. While there are retrospective metabolomic studies reporting correlative changes in host and microbiota-derived metabolites with aGVHD, prospective trials in various patient groups and treatment regimens are needed to identify metabolic pathways and targets for interventional trials. The clinicaltrials.gov website lists a limited number of clinical studies that have metabolism as one of the readouts. However, there is only one trial on clinicaltrials.gov (NCT02763033) specifically designed to prospectively alter metabolism for aGVHD prophylaxis and none are listed for aGVHD therapy or cGVHD prophylaxis or therapy. In the aGVHD prophylaxis study, allo-HSCT patients are being given potato-based resistant starch capable of increasing butyrate levels within the intestines to reduce rates of aGVHD (79). Future studies should focus on unraveling the relationship between metabolism and GVHD and of the metabolism-microbiota axis in order to select appropriate targets for intervention and to assess the safety and long-term effects of such metabolic interventions on infection risk and GVL in allo-HSCT clinical settings. Such deeper understanding of metabolic pathways and associated genes involved in immune cell dysregulation and non-hematopoietic cell damage during allo-HSCT should pave the way to provide novel therapies to prevent and treat GVHD.

## AUTHOR CONTRIBUTIONS

FM and GT reviewed the literature, drafted, and edited the manuscript. SR, PS, RO'C, JR, and BB reviewed, edited the manuscript draft, and critically revised the final manuscript. All authors contributed to the article and approved the submitted version.

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# Graft-Versus-Host Disease Prevention by *In Vitro*-Generated Myeloid-Derived Suppressor Cells Is Exclusively Mediated by the CD11b+CD11c+ MDSC Subpopulation

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Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of myeloid progenitor cells that dampen overwhelming adaptive immune responses through multiple mechanisms and are recognized as an attractive novel immune intervention therapy for counteracting the destructive effects of graft-versus-host disease (GVHD) developing after allogeneic bone marrow transplantation (BMT). MDSCs can be produced in great numbers for cellular therapy, but they present a mixture of subsets whose functions in GVHD prevention are undefined. Here, we generated MDSCs *in vitro* from murine BM cells in the presence of GM-CSF and defined the integrin CD11c as a marker to subdivide MDSCs into two functional subgroups: CD11b+CD11c+ and CD11b+CD11c– MDSCs. Isolated CD11b+CD11c+ and CD11b+CD11c– MDSCs both inhibited alloantigen-stimulated T-cell proliferation *in vitro*, although CD11b+CD11c+ MDSCs were more efficient and expressed higher levels of different immunosuppressive molecules. Likewise, expression of surface markers such as MHC class II, CD80, CD86, or PD-L1 further delineated both subsets. Most importantly, only the adoptive transfer of CD11b+CD11c+ MDSCs into a single MHC class I-disparate allogeneic BMT model prevented GVHD development and strongly decreased disease-induced mortality, while CD11b+CD11c– MDSCs were totally ineffective. Surprisingly, allogeneic T-cell homing and expansion in lymphatic and GVHD target organs were not affected by cotransplanted CD11b+CD11c+ MDSCs indicating a clear contradiction between *in vitro* and *in vivo* functions of MDSCs. However, CD11b+CD11c+ MDSCs shifted immune responses towards type 2 immunity reflected by increased Th2-specific cytokine expression of allogeneic T cells. Induction of type 2 immunity was mandatory for GVHD prevention, since CD11b+CD11c+ MDSCs were ineffective if recipients were reconstituted with STAT6-deficient T cells unable to differentiate into Th2 cells. Most importantly, the beneficial graft-versus-tumor (GVT) effect was maintained in the presence of CD11b+CD11c+ MDSCs since syngeneic tumor cells were efficiently eradicated. Strong differences in the transcriptomic

landscape of both subpopulations underlined their functional differences. Defining CD11b+ CD11c+ MDSCs as the subset of *in vitro*-generated MDSCs able to inhibit GVHD development might help to increase efficiency of MDSC therapy and to further delineate relevant target molecules and signaling pathways responsible for GVHD prevention.

**Keywords: graft-versus-host disease, prophylaxis, myeloid-derived suppressor cells, allogeneic bone marrow transplantation, mouse model, type 2 immune response, GVT effect**

## INTRODUCTION

In the year 2007, myeloid-derived suppressor cells (MDSCs) were introduced as a heterogeneous population of myeloid progenitors with potent immunosuppressive functions (1, 2) that expand under various inflammatory pathological conditions such as chronic inflammation, autoimmune diseases, infections, and cancer. Under inflammatory conditions, MDSCs fail to complete their regular differentiation into mature macrophages, granulocytes, or dendritic cells and are phenotypically and functionally distinct from normal myeloid cells. T cells are the preferred and major targets of MDSCs. MDSCs inhibit T-cell responses by versatile mechanisms including nutrient depletion, nitrosylation, apoptosis, or blockade of lymphocyte homing or induction of Tregs (3, 4). By studying the role of MDSCs in different disease entities, it turned out that they strongly contribute to the decision whether immune responses develop towards type 1 or type 2 immunity. MDSCs shift the balance towards Th2 immunity in pathologies such as sepsis, viral infections, or certain types of cancers (5–8), while they support Th1 immunity in Th2-driven asthma-related airway inflammation (9, 10).

Considering their immunomodulatory functions, MDSCs represent attractive candidates to counterbalance overwhelming immune responses associated with T-cell-mediated diseases. Graft-versus-host disease (GVHD) represents a disease which develops after allogeneic bone marrow (BMT) transplantation and is induced by activation and expansion of alloantigen-activated mature transplant-derived T cells. These donor T cells attack and destroy recipient tissue finally leading to life-threatening posttransplantation complications, which dramatically limit the success of allogeneic stem cell transplantation for treatment of hematological malignancies and genetic disorders (11). However, transplanted allogeneic T cells also mediate the graft-versus-tumor effect (GVT), which ensures eradication of alloantigen expressing residual tumor cells. Therefore, GVHD treatment strategies aiming to interfere with allogenic T-cell activation, proliferation, and function should be balanced in terms to prevent allogeneic T-cell-mediated tissue destruction while simultaneously guaranteeing efficient T-cell immunity to cope with infections and destroy residual tumor cells (12).

MDSCs for adoptive cell therapy of GVHD can be successfully generated in mice. MDSCs can be directly isolated from tumor-bearing mice (13) or after *in vivo* administration of 3′5′-cytidylylguanosine (CpG), granulocyte-colony stimulating factor (G-CSF), or a synthetic G-CSF/Flt-3 ligand. Subsequent adoptive transfer of the isolated MDSCs in allogeneic BM recipients efficiently prevents GVHD development (13–16). Different precursor cells and cytokine combinations are suitable for *in vitro* induction and expansion of MDSCs. While Zhou et al. used mouse embryonic stem cells activated with a mixture of cytokines in a three-step differentiation strategy (17), MDSCs currently are mostly induced by culturing unseparated BM cells in GM-CSF alone or in combinations with cytokines such as G-CSF or IL-13 (18–20). Independent of the cytokine combination used, adoptive transfer of *in vitro*-generated MDSCs efficiently prevents GVHD induction, while tumor reactivity in MDSC-treated mice is maintained. Although randomized trials proving therapeutic potential of MDSCs in humans are lacking, promising results are obtained from humanized mouse models receiving *in vitro*-expanded human MDSCs for prevention of xenogeneic GVHD (21, 22).

MDSCs either isolated *ex vivo* from tissues or generated *in vitro* from hematopoietic precursor cells always present a mixture of cells. Classically, unseparated murine MDSCs coexpress CD11b and Gr-1 and expression of Ly-6C and Ly-6G further subdivides MDSCs into the two major subpopulations: monocytic (M) MDSCs ($CD11b^{+}Ly\text{-}6G^{-}Ly\text{-}6C^{high}$) and polymorphonuclear (PMN) MDSCs ($CD11b^{+}Ly\text{-}6G^{-}Ly\text{-}6C^{high}$) (23, 24). Since Ly-6G and Ly-6C are also expressed on differentiated monocytes and mature neutrophils, further marker panels have been designed including transcription factors, cytokines, and effector molecules to distinguish MDSC subsets from fully matured myeloid cells (25), but the final identification as MDSCs is always designated by their ability to mediate T-cell suppressive functions (26, 27).

Currently, it is not defined which subset of MDSCs contribute to immunosuppression and GVHD prevention in the context of allogeneic BMT. In recent work, we could show that adoptive transfer of CD11b+Gr-1+ MDSCs induced from BM cells in the presence of GM-CSF efficiently prevented GVHD development in two different allogeneic BMT models. GVHD inhibition was mostly attributed to the ability of MDSCs to shift the immune response in the transplanted recipients towards type 2 immunity (18). Interestingly, phenotypic characterization of the *in vitro*-generated MDSCs indicated that these cells were not a unique population but could be distinguished by the expression of the integrin CD11c. CD11b+CD11c+ MDSC subpopulations exhibited increased expression of CD301b, which expression is linked to the Th2-inducing abilities of DCs (28). Additionally, transcription factors IRF4 and Klf4 also associated with Th2 induction (29, 30) were upregulated compared with CD11b+CD11c– MDSCs indicating that both subpopulations might exhibit different properties in GVHD prevention. In the current study, we therefore aimed to

further characterize CD11b+CD11c+ and CD11b+CD11c− MDSCs for their T-cell suppressive capacities and their function in GVHD prevention. Although both subsets suppressed T-cell proliferation *in vitro*, only CD11b+CD11c+ MDSCs prevented GVHD development after allogeneic BMT while maintaining tumor cytotoxicity. GVHD prevention was totally dependent on the ability of CD11b+CD11c+ MDSCs to shift the immune response towards type 2 immunity. Strong differences in the transcriptomic landscape of both MDSC subsets further underlined their functional differences and might be used in further studies to delineate molecules and pathways responsible for MDSC-mediated GVHD inhibition.

# MATERIAL AND METHODS

## Tissue Preparation

### Bone Marrow

Bone marrow (BM) cells were isolated with 26-gauge needle from femurs and tibias. Single-cell suspensions were prepared using a syringe with 20-gauge needle, and erythrocytes were depleted.

### Spleen

Splenic single-cell suspensions were prepared by pouring the spleen through a 70-µm cell strainer followed by erythrocyte depletion.

### Liver

Liver was perfused by the injection of 5 ml liver perfusion medium (Gibco, Carlsbad, CA, USA), followed by 5 ml liver digest medium (Gibco) into the vena cava inferior. Without the gall bladder, liver was digested for 30 min at 37°C in 10 ml liver digest medium. Single-cell suspensions were prepared by pouring the liver through a 70-µm cell strainer. Liver cells were suspended in 35% Percoll (Sigma-Aldrich, St. Louis, MO, USA), followed by overlaying cells onto 70% Percoll. The gradient was centrifuged at 2,000 rpm for 20 min. Interfaces containing liver leukocytes were collected, and residual erythrocytes were depleted.

### Serum

Serum was collected from submandibular blood. Serum was stored at −80°C in cytokine stabilization buffer (U-CyTech Biosciences, Utrecht, Netherlands) (1:20 of collected serum volume) until ProcartaPlex Multiplex immunoassays (ThermoFisher Scientific, Waltham, MA, USA) were performed.

## MDSC *In Vitro* Generation

MDSCs were generated *in vitro* by incubating freshly isolated BM cells with 250 U/ml murine GM-CSF for 4 days at 37°C in an atmosphere with 7.5% $CO_2$. BM cells at $9 \times 10^6$–$1 \times 10^7$ were cultured in α-minimum essential medium (Lonza, Basel, Switzerland), 10% fetal calf serum (Sigma Aldrich), 2 mM l-glutamine (Gibco), 1 mM sodium-pyruvate (Gibco), 100 U/ml penicillin-streptomycin (Gibco), and 0.05 mM 2-mercaptoethanol (Gibco) in Ø 15 cm culture dishes "Cell+" (Sarstedt, Germany).

## Isolation of CD11b+CD11c+ and CD11b+CD11c− MDSCs

CD11b+CD11c+ MDSCs were positively isolated by magnetic-activated cell sorting using anti-CD11c MicroBeads (Miltenyi, Bergisch Gladbach, Germany) according to manufacturer's protocol. CD11b+CD11c- MDSCs were isolated from the flow-through of CD11c isolation by loading the flow-through on a depleting LD column (Miltenyi). Purity of both MDSC subpopulations ranged between 85% and 99%.

## Isolation of CD3+ T Cells

CD3+ T cells were positively isolated from splenic single-cell suspensions by magnetic-activated cell sorting using the CD3ϵ MicroBead Kit (Miltenyi) according to manufacturer's protocol. Purity of isolated T cells was over 70%.

## Mice and Bone Marrow Transplantation

### Mice

Mouse strains used are listed in **Supplementary Table S1**.

### BMT

One day before BMT, B6.bm1 recipient mice received total body irradiation with 12 Gy split in two doses 3 h apart from a $^{137}$Cs source. BM cells were depleted from T cells as described previously (18, 19). Mice were intravenously reconstituted with $5 \times 10^6$ T-cell-depleted BM (TCD-BM) in the presence or absence of $2 \times 10^7$ spleen cells (SC). *In vitro*-generated CD11b+CD11c+ or CD11b+CD11c− MDSCs at $1 \times 10^6$ were coinjected with the transplant. In studies analyzing the GVT effect $5 \times 10^4$ JM6 thymoma (18) were coinjected with the transplant. Clinical GVHD was evaluated according to Cooke et al. (31) by evaluating the parameters weight loss, activity, posture, fur texture, and skin integrity. Animals euthanized during the experiment due to their moribund state remained included in the calculation until the end of experiment with their final GVHD scores. All animal experiments were performed according to the international regulations for the care and use of laboratory animals and were approved by the local ethical committee Regierungspräsidium Tübingen, Germany.

## Carboxyfluorescein Diacetate Succinimidyl Ester Labeling

Cells at $2 \times 10^7$ in 10 ml PBS containing 5% FCS were labeled with 50 µM carboxyfluorescein diacetate succinimidyl ester (CFSE) (ThermoFisher Scientific, MA, USA) for 10 min at 37°C in the dark.

## Mixed Lymphocyte Reaction

CFSE-labeled B6.SJL-derived SCs at $2.5 \times 10^5$ were stimulated with $2.5 \times 10^5$ irradiated (33 Gy) DBA/2-derived SCs in the absence or presence of B6-derived CD11b+CD11c+ or CD11b+CD11c− MDSCs. iNOS was inhibited using 500 µM L-$N^G$-monomethyl-arginine-citrate (L-NMMA) (Merck, Darmstadt, Germany) and PD-L2 was blocked using 10 µg/ml antimouse PD-L2 antibodies (Biocell, St. Irvine, CA, USA). Mixed lymphocyte reactions

(MLRs) were cultured in α-MEM medium (Lonza) supplemented with 10% FCS (Sigma Aldrich), 2 mM l-glutamine (Gibco), 1 mM sodium-pyruvate (Gibco), 100 U/ml penicillin-streptomycin (Gibco), and 0.05 mM 2-mercaptoethanol (Gibco) for 4 days at 37°C in an atmosphere with 7.5% $CO_2$. After 4 days, T-cell proliferation was determined using flow cytometry and percentage of T-cell suppression was calculated.

## Flow Cytometry

Cells at $5 \times 10^5$–$1 \times 10^6$ were stained with respective fluorochrome-conjugated antibodies. Antibodies used are listed in **Supplementary Table S2**. Flow cytometric analyses were performed on a LSR II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

## Quantitative Reverse-Transcription Polymerase Chain reaction

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed using the SsoAdvanced™ Universal SYBR® Green Supermix (BIO-RAD, Irvine, CA, USA) and analysis was performed on a CFX Connect Optics Module (BIO-RAD). Relative expression was determined using the comparative $C_T$ method. Mouse aryl hydrocarbon receptor-interacting protein (AIP) was used as a housekeeping gene. Primer sets used are listed in **Supplementary Table S3**.

## Cytokine Analysis Using ProcartaPlex™ Multiplex Immunoassay

Cytokine concentrations of 12.5 µl blood serum or 25 µl cell culture supernatant were analyzed by ProcartaPlex™ multiplex immunoassays (ThermoFisher Scientific) according to manufacturer's protocol. Analyses were performed on a BIO-RAD Bioplex 200 system (BIO-RAD).

## RNA Isolation and Quality Control

FACS-sorted CD11b+CD11c+ and CD11b+CD11c− MDSCs were collected in RLT buffer (QIAGEN, Hilden, Germany) supplemented with 2-mercaptoethanol followed by RNA extraction using the RNeasy Mini Kit (Qiagen) and adding an on-column DNA digestion step according to manufacturer's instructions. Total RNA was quantitatively and qualitatively assessed using the absorbance-based Take3 microvolume plate system on a Cytation 5 instrument (BioTek, Bad Friedrichshall, Germany) and the Standard Sensitivity RNA Analysis DNF-471 Kit on a 12-channel Fragment Analyzer (Agilent Technologies, Santa Clara, CA, USA), respectively. Concentrations averaged at 310 ng/µl while RIN values ranged from 8.6 to 10, with an average of 9.8.

## Whole Transcriptome Profiling With PolyA Enrichment (mRNA-Seq)

MDSC-derived RNA samples were normalized, and a RNA input of 100 ng was used for library construction with the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina #E7760, together with the NEBNext Poly(A) mRNA Magnetic Isolation Module #E7490 upstream and the NEXNext Multiplex Oligos for Illumina #E7600 downstream (New England Biolabs, Frankfurt am Main, Germany). Ampure XP beads (Beckman Coulter, Brea, CA, USA) were used for double-stranded cDNA purification. mRNA sequencing libraries were quantified by the High Sensitivity dsDNA Quanti-iT Assay Kit (ThermoFisher Scientific) on a Synergy HTX (BioTek). Library molarity averaged at 134 nM. Final library size distribution was assessed (smear analysis of 364 bp average and adapter dimer presence <0.5%) by the High Sensitivity Small Fragment DNF-477 Kit on a 12-channel Fragment Analyzer (Agilent Technologies). All sequencing libraries passed quality check, were normalized, pooled, and spiked in with PhiX Control v3 (Illumina, San Diego, CA, USA). The library pool was subsequently clustered with the HiSeq 3000/4000 SR Cluster Kit on a cBot and sequenced on a HiSeq 3000 Sequencing System (Illumina) with single index, single read at 85 bp length (Read parameters: Rd1: 85, Rd2: 8), reaching an average depth of 29 million Pass-Filter reads per sample (11% CV).

## mRNA-Seq Computational Analysis

Illumina reads were converted to FASTQ files and aligned to the mouse reference genomes from Ensembl 70 (http://www.ensembl.org) using the STAR v2.5.2 program on default settings (32). SAM files were converted by samtools v0.1.18 (33) to BAM files. Sequenced read quality was checked with FastQC v0.11.2 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and alignment quality metrics were calculated using the RNASeQC v1.1.8 (34). Duplication rates were assessed with bamUtil v1.0.11 (35) and dupRadar v1.4 (36). Gene expression levels were quantified by Cufflinks v2.2.1 (37) to get reads per kilobase per million mapped reads (RPKM) as well as FeatureCounts (38) to get read counts. Differential expression analysis was performed based on voom-normalized (39) read counts as input for the Bioconductor R package LIMMA (40). The batch number was used as a factor in the LIMMA linear regression model. *p*-values were corrected for multiple testing by Benjamini–Hochberg. Complete mRNA-sequencing (mRNA-Seq) data are available at Gene Expression Omnibus (GEO accession number: GSE182262).

## Statistics

Data were analyzed using Mann-Whitney *U* test or unpaired Student's *t*-test. For multiple comparisons ANOVA Tukey multiple comparison test or Kruskal-Wallis test were used. Survival studies were analyzed using Log-Rank (Mantel-Cox) test. Results were considered significant if $p < 0.05$. Statistical tests were performed with GraphPad Prism 8.

# RESULTS

## Expression of CD11b and CD11c Distinguishes Two Subpopulations of *In Vitro*-Generated MDSCs

MDSCs were generated from BM cells in the presence of GM-CSF. After 4 days, more than 90% of cells expressed CD11b and Gr-1

indicative for successful MDSC generation *in vitro* (**Figure 1A**). By staining CD11b and CD11c *in vitro*-generated MDSCs could be separated into two major subpopulations. Eighty percent of MDSCs exhibited solely CD11b positivity, while 20% coexpressed CD11b and CD11c (**Figure 1B**). CD11b+CD11c+ MDSCs could be distinguished from CD11b+CD11c– MDSCs by decreased Gr-1 expression. To assign both MDSC subsets to M-MDSCs (Ly-6C$^{high}$Ly6G$^{-}$) or PMN-MDSCs (Ly-6C$^{low}$Ly6G$^{+}$), we costained for Ly-6C and Ly-6G. CD11b+CD11c+ MDSCs consist of about 60% M-MDSCs and low percentage of PMN-MDSCs, while 20% of the cells neither expresses Ly-6G and Ly-6C. CD11b+CD11c– MDSCs represent a mixture of M-MDSCs (about 50%) and PMN-MDSCs (40%) (**Figure 1C**). To further define differences between both MDSC subpopulations, we analyzed expression of surface markers often coexpressed on CD11c-positive cells. CD11b+CD11c+ MDSCs exhibited increased expression of antigen-presenting cell (APC)-associated markers MHC class II (I-A$^{b}$), F4/80, CD40, the activating costimulatory molecules CD80 and CD86, as well as the inhibitory molecules PD-L1 (CD274) and PD-L2 (CD273) (**Figure 1D**). These results clearly show that by using GM-CSF for MDSC generation *in vitro*, most of the cells exhibit the classical CD11b+CD11c– phenotype, while about 20% of cells showed coexpression of CD11c and APC-associated markers.

## CD11b+CD11c+ MDSCs Exhibit Increased Immunosuppressive Capacity Compared With CD11b+CD11c– MDSCs *In Vitro* and Shift the T-Cell Response Towards Type 2 Immunity

Since CD11c is expressed on APCs such as dendritic cells, macrophages, and a small subset of B cells, we next defined whether CD11b+CD11c+ MDSCs exhibit immune-activating or suppressing functions. Therefore, B6-derived *in vitro*-generated MDSCs were separated by CD11c Micro Beads into CD11b+CD11c+ and CD11b+CD11c– MDSCs (**Supplementary Figure S1A**) with a purity of about 95% for both populations (**Supplementary Figure 1B**). Purified MDSCs were added at different numbers to CFSE-labeled B6-SJL (H-2$^{b}$, CD45.1+) spleen cells, which were activated by DBA/2-derived (H-2$^{d}$, CD45.2+) irradiated spleen cells. Using the congenic marker CD45.1 expressed solely on CFSE-labeled effector cells, proliferation of CD45.1+CD4+ and CD45.1+CD8+ T cells was determined. Both MDSC subpopulations efficiently suppressed T-cell proliferation, but CD11b+CD11c+ MDSCs exhibited strongly increased inhibitory capacity especially towards CD8+ T-cell proliferation (**Figure 2A**). Due to the differences in the

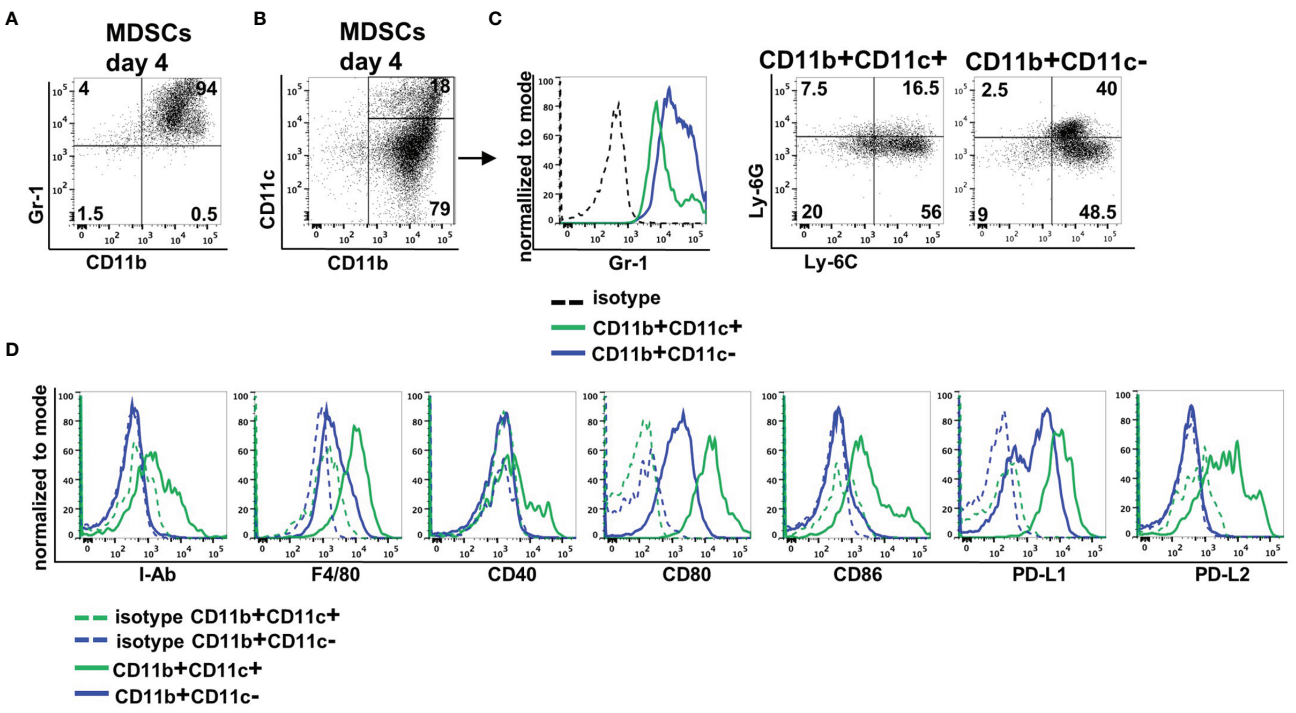


**FIGURE 1** | *In vitro*-generated MDSCs consist of CD11b+CD11c+ and CD11b+CD11c– MDSCs. MDSCs were generated from BM cells in the presence of GM-CSF. **(A)** After 4 days, MDSCs were stained for CD11b and Gr-1 expression or **(B)** for CD11b and CD11c expression. **(C)** Expression of Gr-1 was determined on CD11b+CD11c+ and CD11b+CD11c– MDSCs, and CD11b+CD11c+ and CD11b+ and CD11c+ MDSCs were designated to M-MDSCs or PMN-MDSCs by staining Ly-6C and Ly-6G. **(D)** Staining of various surface markers to define differences in the expression on CD11b+CD11c+ and CD11b+CD11c– MDSCs. FACS diagrams show one representative experiment out of at least three experiments performed.

immunosuppressive capacity, expression of molecules attributed to mediate suppression was defined in the isolated MDSC subsets by qRT-PCR. iNOS, IDO, and HO-1 expression were increased in CD11b+CD11c+ MDSCs while expression of arginase-1 and the anti-inflammatory modulators TGF-β and IL-10 were similar in both MDSC subsets (**Figure 2B**). By using the iNOS inhibitor L-NMMA, the immunosuppressive capacity of CD11b+CD11c− MDSCs towards CD4+ and CD8+ T cells was abolished to nearly 100%. L-NMMA-treated CD11b+CD11c+ MDSCs, however, maintained about 40% of their inhibitory function towards both T-cell subsets (**Figure 2C**). IDO or HO-1 inhibitors, however, did not affect the inhibitory capacity of CD11b+ CD11c+ MDSCs (data not shown), indicating that immunosuppression is mediated by iNOS activity and a not yet identified mechanism. Since PD-L1 and PD-L2 were strongly upregulated on CD11b+CD11c+ MDSCs (**Figure 1A**) and are known to inhibit T-cell activation by binding to PD-1, impact of PD-L1 and PD-L2 on the suppressive function of CD11b+ CD11c+ MDSCs was defined. PD-L1 function was abrogated by using isolated CD11b+CD11c+ MDSCs generated from BM cells of PD-L1$^{-/-}$ mice and PD-L2 blocking was achieved by antagonistic antibodies. Purified PD-L1$^{-/-}$ CD11b+CD11c+ MDSC added to allogeneic-activated spleen cells suppressed T-cell proliferation of CD4+ and CD8+ T cells comparable with PD-L1 expressing MDSCs derived from B6 wildtype (WT) mice (**Figure 2D**). Likewise, adding PD-L2 antagonistic antibodies to allogeneic-activated T cells in the presence of B6-derived CD11b+ CD11c+ MDSCs did not impair T-cell suppression (**Figure 2E**) clearly showing that neither PD-L1 or PD-L2 contribute to CD11b+ CD11c+ MDSC-mediated immunosuppression *in vitro*.

Although inhibition of T-cell expansion designates the main feature of MDSCs, MDSCs functions are also attributed to modulate the Th1/Th2 induction especially *in vivo*. To define the T-cell polarizing capacity of MDSC subsets *in vitro*, supernatants of allogeneic MLRs performed in the presence of CD11b+CD11c+ or CD11b+CD11c− were analyzed for composition of type 1- and type 2-asssociated cytokines. Of the type 2-specific cytokines analyzed, IL-5 secretion was strongly upregulated by CD11b+CD11c+ MDSCs, while IL-4 and IL-13 was unaffected. Th1-specific IFN-γ production was similar in CD11b+CD11c+- and CD11b+CD11c−-treated cultures (**Figure 2F**). In summary, these results show that CD11b+CD11c+ and CD11b+CD11c− MDSCs can be distinguished phenotypically and functionally.

## Exclusively the CD11b+CD11c+ MDSC Subset Prevents GVHD While Maintain the GVT Effect

Due to functional differences between both MDSC subsets *in vitro*, we tested their potential to block GVHD development after allogeneic BMT. We used the single MHC class I-disparate allogeneic BMT model, B6 (H-2K$^{b}$)→B6.bm1 (H-K$^{bm1}$), in which lethally irradiated B6.bm1 mice were reconstituted with TCD-BM and SCs from B6 mice. At the day of BMT, isolated CD11b+CD11c+ or CD11b+CD11c− MDSCs were cotransplanted together with TCD-BM and SCs. While 52% of mice transplanted with TCD-BM and SCs succumbed to the disease associated with high GVHD scores and weight loss of about 20%, CD11b+CD11c+ co-transplantation rescued 78% of the mice from disease-induced mortality reflected by a reduced GVHD score and less weight loss. In contrast, cotransplantation of CD11b+CD11c− MDSCs totally failed to prevent GVHD development. Surviving rates and GVHD scores were undistinguishable in mice receiving TCD-BM and SC and mice cotreated with CD11b+CD11c− MDSCs. Control mice receiving TCD-BM survived and did not develop GVHD (**Figures 3A–C**).

Maintenance of the GVT effect is a basic requirement for the application of allogeneic stem cell transplantation in the treatment of hematological malignancies. Therefore, the impact of MDSC subpopulations on the GVT effect was determined by coinjecting the CD8+CD4− syngeneic thymoma cell line JM6 in BM-reconstituted mice. All mice receiving only BM cells and JM6 died between 20 and 24 days after BMT from tumor development (**Figure 3D**) reflected by high numbers of tumor cells in spleen and liver (**Figure 3E**). Although transplantation of TCD-BM and SC totally prevented tumor growth in all mice due to the presence of tumor-reactive splenic mature T cells, 50% of the mice died by GVHD development. Most importantly, about 80% of the mice cotreated with CD11b+CD11c+ MDSCs survived reflected by the absence of tumor cells in spleen and liver. Five mice from this group died during the experiment. They were all tumor free but succumbed GVHD-induced death. Although all mice transplanted with CD11b+CD11c− MDSCs did not develop spleen or liver tumors, only 50% of the mice survived due to GVHD development, as shown in **Figure 3A**. Thus, our experiments define CD11b+CD11c+ MDSCs as the subpopulation of *in vitro*-generated MDSCs able to protect BMT mice from GVHD development without impairing antitumor cytotoxicity.

## CD11b+CD11c+-Mediated GVHD Inhibition Does Not Prevent Expansion and Homing of Allogeneic T Cells *In Vivo* But Requires Induction of Type 2 Immunity

Next, we questioned whether GVHD prevention by CD11b+CD11c+ MDSCs was due to impaired expansion of allogenic GVHD-inducing T cells, since CD11b+CD11c+ MDSCs most efficiently blocked allogeneic T-cell proliferation *in vitro*. By transplanting SCs from B6.SJL (CD45.1+) mice together with B6-derived TCD-BM (CD45.2+) into irradiated B6.bm1 (CD45.2+) mice, homing and expansion of allogeneic GVHD-inducing T cells were followed by staining the congenic marker CD45.1 in spleen and liver of transplanted mice. CD45.1+ T cells were detectable in spleen and the GVHD target organ liver already at day 3 after BMT in mice transplanted with TCD-BM and SCs. An increase of about 200-fold was achieved 10 days after BMT in both organs. However, cotransplantation of MDSCs did not prevent invasion and expansion of allogeneic T cells independent whether isolated CD11b+CD11c+ or CD11b+CD11c− MDSCs were transferred (**Figure 4A**). Ten days after BMT, allogeneic T-cell numbers continuously decreased and mice became lymphopenic at the time when clinical signs of GVHD were manifested (data not shown).

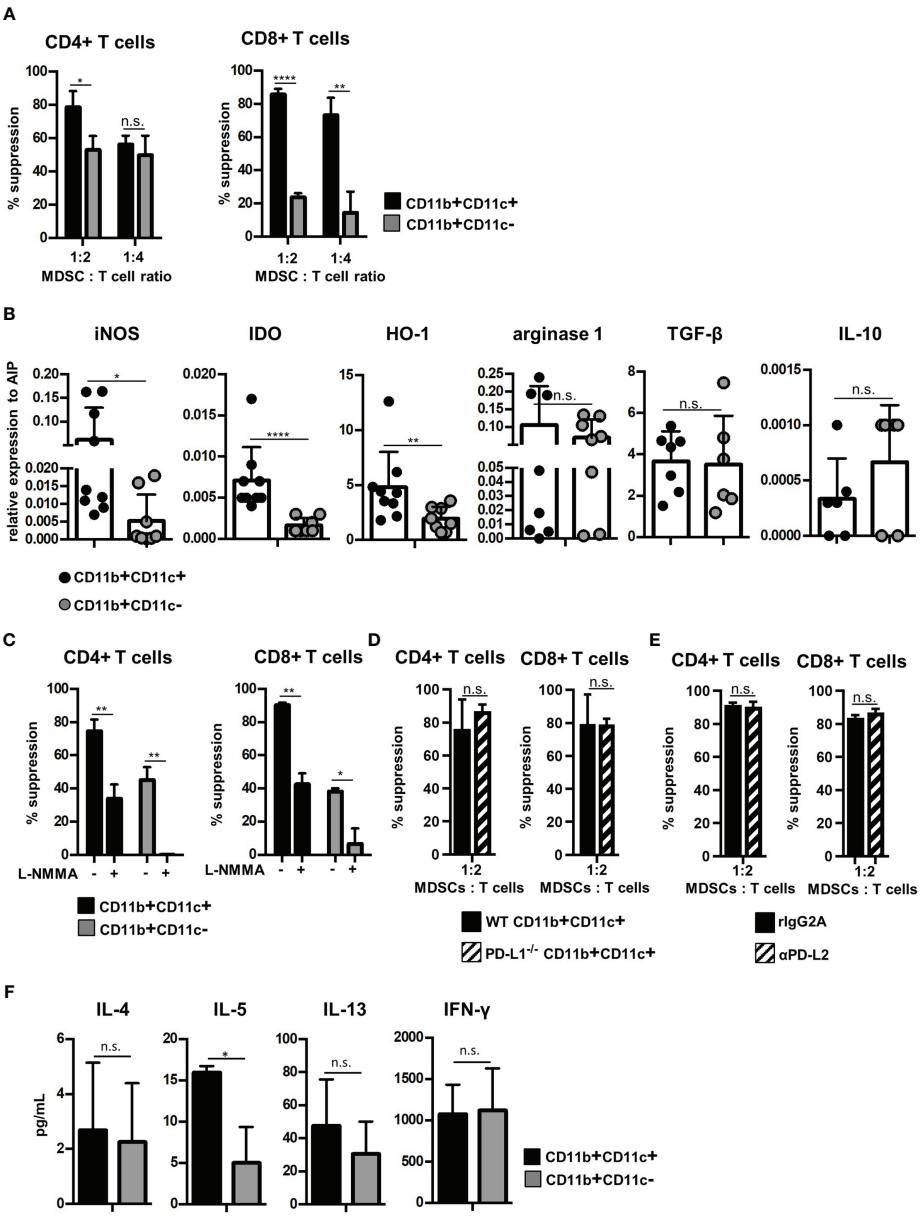


**FIGURE 2** | CD11b+CD11c+ MDSCs exhibit increased immunosuppressive capacity than CD11b+CD11c– MDSCs and induce type 2 immunity *in vitro*. CD11b+CD11c+ and CD11b+CD11c– MDSCs were isolated from B6-derived (H-2$^b$, CD45.2+) *in vitro*-generated MDSCs. **(A)** CD11b+CD11c+ or CD11b+CD11c– MDSCs were cocultivated with B6.SJL-derived (H-2$^b$, CD45.1+) CFSE-labeled spleen cells stimulated by irradiated allogeneic DBA/2-derived (H-2$^d$, CD45.2+) spleen cells. After 4 days, CD45.1+ T cells were stained for CD3, CD4, and CD8 and suppression of CD4+ and CD8+ T-cell proliferation was calculated. **(B)** CD11b+CD11c+ and CD11b+CD11c– MDSC subpopulations were analyzed for relative expression of immunosuppressive molecules by qRT-PCRs. **(C)** CD11b+CD11c+ or CD11b+CD11c– MDSCs were cocultivated with B6.SJL-derived CFSE-labeled spleen cells stimulated by irradiated allogeneic DBA/2-derived spleen cells in the absence or presence of iNOS inhibitor L-NMMA (500 μM). After 4 days, suppression of CD4+ and CD8+ T-cell proliferation was determined. **(D)** B6-derived wildtype (WT) and PD-L1$^{-/-}$ CD11b+CD11c+ or CD11b+CD11c– MDSCs were cocultured with CFSE-labeled B6.SJL-derived spleen cells stimulated with irradiated allogeneic DBA/2 spleen cells. **(E)** To block PD-L2, antagonistic PD-L2 antibodies or recombinant isotype control were added to MLRs, in which CFSE-labeled B6.SJL-derived spleen cells were stimulated with irradiated allogeneic DBA/2 spleen cells in the presence of CD11b+CD11c+ MDSCs. **(F)** CD11b+CD11c+ or CD11b+CD11c– MDSCs were cocultivated with B6.SJL-derived CFSE-labeled spleen cells stimulated by irradiated allogeneic DBA/2-derived spleen cells. After 4 days, secretion of cytokines associated with type 2 T-cell immunity (IL-4, IL-5, and IL-13) or type 1 T-cell immunity (IFN-γ) were determined in the supernatants. **(A)** Data represent the mean value ± SD of triplicates of one representative experiment out of four experiments performed. **(B)** Data represent the mean value ± SD of six to nine samples. **(C)** Data represent the mean value ± SD of triplicates of one representative experiment out of three experiments performed. **(D)** Data represent the mean value ± SD of $n = 3$ PD-L1$^{-/-}$ and WT mice. **(E)** One experiment out of two experiments performed. Values present the mean value ± SD of triplicates. **(F)** Data represent the mean value ± SD of $n = 3$ experiments. **(A, C–F)** Student's *t*-test. **(B)** Mann-Whitney *U* test. *$p \leq 0.05$; **$p \leq 0.01$; ****$p \leq 0.0001$. n.s., not significant.

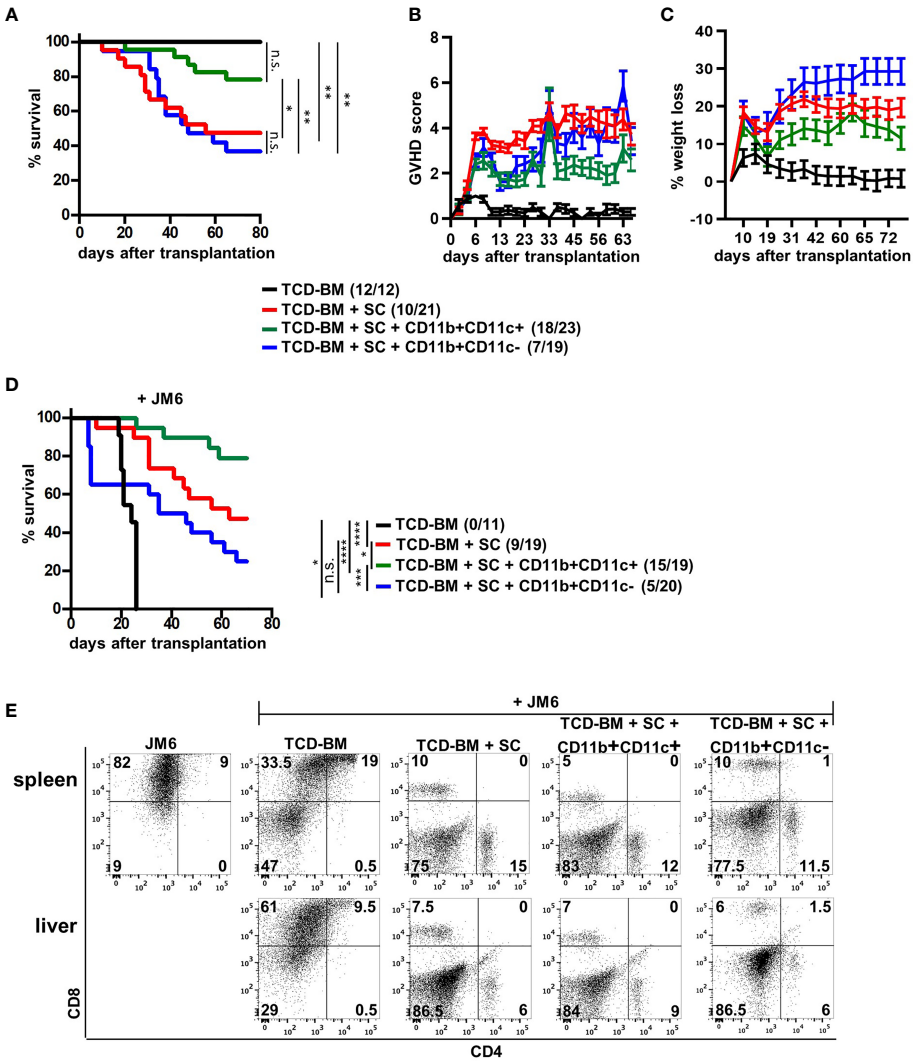


**FIGURE 3** | CD11b+CD11c+ expression defines the subpopulation of MDSCs able to prevent GVHD without disabling the GVT effect. **(A–E)** Lethally irradiated B6.bm1 mice (H-2K$^{bm1}$) were reconstituted with B6-derived (H-2K$^{b}$) TCD-BM and SCs with or without B6-derived CD11b+CD11c+ or CD11b+CD11c– MDSCs. **(D, E)** Mice were additionally coinjected with the CD8+CD4– syngeneic thymoma tumor cell line JM6 at day of transplantation. **(A, D)** Survival was determined. Surviving animals/total animals treated are indicated in brackets. **(B)** Clinical GVHD scores **(C)** and percentage of weight loss were determined. **(E)** Presence of tumor cells was analyzed in spleens and livers by staining for CD4 and CD8 at day mice were sacrificed due to their moribund state or at the end of the experiment. **(A, C)** Kaplan-Meier method and Log-rank test. **(B, C)** Data represent the mean value ± SEM. **(D)** Representative FACS diagrams of one mouse/group are displayed. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$; n.s., not significant.

Since CD11b+CD11c+-mediated inhibition of GVHD did not impair allogeneic T-cell expansion, we determined whether CD11b+CD11c+ MDSCs support T-cell polarization towards Th2 immunity known to be advantageous for GVHD inhibition. Serum level of Th2-specific cytokine IL-5 was only elevated in mice treated with CD11b+CD11c+ MDSC. Type 2-specific cytokines such as IL-4 and IL-13 were not detectable, probably due to concentrations below the detection level of the kit used or degradation after freezing and thawing (41). Serum levels of Th1-associated cytokines IFN-γ or TNF-α were not altered by MDSC treatment (**Figure 4B**). To further prove CD11b+CD11c+-mediated type 2 polarization, mRNA expression of allogeneic T cells isolated from mice reconstituted with TCD-BM and SCs or cotransplanted with either CD11b+CD11c+ or CD11b+CD11c– MDSCs was determined. T cells isolated from CD11b+CD11c+ MDSC-treated mice expressed significantly increased levels of IL-4 and IL-5, while IL-13 was only slightly upregulated (**Figure 4C**). While TNF-α expression was unaffected by MDSC treatment, IFN-γ levels increased in T cells from CD11b+CD11c-treated mice further indicating that CD11b+CD11c– MDSCs support Th1 immunity and GVHD induction.

To prove the indispensability of type 2 polarization for GVHD prevention in mice treated with CD11b+CD11c+ MDSCs, we reconstituted B6.bm1 mice with TCD-BM and

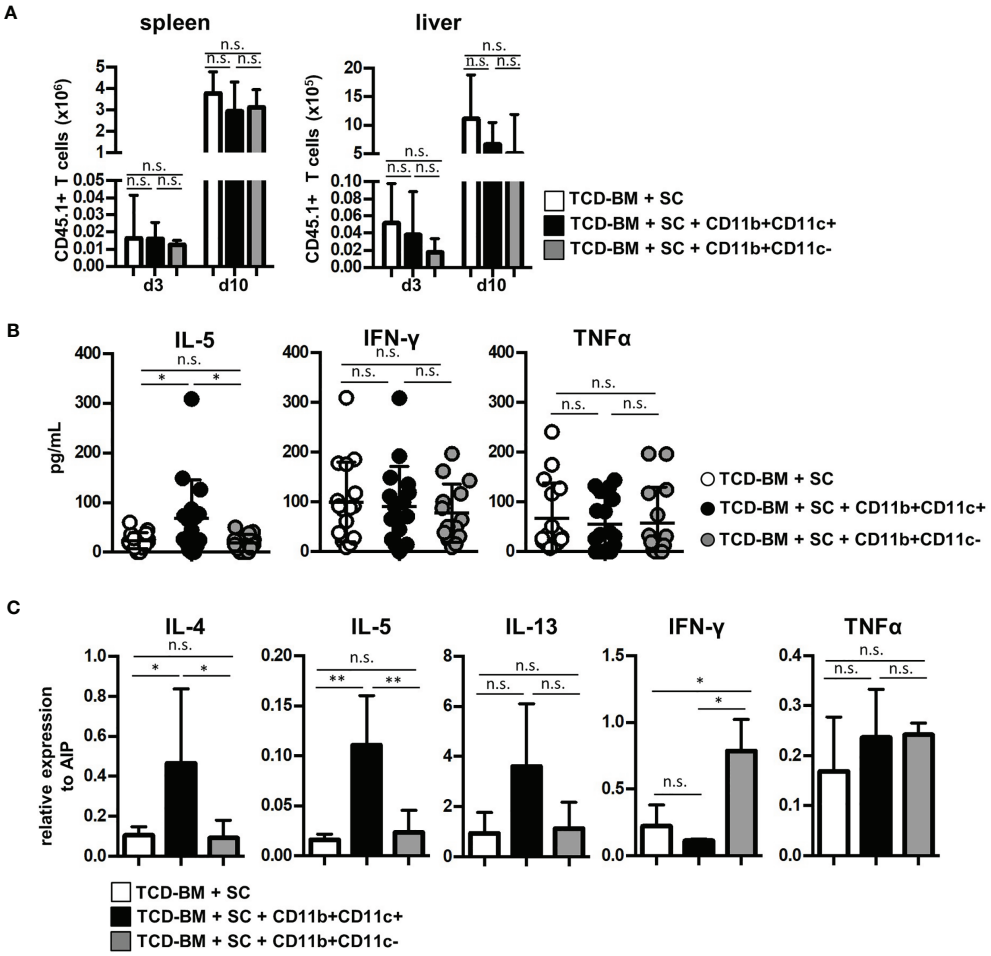


**FIGURE 4** | Cotransplantation of CD11b+CD11c+ MDSCs does not prevent allogeneic T-cell expansion and homing but induces type 2 immunity. **(A–C)** Lethally irradiated B6.bm1 (H-2K$^{bm1}$, CD45.2) mice were reconstituted with B6-derived (H-2K$^{b}$, CD45.2) TCD-BM and B6.SJL-derived (H-2K$^{b}$, CD45.1) spleen cells in the presence or absence of B6-derived (H-2K$^{b}$; CD45.2) CD11b+CD11c+ or CD11b+CD11c– MDSCs. **(A)** Spleen and liver were analyzed for infiltrated allogeneic CD45.1+ T cells 3 and 10 days after transplantation. **(B)** Ten days after transplantation, serum cytokine concentrations of Th2- (IL-5) and Th1-associated (IFN-γ and TNF-α) cytokines were determined. **(C)** Ten days after transplantation, splenic T cells were isolated and relative mRNA expression of Th2- (IL-4, IL-5, IL-13) and Th1-associated (TNF-α, IFN-γ) cytokines was analyzed by qRT-PCRs. **(A)** Data represent the mean value ± SD of three mice/group. Kruskal-Wallis test. **(B)** Data represent the mean value ± SD of 14–16 mice/group. **(C)** Data represent the mean value ± SD of $n$ = 3–5 samples with cells from three to five pooled mice/sample. **(B, C)** ANOVA Tukey multiple comparison test. *$p \leq 0.05$; **$p \leq 0.01$. n.s., not significant.

allogeneic SCs either derived from STAT6-deficient (STAT6$^{-/-}$) or B6 WT mice and CD11b+CD11c+ MDSCs. STAT6$^{-/-}$ splenic T cells are unable to differentiate into type 2 T cells but retain their ability to turn into type 1 T cells and to induce GVHD indistinguishable from STAT6-expressing WT T cells as shown previously (18). Cotransplantation of CD11b+CD11c+ MDSCs in mice reconstituted with STAT6$^{-/-}$ SCs failed to prevent GVHD and 64% of the mice succumbed to the disease, while only 18% of the mice receiving B6-derived WT SCs and CD11b +CD11c+ MDSCs developed lethal GVHD associated with increased GVHD scores (**Figures 5A, B**). In accordance to the survival data, IL-5 levels were only increased in mice reconstituted with B6-derived WT SCs, while IFN-γ levels were elevated in serum from mice reconstituted with STAT6$^{-/-}$ SCs. Differences in the concentration of TNF-α levels were not detected (**Figure 5C**). In summary, these results clearly show that CD11b+CD11c+ MDSCs do not impair the expansion and homing of allogeneic T cells in lymphatic and GVHD target organs but prevent GVHD induction by shifting the T-cell response towards type 2 immunity.

## Comparative Transcriptome Analysis Between CD11b+CD11c+ and CD11b+ CD11c– MDSCs

Since CD11b+CD11c+ and CD11b+CD11c– MDSCs can be clearly distinguished by their ability to interfere with GVHD development, we aimed to define genes and signaling pathways mediating immunosuppressive functions of CD11b+CD11c+ MDSCs in the context of BMT. Comparison of the transcriptome between CD11b+CD11c+ and CD11b+CD11c–

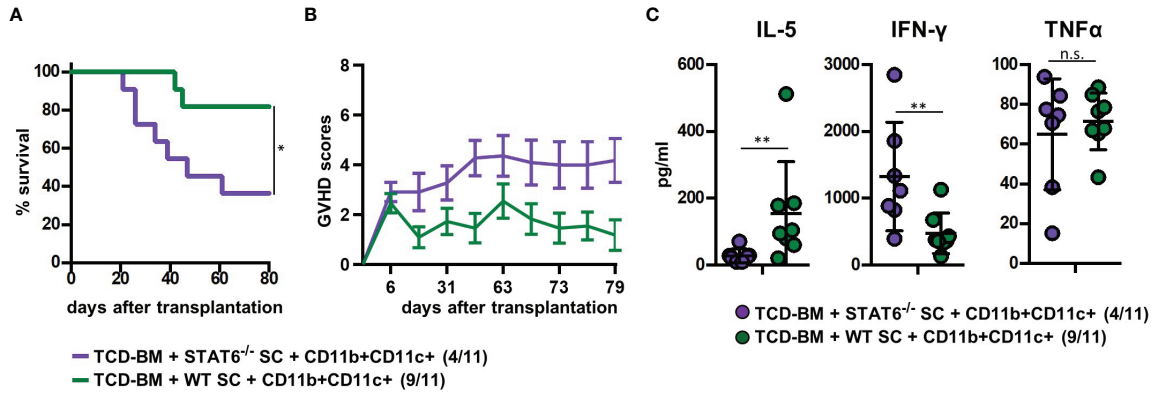


**FIGURE 5** | Type 2 immune induction by cotransplanted CD11b+CD11c+ MDSCs is required for GVHD prevention. Lethally irradiated B6.bm1 (H-2K$^{bm1}$) recipient mice were reconstituted with B6-dervied (H-2K$^{b}$) T-cell-depleted bone marrow (TCD-BM) and SCs either derived from B6 wild-type (WT) mice (H-2K$^{b}$) or STAT6$^{-/-}$ mice (H-2K$^{b}$). B6-derived (H-2K$^{b}$) CD11b+CD11c+ MDSCs were cotransplanted at day of transplantation. **(A)** Survival and **(B)** GVHD scores were analyzed. Surviving animals/total animals treated are indicated in brackets. **(C)** Ten days after transplantation, serum cytokine concentrations of Th2- (IL-5) and Th1-associated (IFN-γ and TNF-α) cytokines were determined. **(A)** Data represent the mean value ± SEM. **(B)** Kaplan-Meier method and Log-rank test. **(C)** Mann-Whitney *U* test. *$p \leq 0.05$; **$p \leq 0.01$; n.s., not significant.

MDSCs was done by mRNA-Seq. Principal component analysis (PCA) of two experiments displays the degree to which the transcriptome of CD11b+CD11c+ MDSCs differs from CD11b+CD11c− MDSCs. In both experiments, PCA analysis clearly separated two clusters corresponding to the CD11b+CD11c+ and CD11b+CD11c− MDSCs (**Supplementary Figure S2**), revealing a totally different transcriptome of both MDSC subpopulations which further underlies the functional differences between both subpopulations. In total, 2,783 differentially expressed genes (*p*-value <0.01, RPKM >5) were identified, from which 1,443 genes were upregulated and 1,340 genes were downregulated in CD11b+CD11c+ MDSCs compared with CD11b+CD11c− MDSCs. Focusing on the transcripts that were highly upregulated or downregulated in the CD11b+CD11c+ MDSC subpopulation, we performed enrichment analysis using GO database. Target genes upregulated in CD11b+CD11c+ MDSCs can be largely grouped into the biological and functional categories (**Table 1**): 1. cell movement and migration, 2. cell adhesion, 3. leukocyte activation and immune response, 4. ERK1 and ERK2 cascade, 5. response to cytokine, and 6. Stress response. Identified GO terms and linked target genes are listed in **Table 1** and **Supplementary Table S4**. Target genes downregulated in CD11b+CD11c+ MDSCs are mostly related to immune and defense response against other organisms such as bacteria or fungi, which might be related to their immunosuppressive phenotype. Identified GO terms and linked target genes are listed in **Table 2** and **Supplementary Table S5**. Focusing on the transcripts that were highly upregulated in CD11b+CD11c+ MDSCs, we ascertained the target genes that were upregulated more than 15-fold in CD11b+CD11c+ compared with CD11b+CD11c− MDSCs (**Table 3**). With a fold change of 57.96, CCL17 was the highest expressed target gene in CD11b+CD11c+ compared with CD11b+CD11c− MDSCs. Together with CCL22, which showed a 24-fold overexpression in CD11b+CD11c+ MDSCs, both chemokines are known to attract CCR4-bearing Th2 cells and serve as markers for the severity of Th2-mediated atopic dermatitis (42, 43). Furthermore, the fatty acid translocase CD36 is 20-fold stronger expressed in CD11b+CD11c+ MDSCs than in CD11b+CD11c− MDSCs and serves in association with the platelet-activating factor receptor as an important mediator of Th2-mediated house dust mite allergy development (44). Increased expression of CCL17, CCl22, and CD36 by CD11b+CD11c+ MDSC in comparison with CD11b+CD11c− MDSCs was confirmed by qRT-PCR. Additionally, CD36 was found to be strongly expressed on the surface of CD11b+CD11c+ MDSCs (**Supplementary Figure S3**). In summary, transcriptome analysis further underlines the functional differences between CD11b+CD11c+ and CD11b+CD11c− MDSCs and indicates candidate genes and pathways, which might contribute to the therapeutic potential of CD11b+CD11c+ MDSCs.

## DISCUSSION

Allogeneic hematopoietic cell transplantation is considered an important treatment strategy to cure life-threatening malignant hematological diseases, however, with the limitation of GVHD development. Initial treatment comprises steroid therapy, while second-line treatment often includes immunomodulatory therapies to dampen the destructive capacity of allogeneic T cells. MDSCs are recognized as strong modulators of T-cell functions and were already applied in preclinical models as cellular therapy for GVHD prevention. Considering the heterogenicity of *in vitro*-generated MDSCs, we aimed to define the MDSC subset responsible for GVHD prevention. To our knowledge, we show here for the first time that only a small proportion of MDSCs, which have been generated *in vitro* form BM cells, fulfills GVHD-

**TABLE 1** | GO term analysis and identification of biological and functional processes activated in CD11b+CD11c+ MDSCs compared with CD11b+CD11c– MDSCs.

| Biological and functional category | GO term | GO ID | *p*-value changed | FDR *q*-value | Number of target genes |
|---|---|---|---|---|---|
| Cell movement and migration | Cell motility | 0048870 | 2.50E–11 | 1.22E–07 | 62 |
| | Cell migration | 0016477 | 4.54E–11 | 1.47E–07 | 59 |
| | Locomotion | 0040011 | 5.08E–11 | 1.23E–07 | 65 |
| | Lymphocyte migration | 0072676 | 1.51E–10 | 2.44E–07 | 12 |
| | Movement of cell or subcellular component | 0006928 | 2.87E–10 | 3.98E–07 | 70 |
| | Lymphocyte chemotaxis | 0048247 | 3.36E–09 | 3.63E–06 | 8 |
| | Chemotaxis | 0006935 | 1.46E–08 | 1.18E–05 | 28 |
| | Taxis | 0042330 | 3.04E–08 | 1.85E–05 | 28 |
| | Cell chemotaxis | 0060326 | 9.10E–08 | 4.92E–05 | 22 |
| | Monocyte chemotaxis | 0002548 | 1.23E–07 | 5.97E–05 | 8 |
| | Leukocyte chemotaxis | 0030595 | 1.61E–07 | 6.80E–05 | 12 |
| | Leukocyte migration | 0050900 | 2.36E–07 | 8.49E–05 | 21 |
| | Mononuclear cell migration | 0071674 | 8.46E–07 | 2.00E–04 | 9 |
| | Myeloid leukocyte migration | 0097529 | 3.72E–06 | 6.57E–04 | 10 |
| Cell adhesion | Biological adhesion | 0022610 | 2.35E–08 | 1.53E–05 | 45 |
| | Cell adhesion | 0007155 | 8.20E–08 | 4.69E–05 | 43 |
| | Positive regulation of cell-cell adhesion | 0022409 | 1.22E–07 | 6.26E–05 | 16 |
| | Positive regulation of cell adhesion | 0045785 | 9.26E–07 | 2.14E–04 | 19 |
| | Regulation of leukocyte cell-cell adhesion | 1903037 | 3.40E–06 | 6.62E–04 | 15 |
| | Regulation of cell-cell adhesion | 0022407 | 4.05E–06 | 7.02E–04 | 39 |
| Leukocyte activation and immune response | Immune response | 0006955 | 1.47E–10 | 2.85E–07 | 55 |
| | lymphocyte migration | 0072676 | 1.51E–10 | 2.44E–07 | 12 |
| | Positive regulation of immune system process | 0002684 | 1.25E–09 | 1.52E–06 | 60 |
| | Lymphocyte chemotaxis | 0048247 | 3.36E–09 | 3.63E–06 | 8 |
| | Monocyte chemotaxis | 0002548 | 1.23E–07 | 5.97E–05 | 8 |
| | Leukocyte chemotaxis | 0030595 | 1.61E–07 | 6.80E–05 | 12 |
| | positive regulation of lymphocyte activation | 0051251 | 1.77E–07 | 7.17E–05 | 31 |
| | Positive regulation of leukocyte activation | 0002696 | 1.84E–07 | 7.14E–05 | 34 |
| | Leukocyte migration | 0050900 | 2.36E–07 | 8.49E–05 | 21 |
| | Defense response | 0006952 | 2.79E–07 | 9.69E–05 | 55 |
| | Antigen processing and presentation of exogenous peptide antigen via MHC class II | 0019886 | 5.15E–07 | 1.43E–04 | 9 |
| | Antigen processing and presentation of peptide antigen via MHC class II | 0002495 | 5.15E–07 | 1.47E–04 | 9 |
| | Antigen processing and presentation of peptide or polysaccharide antigen via MHC class II | 0002504 | 5.15E–07 | 1.52E–04 | 9 |
| | Positive regulation of leukocyte cell-cell adhesion | 1903039 | 5.40E–07 | 1.46E–04 | 14 |
| | Negative regulation of immune system process | 0002683 | 7.14E–07 | 1.78E–04 | 21 |
| | Positive regulation of T-cell activation | 0050870 | 8.39E–07 | 2.04E–04 | 13 |
| | Mononuclear cell migration | 0071674 | 8.46E–07 | 2.00E–04 | 9 |
| | Adaptive immune response | 0002250 | 1.40E–06 | 3.17E–04 | 12 |
| | Humoral immune response | 0006959 | 3.25E–06 | 6.45E–04 | 15 |
| | Regulation of leukocyte cell-cell adhesion | 1903037 | 3.40E–06 | 6.62E–04 | 15 |
| | Positive regulation of leukocyte differentiation | 1902107 | 3.58E–06 | 6.82E–04 | 23 |
| | Myeloid leukocyte migration | 0097529 | 3.72E–06 | 6.57E–04 | 10 |
| | Inflammatory response | 0006954 | 4.71E–06 | 7.89E–04 | 35 |
| | Regulation of leukocyte differentiation | 1902105 | 5.18E–06 | 8.53E–04 | 12 |
| | Antigen processing and presentation of exogenous peptide antigen | 0002478 | 6.45E–06 | 1.03E–03 | 9 |
| | Myeloid leukocyte migration | 0097529 | 3.72E–06 | 6.57E–04 | 10 |
| ERK1 and ERK2 cascade | Regulation of ERK1 and ERK2 cascade | 0070372 | 3.59E–06 | 6.71E–04 | 16 |
| | Positive regulation of ERK1 and ERK2 cascade | 0070374 | 2.31E–08 | 1.60E–05 | 16 |
| Response to cytokine | Chemokine-mediated signaling pathway | 0070098 | 8.52E–09 | 7.53E–06 | 11 |
| | Response to interferon-gamma | 0034341 | 2.09E–08 | 1.57E–05 | 20 |
| Response to stress | Response to tumor necrosis factor | 0034612 | 1.94E–06 | 4.29E–04 | 9 |

inhibiting functions. This subset is characterized by the coexpression of Gr-1, CD11b, and CD11c. Gr-1+CD11b+CD11c+ MDSCs effectively prevent GVHD development and maintain antitumor cytotoxicity of allogeneic T cells, while the majority of the *in vitro*-generated MDSCs expressing Gr-1+CD11b+CD11c– are totally inefficient to dampen GVHD, although they block T-cell expansion *in vitro*. Extensive differences in the transcriptomic landscape of both populations underlined their various *in vivo* functions, indicating that the success of cellular therapies using MDSCs requires a thoughtful characterization of MDSC subset functions *in vitro* and *in vivo*.

In a clinically relevant BMT model with disparity in only one MHC molecule (B6 into B6.bm1), we defined which subset of *in vitro*-generated MDSCs prevents GVHD. MDSC were generated

**TABLE 2 |** GO term analysis and identification of biological and functional processes downregulated in CD11b+CD11c+ MDSCs compared with CD11b+CD11c– MDSCs.

| Biological and functional category | GO term | GO ID | *p*-value changed | FDR *q*-value | Number of target genes |
|---|---|---|---|---|---|
| Immune and defense response | Defense response | 0006952 | 1.41E–09 | 1.37E–05 | 20 |
| | Defense response to fungus | 0050832 | 1.31E–07 | 6.37E–04 | 5 |
| | Defense response to bacterium | 0042742 | 3.49E–07 | 8.48E–04 | 9 |
| | Antifungal humoral response | 0019732 | 5.40E–07 | 1.05E–03 | 2 |
| | Response to bacterium | 0009617 | 1.13E–06 | 1.83E–03 | 11 |
| | Response to fungus | 0009620 | 1.57E–06 | 2.18E–03 | 5 |
| | Disruption of cells of other organism | 0044364 | 1.94E–06 | 2.36E–03 | 6 |
| | Killing of cells of other organism | 0031640 | 1.94E–06 | 2.10E–03 | 6 |
| | Regulation of inflammatory response | 0050727 | 2.09E–06 | 2.03E–03 | 28 |
| | Organ- or tissue-specific immune response | 0002251 | 2.41E–06 | 1.95E–03 | 5 |
| | Mucosal immune response | 0002385 | 2.41E–06 | 2.13E–03 | 5 |
| | Innate immune response in mucosa | 0002227 | 2.45E–06 | 1.83E–03 | 3 |
| | Regulation of defense response | 0031347 | 2.53E–06 | 1.75E–03 | 42 |
| | Humoral immune response | 0006959 | 4.08E–06 | 2.64E–03 | 7 |

**TABLE 3 |** Most strongly upregulated genes (>15-fold increase) in CD11b+CD11c+ MDSCs compared with CD11b+CD11c– MDSCs.

| | Target gene | Name | Fold change | Adjusted *p*-value |
|---|---|---|---|---|
| 1. | CCL17 | Chemokine (C–C motif) ligand 17 | 57.96 | 9.55E–08 |
| 2. | Plet1 | Placenta-expressed transcript 1 protein | 43.11 | 2.33E–07 |
| 3. | Hepacam2 | HEPACAM family member 2 | 42.23 | 4.02E–05 |
| 4. | Klrb1b | Killer cell lectin-like receptor subfamily B member 1B allele B | 36.63 | 5.39E–06 |
| 5. | Hr | Lysine-specific demethylase hairless | 34.35 | 8.50E–08 |
| 6. | H2-Eb1 | H-2 class II histocompatibility antigen, I-A beta chain | 32.28 | 9.07E–07 |
| 7. | Emp2 | Epithelial membrane protein 2 | 30.40 | 3.74E–04 |
| 8. | C1qc | Complement C1q subcomponent subunit C | 28.67 | 5.17E–03 |
| 9. | H2-Aa | H-2 class II histocompatibility antigen, A-B alpha chain | 28.65 | 5.25E–07 |
| 10 | Kcp | Kielin/chordin-like protein | 27.77 | 3.52E–03 |
| 11. | Flnc | Filamin-C | 26.75 | 2.97E–06 |
| 12. | Aldh1a2 | Retinal dehydrogenase 2 | 26.16 | 3.67E–04 |
| 13. | H2-Ab1 | H-2 class II histocompatibility antigen, A beta chain | 25.73 | 2.51E–07 |
| 14. | Adam23 | Disintegrin and metalloproteinase domain-containing protein 23 | 25.64 | 3.40E–05 |
| 15. | Speg | Striated muscle-specific serine/threonine-protein kinase | 25.16 | 9.10E–06 |
| 16. | C1qb | Complement C1q subcomponent subunit B | 24.72 | 2.47E–03 |
| 17. | CCL22 | Chemokine (C–C motif) ligand 22 | 24.45 | 8.61E–05 |
| 18. | Mmp12 | Macrophage metalloelastase 12 | 24.12 | 3.21E–06 |
| 19. | Sema6d | Semaphorin-6D | 24.00 | 1.74E–08 |
| 20. | Tnfaip8l3 | Tumor necrosis factor alpha-induced protein 8-like protein 3 | 23.94 | 6.67E–07 |
| 21. | Itgae | Integrin alpha-E | 23.02 | 6.81E–03 |
| 22 | Dcstamp | Dendritic cell-specific transmembrane protein | 22.63 | 1.83E–06 |
| 23. | Nr4a3 | Nuclear receptor subfamily 4 group A member 3 | 22.28 | 5.09E–04 |
| 24. | Fscn1 | Fascin | 22.01 | 1.21E–03 |
| 25. | Ciita | MHC class II transactivator | 21.69 | 8.50E–08 |
| 26. | CCR7 | C–C chemokine receptor type 7 | 21.55 | 4.26E–03 |
| 27 | Tnfrsf9 | Tumor necrosis factor receptor superfamily member 9 | 21.35 | 3.11E–06 |
| 28 | Asgr2 | Asialoglycoprotein receptor 2 | 21.24 | 2.30E–03 |
| 29. | Anpep | Aminopeptidase N | 21.00 | 1.94E–05 |
| 30. | Hgfac | Hepatocyte growth factor activator | 20.47 | 1.59E–04 |
| 31. | Ptx3 | Pentraxin-related protein PTX3 | 20.36 | 1.60E–04 |
| 32. | CD36 | Platelet glycoprotein 4 | 19.65 | 1.10E–07 |
| 33. | IL7r | Interleukin-7 receptor subunit alpha | 19,36 | 9.62E–04 |
| 34. | P2rx5 | Purinergic receptor P2X ligand-gated ion channel 5 | 18.08 | 3.51E–08 |
| 35. | Tspan33 | Tetraspanin-33 | 17.87 | 2.09E–05 |
| 36. | Blnk | B-cell linker | 17.66 | 8.97E–05 |
| 37. | Il4i1 | Interleukin 4 induced 1 | 16.61 | 2.46E–04 |
| 38. | Zbtb46 | Zinc finger and BTB domain containing 46 | 16.35 | 4.66E–06 |
| 39. | Sdc3 | Syndecan 3 | 15.04 | 2.41E–07 |

from BM cells in the presence of GM-CSF. More than 90% of the cells exhibited Gr-1 and CD11b expression, but only a small proportion of about 10%–25% cells showed coexpression of the integrin alphaX CD11c, which is also found at high levels not only on the surface of dendritic cell, but also on monocytes, macrophages, neutrophils, and subsets of NK, B, and T cells. By separating MDSCs into CD11b+CD11c+ and CD11b+CD11c– subsets, a clear correlation with the classically defined M-MDSCs

and PMN-MDSCs was not observed. While CD11b+CD11c− MDSCs represented a mixture of M-MDSCs and PMN-MDSCs, CD11b+CD11c+ MDSCs consisted mainly of M-MDSCs, a small proportion of PMN-MDSCs and cells, which neither expressed Ly-6C nor Ly-6G. CD11b+CD11c+ MDSCs further expressed higher levels of APC-associated markers such as CD80, CD86, MHC class II, and F4/80 compared with their CD11c-negative counterparts. Importantly, a single injection of CD11b+CD11c+ MDSCs inhibited GVHD development in about 80% of the BM-transplanted mice, while adoptive transfer of CD11b+CD11c− MDSCs had no impact on disease development. CD11b+CD11c+ -treated mice, however, remain immunosufficient since syngeneic tumor cells were efficiently eradicated in 100% of the mice. Although BMTs are routinely applied to abrogate residual B-cell lymphoma cells, we used the $CD8^+CD4^-$ JM6 thymoma cell line. To our knowledge, JM6 is currently the only available syngeneic tumor cell line for B6.bm1 mice. By using JM6 cells, we cannot totally exclude that MDSCs interact with JM6 tumor growth in transplanted mice. However, in a previous work, unseparated MDSCs, which represent a mixture of CD11b+CD11c+ and CD11b+CD11c− MDSCs, did not abrogate the GVT effect in a parent into F1 BMT model (18), indicating that none of the MDSC subpopulations reduce the capacity of allogeneic T cells to attack residual tumor cells. Furthermore, the GVHD-inhibiting capacity of CD11b+CD11c+ MDSC requires confirmation in other BMT models with disparities also in only MHC class II genes or disparities in MHC class I and II genes to exclude that the observed effects are model dependent.

*In vitro* or *in vivo* induction of MDSCs for cellular therapy of GVHD have been performed by using various approaches with different effectiveness (45); however, the ability of different MDSC subpopulations have not been analyzed for their GVHD-inhibiting potential. Treatment of donor mice with CpG and incomplete Freund's adjuvant (IFA), G-CSF, or recombinant G-CSF/Flt-3 ligand + G-CSF results in increase of splenic CD11b+Gr1+ cells preventing GVHD after cotransplantation with allogeneic T cells (14–16). While a proportion of CpG+IFA-induced Gr-1 cells coexpress CD80, CD86, and CD11c (15), CD11c expression is absent on *in vivo*-generated G-CSF-or G-CSF/Flt-3 + G-CSF-induced MDSCs (14, 16). MDSCs induced *in vitro* from BM cells by GM-CSF, G-CSF, and IL-13 exhibited expression of CD11c on about 15% of the cells, but adoptive transfer into BMT mice was performed solely with unseparated MDSCs (20). However, GM-CSF+G-CSF+ IL-13-induced MDSCs upregulated CD11c, MHC class II, and F4/80 in the inflammatory GVHD environment. Re-isolation of CD11c+, MHC class $II^{high}$, and $F4/80^{high}$ cells from GVHD mice showed a loss in immunosuppressivity *in vitro* (46). Despite the expression of surface makers similar to *in vitro*-generated CD11b+CD11c+ MDSCs, functional properties are different. While *ex vivo*-isolated CD11c+ MDSCs mediate T-cell suppression by arginase-1, suppressive ability by *in vitro*-generated CD11b+CD11c+ MDSCs was mainly attributed to iNOS activity and a not yet defined mechanism, which does not involve IDO, arginase-1, or HO-I activity. Even the role of PMN-MDSCs and M-MDSCs for GVHD development is not defined since we are not aware of BMT experiments using isolated PMN- or M-MDSCs as suppressor cells. G-CSF treatment of donor mice induced low-density splenic granulocytes, which inhibit experimental GVHD (47) and the administration of GVHD-suppressing drug rapamycin, results in expansion of PMN-MDSCs (48) indicating that PMN-MDSCs are the major suppressor population. On the other hand, the presence of G-CSF-induced M-MDSCs correlates with a lower GVHD incidence in humans and humanized GVHD models (21, 49, 50). These findings might reflect species-specific differences in the dependence on MDSC subsets for GVHD inhibition, but extracorporeal photopheresis promotes protective PMN-MDSC expansion in GVHD patients (51).

Striking differences were observed in the *in vitro* and *in vivo* activity of MDSC subsets. While CD11b+CD11c− MDSCs suppressed allogeneic T-cell expansion *in vitro* although to a lesser extent than CD11b+CD11c+ MDSCs, they totally failed to prevent GVHD induction. Immunosuppressive mechanisms differ in both subpopulations since CD11b+CD11c− MDSCs inhibit T-cell proliferation *in vitro* exclusively by iNOS activity, while function of CD11b+CD11c+ MDSCs depends only half on iNOS. Despite upregulation of PD-L1 and PD-L2, both molecules are neglectable for immunosuppression *in vitro* by CD11b+CD11c+ MDSCs. The discrepancy of *in vitro* and *in vivo* action of MDSCs is supported by our work. Unseparated *in vitro*-generated MDSCs induced from BM cells by GM-CSF strongly suppressed T-cell proliferation *in vitro*, but act immunostimulatory in mice receiving blunt chest trauma (TxT). MDSC treatment of TxT mice strongly increased splenic T-cell numbers and proliferative capacity without impairing antigen reactivity (52). Studies by Schmidt et al. also show that tumor-induced MDSCs prevent cytotoxic T lymphocyte (CTL) functions *in vitro* but not *in vivo* following adoptive transfer (53) strongly indicating an important effect on MDSC functions by the interacting microenvironment. Likewise, MDSCs isolated from septic mice at different time points after sepsis induction and transferred into septic mice either deteriorate or ameliorate disease development (54).

Microenvironmental influence on MDSC function is further underlined by the finding that CD11b+CD11c+ MDSCs prevent GVHD development by inducing Th2 immunity without altering allogeneic T-cell expansion and homing, although T-cell expansion was severely blocked by this subpopulation *in vitro*. MDSC-mediated type 2 immunity induction is reported also in the context of cancer, sepsis, pregnancy, and virus infection (5, 6, 8, 55). On the other hand, the transfer of MDSCs in models of Th2-mediated diseases such as asthma-related airway inflammation dampens disease development by shifting immune responses towards Th1 immunity (9, 10). Interestingly, Th1 immunity induction by MDSCs in asthma-related models is found independent whether MDSCs were derived from LPS-treated or tumor-bearing mice, although MDSCs in the context of cancers are known to promote Th2 immunity. Inhibiting T-cell proliferation *in vitro* is indispensable for their assignment as MDSCs (26) but is not necessarily indicative for their *in vivo* functions.

Defining MDSC subsets either able to prevent GVHD or being totally inefficient in blocking GVHD development opens up the possibility to define molecules and molecular pathways contributing to MDSC-mediated GVHD inhibition. mRNA-Seq analysis showed that CD11b+CD11c+ and CD11b+CD11c−

MDSCs had a totally different transcriptomic landscape differing in more than 2,500 genes. Upon the most strongly upregulated genes (>15-fold increase), the fatty acid translocase CD36 or the chemokines CCL17 and CCL22 were identified. Although CD36 expression is not directly linked to elevated immunosuppressivity, increased lipid contents are reported to augment the immunosuppressive functions of MDSCs (56–58), and Baumann et al. recently reported that human MDSCs derived from isolated CD14+ blood monocytes downregulate glycolysis-related enzymes (59). CCL17 and CCL22 are key chemokines inducing Th2 chemotaxis and are strongly elevated in the serum of patients with Th2-driven atopic dermatitis (42, 43). Possibly, Th2 cells are attracted into lymphatic areas invaded by CCL17/CCL22 expressing CD11b+CD11c+ MDSCs and stimulated for increased expansion. However, only adoptive transfer experiments with MDSCs derived from CD36 or CCL17/CCL22-deficient mice will clarify their substantial role in GVHD prevention. Due to the high numbers of differentially expressed genes, it might be worthwhile to re-isolate adoptively transferred CD11b+CD11c+ and CD11b+CD11c− MDSCs from BM-transplanted mice for transcriptome analysis. Defining the intersection of genes differentially expressed by *in vitro* and *ex vivo* isolated CD11b+CD11c+ MDSCs might narrow down the number of possible candidates responsible for GVHD prevention.

Taken together, we could define a small subset of GM-CSF-induced MDSCs characterized by the coexpression of Gr-1+CD11b+CD11c+ as the MDSC subpopulation able to prevent GVHD while maintaining T-cell reactivity and cytotoxicity. This might offer the possibility to identify key molecules and signaling pathways involved in disease prevention with the future perspective to substitute cellular MDSC therapy by pharmacological approaches. Furthermore, the clear discrepancy between *in vitro* and *in vivo* functions of MDSCs requires thoughtful testing of MDSC functions in the relevant disease context.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available and are based on JS's dissertation: (60). mRNA Seq data can be found here: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE182262.

## ETHICS STATEMENT

The animal study was reviewed and approved by the ethical committee Regierungspräsidium Tübingen, Germany.

## AUTHOR CONTRIBUTIONS

JS performed experiments, analyzed data, and generated figures. KK, HH, SP, and KF-C performed mRNA-Seq analysis and analyzed transcriptomic data. K-MD contributed to the study design and interpreted data. GS created the study design, performed data interpretation, and wrote the manuscript together with JS. All authors approved the final version of the manuscript, revised the manuscript, and are accountable for all respects of the work.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.754316/full#supplementary-material

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# GPR Expression in Intestinal Biopsies From SCT Patients Is Upregulated in GvHD and Is Suppressed by Broad-Spectrum Antibiotics

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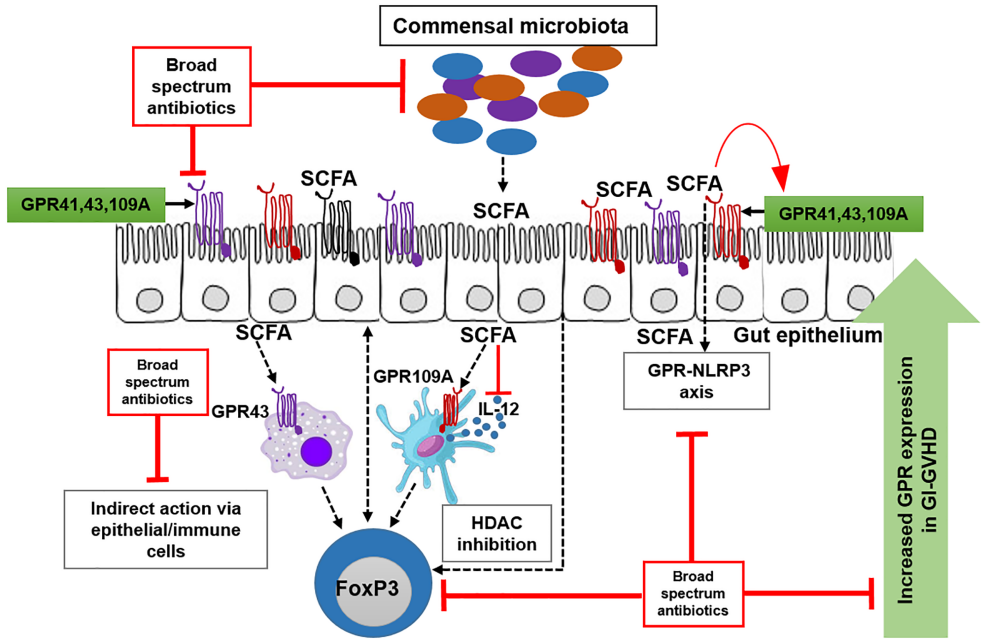
Microbiota can exert immunomodulatory effects by short-chain fatty acids (SCFA) in experimental models of graft-versus-host disease (GvHD) after allogeneic hematopoietic stem cell transplantation (allo-SCT). Therefore we aimed to analyze the expression of SCFAs sensing G-protein coupled receptor GPR109A and GPR43 by quantitative PCR in 338 gastrointestinal (GI) biopsies obtained from 199 adult patients undergoing allo-SCT and assessed the interaction of GPR with FOXP3 expression and regulatory T cell infiltrates. GPR expression was strongly upregulated in patients with stage II-IV GvHD (p=0.000 for GPR109A, p=0.01 for GPR43) and at the onset of GvHD (p 0.000 for GPR109A, p=0.006 for GPR43) and correlated strongly with FOXP3 and NLRP3 expression. The use of broad-spectrum antibiotics (Abx) drastically suppressed GPR expression as well as FOXP3 expression in patients' gut biopsies (p=0.000 for GPRs, FOXP3 mRNA and FOXP3+ cellular infiltrates). Logistic regression analysis revealed treatment with Abx as an independent factor associated with GPR and FOXP3 loss. The upregulation of GPRs was evident only in the absence of Abx (p=0.001 for GPR109A, p=0.014 for GPR43) at GvHD onset. Thus, GPR expression seems to be upregulated in the presence of commensal bacteria and associates with infiltration of FOXP3+ T regs, suggesting a protective, regenerative immunomodulatory response. However, Abx, which has been shown to induce dysbiosis, interferes with this protective response.

**Keywords: broad-spectrum antibiotics, GPR, Foxp3, GvHD, microbiota, SCFA**




**GRAPHICAL ABSTRACT** | G-protein coupled receptor (GPR109A, GPR43 and GPR41) is predominantly expressed on epithelial and immune cells. GPR is activated by its ligand short chain fatty acids (SCFAs). In absence of broad-spectrum antibiotics (Abx), beneficial commensals produce SCFAs that activate GPR pathway. SCFA engage GPR-NLRP3 pathway for the maintainance of epithelial barrier. SCFA also engage GPR on immune cells to induce regulatory T cells. Patients who do not receive Abx show an upregulation of GPR expression in the presence of aGvHD suggesting a counterregulatory mechanism. Abx suppresses commensals leading to reduced SCFA hence less GPR. GPR-NLRP3 axis and GPR-Tregs axis are strongly abrogated by Abx. Abx also interfere with the upregulation of GPR during aGvHD.

## INTRODUCTION

Acute Graft versus host disease (aGvHD) is the major cause of transplant-related mortality (TRM) and morbidity following allogeneic stem cell transplantation (SCT). Current treatment options for this complication are poor if initial treatment with steroids has failed (1). Landmark studies in the early 70s by van Bekkum already pointed to a role of the intestinal microflora in gastrointestinal (GI) aGvHD (2) and suggested protection of germfree mice from GvHD. Preclinical and clinical studies therefore introduced prophylactic use of decontamination as an approach to reduce GvHD (3) and together with the concept of prevention of neutropenic gram-negative infections, antibiotic prophylaxis has become standard of care (4) With the introduction of next-generation sequencing technologies including 16s rRNA, it now became evident that the intestinal microbiota is an important modulator of aGvHD. Since 2012, several studies using this technique in experimental (5) and clinical settings (6, 7) reported a strong loss of commensal bacteria (dysbiosis) but no complete decontamination and an association of dysbiosis with the occurrence of GI aGvHD as well as several severe infectious complications following allogeneic SCT. Prophylactic and therapeutic antibiotics were even identified as the major driver of dysbiosis (8, 9) and these findings more and more questioned at least prophylactic concepts. Recent reports also suggested that even the reconstitution of commensal bacteria by fecal microbiota transfer (FMT) contributes favorably to the treatment of patients (pts) with steroid-refractory aGvHD (10–12).

The mechanisms of how commensal intestinal microbiota dampens intestinal inflammation in general and in the setting of aGvHD are still poorly understood. Microbial metabolites that are produced by commensal bacteria after digestion of dietary fibers, tryptophan, and other sources have been identified as major protective molecules that act as mediators of pathogen-host interaction and exert protective functions. In this context, short-chain fatty acids (SCFA) like butyrate and propionate are not only a major energy source for colonocytes but also stabilize the epithelium and dampen immune reactions by multiple mechanisms including regulation of Nlrp3-inflammasome dependent inflammation (13) and by induction of regulatory T cells (T regs) (14, 15). Indoles derived from dietary tryptophan stabilize the epithelium *via* induction of interleukin 22 in innate lymphoid cells and modulate inflammation by inducing anti-inflammatory cytokines such as interleukin 10 (16, 17). Strong protection against aGvHD by the tryptophan-metabolite Indol-3-carboxaldehyde (ICA) was observed in Swimm´s study (18) as gavage with ICA reduced aGvHD mortality to a large extent in a type I Interferon (IFN-I) dependent manner while maintaining graft-versus-leukemia activity.

As all these mechanisms have been reported to modify GvHD, it is not surprising that experimental reports found

significant protection from GvHD by these metabolites. Mathewson and colleagues applied butyrate gavage and a cocktail of commensal clostridia known to be high SCFA producers in a murine model of GvHD and reported strong protection (19). Recently, the same group addressed the role of SCFA by using knockout mice for one of the receptors of SCFA, G-protein coupled receptor (GPR) 43, and reported that GPR43 knockout on non-hematopoietic cells led to accelerated and increased GvHD related mortality (20).

In humans, a comparable role of SCFA is likely and suggested by a recent analysis of Romick-Rosendale et al. (21) who reported reduced stool SCFAs after exposure to Abx suppressing commensals in children receiving HSCT but so far no data have been reported regarding the expression of GPR in adult human GvHD. We, therefore, performed an analysis of expression of the major SCFA receptors GPR43 and GPR109A by quantitative PCR in intestinal biopsies obtained from pts receiving allogeneic SCT at our unit. We observed upregulation of GPR in aGvHD which was strongly suppressed by broad-spectrum antibiotics.

# MATERIAL AND METHODS

## Patient Characteristics

338 serial biopsies were obtained and analyzed from a total of 199 adult patients (pts) receiving allogeneic SCT between Nov 2008 and Nov 2015. Patient characteristics are summarized in **Supplementary Table 1**. The disease status was defined according to the EBMT score (22). All pts gave informed consent, the biopsy studies and scientific analyses were approved by the local ethical review board (approval no 02/220 and 09/059). All studies were performed in accordance with the regulations of Helsinki. Serial biopsies were either obtained i) in the course of a screening study in asymptomatic, clinically aGvHD free pts or ii) because of clinical symptoms indicative of *de novo* onset or iii) persistence or recurrence of GI aGvHD. Biopsies were obtained through upper or lower GI endoscopy.

## Quantitative Real-Time PCR (qPCR)

qPCR on intestinal biopsies was performed according to RNA availability. 338 serial biopsies for *GPR109A*, 263 biopsies for *GPR43*, 103 biopsies for *NLRP3*, 281 biopsies for *FOXP3* mRNA and 240 biopsies for FOXP3 immunohistochemistry were available. Intestinal biopsies were immediately transferred to 500 µl RNA later (QIAGEN) and were stored at -80°C until RNA extraction. RNA was extracted using RNeasy Mini Kit (QIAGEN) as per manufacturer's recommendation. RNA concentration and purity was monitored by NanoDrop and Bioanalyzer respectively. 1 µg of RNA was reverse transcribed to cDNA using *moloney murine leukemia virus* reverse transcriptase (Promega) following the manufacturer's instructions. qPCR was performed on a Mastercycler Ep Realplex (Eppendorf) using QuantiFast SYBR Green PCR Kit (QIAGEN). *18S* ribosomal RNA was used as reference gene. Gene of interest was normalized to the reference gene.

Gene-specific primer sequences are as follows: *GPR109A*, forward: 5' GCG-TTG-GGA-CTG-GAA-GTT-TG-3', reverse: 5'- GCG-GTT-CAT-AGC-CAA-CAT-GA-3'; *GPR43*, forward: 5'- GTA-GCT-AAC-ACA-AGT-CCA-GTC-CT -3', reverse: 5-CTA-GGT-GTT-GCT-TTG-AAG-CTT-GT -3'; *FOXP3*, forward: 5'-GAA-ACA-GCA-CAT-TCC-CAG-AGT-TC -3'; reverse: 5'- ATG-GCC-CAG-CGG-ATG-AG-3'; *NLRP3*, forward: 5'-GGA-CTG-AAG-CAC-CTG-TTG-TGC-A-3', reverse: 5'- TCC-TGA-GTC-TCC-CAA-GGC-ATT-C-3'; *18S*, forward: 5'-ACC-GAT-TGG-ATG-GTT-TAG-TGA-G-3', reverse: 5'-CCT-ACG-GAA-ACC-TTG-TTA-CGA-C-3'.

## Immunohistological Analysis

The same pathologist blinded to the clinical data assessed serial biopsies. GI-aGvHD was graded according to the Lerner grading system (23). The number of FOXP3 positive cells was determined by immunohistochemistry, analyzed with a Zeiss Axioskop 40 microscope. 2-3µm thick slides sectioned from the formalin-fixed and paraffin-embedded (FFPE) biopsies were deparaffined and stained automatically (Ventana Benchmark Ultra). After pre-treatment with CC1 buffer the immunohistochemical staining was performed with a monoclonal mouse antibody (1:120, eBioscience 14-4777, clone 236A/E7) and OptiView DAB IHC Detection Kit (Ventana). The mean number of FOXP3 positive stromal cells was determined microscopically per high power field (HPF), counting 3-12 HPF exhibiting the highest histological aGvHD damage.

## Immunofluorescence of Biopsies

FFPE biopsies were cut 2-3 µm thick and were incubated at 80°C for 30 minutes followed by immersing in Xylol twice for 10 minutes each following descending alcohol line for 5 minutes each. Sections blocked with 20% Bovine Serum Albumin (BSA) for 20 min at room temperature (RT). Single immunofluorescence was performed for GPR43 (Biozol, LSA1578-50, rabbit polyclonal). Double immunofluorescence was performed for GPR43 (Biozol, LSA6599, rabbit polyclonal) and CD68 (Dako, PG-M1, mouse monoclonal). Primary antibodies were diluted in 1% BSA and were applied to biopsy section at the dilution of 1:50 for 1 hour at RT followed by secondary antibodies Alexa Flour (AF) 488 and Alexa Flour 594 (Invitrogen) for 30 minutes (dilution 1:100) in the dark at RT. CD68 was conjugated with AF488 and GPR43 was conjugated with AF594. Sections were counterstained with DAPI and were sealed with mounting media. Biopsy sections were washed three times with PBS after every step. GPR43 positive cells were observed and images were taken at 200X magnification using Zeiss epifluorescence microscope.

## Dendritic Cell (DC) Culture and Determination of Cytokines

Monocytes were isolated from PBMC of healthy donors after leukapheresis followed by density gradient centrifugation over Ficoll/Hypaque as described previously (24). All healthy volunteers consented to the study. Freshly isolated monocytes were differentiated into DCs as previously described (17). On day 7, 100 ng/mL LPS (Enzo) was added to induce maturation of

immature DCs (iDCs) in absence or presence of 5 mM sodium acetate (NaA), 2.5 mM sodium propionate (NaP) and 0.5 mM sodium butyrate (NaB) for another 48 hours. NaA, NaP and NaB were purchased form Sigma-Aldrich. On day 9, mature DCs (mDCs) were harvested for RNA extraction, cDNA synthesis and qPCR as described above. Supernatants were collected for cytokine determination and were stored at -20°C until further analysis. IL-12 and IL-10 cytokines were analyzed using enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's recommendation (R&D).

## Caco-2 Cell Culture

The human intestinal Caco-2 cell line was purchased form CLS Germany. Cells were maintained in DMEM low glucose media (Sigma) supplemented with 10% FCS (Sigma), 1% NEAA, 1% NaP and 0.5% P/S (Gibco) in a collagen (5 μg/cm$^2$) coated T75 flask. Cells were seeded at 9.3x10$^3$ cells/cm$^2$ and subcultured after 60-80% confluency for a maximum of 10 passages, changing media every two days. For differentiation, Caco-2 cell monolayer was grown at a density of 3x10$^5$ cells/cm$^2$ on 0.4 μm collagen coated polyster membrane 12 well transwell (1.12 cm$^2$ area) for 3 weeks. The monolayer was monitored by measuring trans epithelial electrical resistance (TEER) with Millicell ERS-2 voltohmmeter (EMD Millipore). Media was changed every two to three days. On day 21, cells were stimulated with 50 ng/ml IL1β and 100 ng/ml TNF (PromoCell) with or without sodium butyrate (Sigma) for 24 hours. Barrier integrity was monitored by TEER measuremernt. Cell supernatants were analysed for IL-6 and IL-8 ELISA as per manufacturer's recommendation (R&D). Immuofluorescent staining of Caco-2 cells were performed as previously described (17).

## Statistical Analysis

Data analysis was done in SPSS v26. Test of normality was performed using Shapiro-Wilk test. Normally distributed data was analysed with t-test or one way ANOVA. Correlation analysis was performed with Pearson test. For non-normal data, Mann-Whitney or Kruskal Wallis tests were performed and Spearman correlation was chosen. For multivariate analysis, results were dichotomized based on median. The Lerner stage of aGvHD and use of Abx prior to biopsy were analyzed using binary logistic regression.

# RESULTS

## GPR Expression Correlates With the Severity and Onset of GI-aGvHD

When we assigned unbiasedly selected serial biopsies based on the determined histological Lerner stage to either aGvHD 0-1 or aGvHD 2-4, we found that patients (pts) with higher Lerner stages showed increased GPR expression (**Figures 1A, B**; p=0.000015 for GPR109A, p=0.008 for GPR43). In accordance with this observation, clinical symptomatic aGvHD pts showed higher GPR expression (p=0.0001 for GPR109A, p=0.006 for GPR43) compared to aGvHD free screening pts or ongoing aGvHD pts (**Figures 1C, D**). In addition, the phenomenon of GPR upregulation was observed in both upper or lower gastro-intestinal (GI) tract (**Table 1**). In summary, GPR expression was upregulated in both histological and clinical aGvHD independent of anatomical section of biopsy.

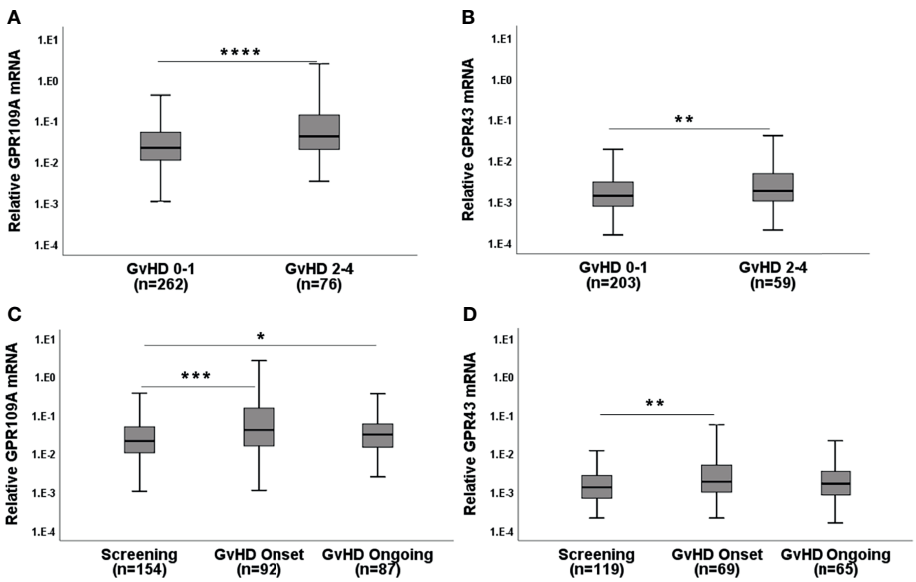



**FIGURE 1** | GPR mRNA expression in the serial biopsies from the gastro-intestinal tract in the course of GvHD. **(A)** GPR109A and **(B)** GPR43 expression with respect to Lerner GI-GvHD. **(C)** GPR109A and **(D)** GPR43 expression in screening biopsies and at the clinical onset of GI-GvHD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, Mann-Whitney U test.

**TABLE 1** | Distribution of GPR109A and GPR43 in the upper and lower gastro-intestinal (GI) tract.

**A. Histological GvHD**

| Genes | Lerner stage | No of samples | Mean rank | P value |
|---|---|---|---|---|
| **Upper Gastrointestinal tract** | | | | |
| GPR109A | 0-1 | 82 | 47.2 | 0.003 |
| | 2-4 | 20 | 69.15 | |
| GPR43 | 0-1 | 70 | 41.06 | 0.058 |
| | 2-4 | 16 | 54.19 | |
| **Lower Gastrointestinal tract** | | | | |
| GPR109A | 0-1 | 179 | 110.56 | 0.002 |
| | 2-4 | 57 | 143.43 | |
| GPR43 | 0-1 | 133 | 83.96 | 0.023 |
| | 2-4 | 44 | 104.43 | |

**B. Clinical GvHD**

| Genes | Clinical character | No of samples | Mean rank | P value |
|---|---|---|---|---|
| **Upper Gastrointestinal tract** | | | | |
| GPR109A | Screening | 51 | 31.69 | 0.005 |
| | Onset | 20 | 47.00 | |
| GPR43 | Screening | 43 | 27.12 | 0.017 |
| | Onset | 17 | 39.06 | |
| **Lower Gastrointestinal tract** | | | | |
| GPR109A | Screening | 103 | 79.37 | 0.007 |
| | Onset | 72 | 100.34 | |
| GPR43 | Screening | 76 | 58.61 | 0.030 |
| | Onset | 52 | 73.12 | |

***(A)*** *GPR distribution in the GI tract according to the Lerner classification of acute GvHD (GvHD 0-1 vs GvHD 2-4).* ***(B)*** *GPR distribution in the GI tract according to the clinical characteristics of acute GvHD (screening vs onset).*

## Broad Spectrum Antibiotics (Abx) Suppress GPR and FOXP3 Expression

Broad spectrum Abx results in rapid loss of microbiota diversity. We, therefore, considered the application of Abx (mainly piperacillin/tazobactam or carbapenems) within 7 days before obtaining biopsies as an indicator of microbiota damage. GPR expression in Abx group was significantly reduced compared to the no Abx group (**Figures 2A, B**, p=0.0004 for GPR109A and

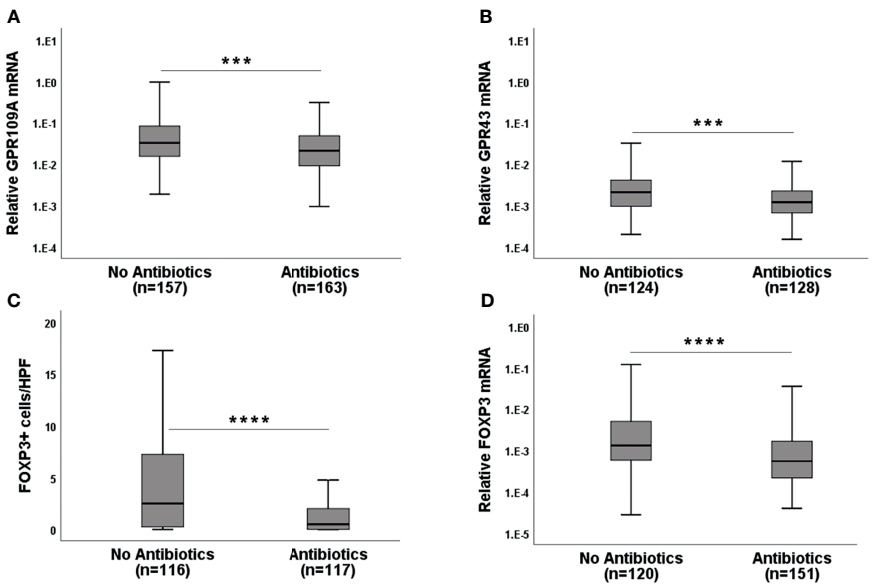



**FIGURE 2** | Effect of broad-spectrum antibiotics (Abx) on GPR and FOXP3 expression in the serial biopsies from the gastro-intestinal tract. **(A)** GPR109A mRNA **(B)** GPR43 mRNA **(C)** FOXP3+ cellular infiltrates and **(D)** FOXP3 mRNA expression in the gut biopsies of patients without or with broad-spectrum antibiotic exposure at the time of biopsy retrieval. HPF-high power field. ***p < 0.001, ****p < 0.0001, Mann-Whitney U test.

p= 0.0001 for GPR43) suggesting that commensal bacteria and their metabolites are needed for optimal GPR induction. Abx suppressed not only GPR, but also FOXP3 mRNA, as well as FOXP3+ regulatory cell (Tregs) infiltrates (**Figures 2C, D**, p<0.0001 forboth FOXP3 mRNA and protein). Following these results, we subsequently classified pts based on cumulative and long-term antibiotic exposure. The first group did not receive early Abx (before or at day 0 of transplantation) or at the time of biopsy. The second group received early Abx but not at the time of biopsy. The third group had Abx at the time of biopsy but no early exposure. The fourth group had both early Abx exposure and at the time of biopsy. The highest GPR expression was observed in the patient group who never had Ab exposure (**Supplementary Figures 1A, B**, p=0.002 for GPR109A, p=0.016 for GPR43, Kruskal-Wallis test). A similar picture was obtained for FOXP3 mRNA and Tregs infiltrates (**Supplementary Figures 1C, D**, p=0.007 for FOXP3+ cellular infiltrates, p=0.0004 for FOXP3 mRNA, Kruskal-Wallis test). Significant loss of GPR was observed in the patient group with early Abx and Abx at biopsy. This may result from previously reported rapid loss of commensals after Abx treatment to pts and is reflected by reduced GPR expression in the gut biopsies. When we grouped pts according to the clinical GI-aGvHD status at the time of biopsies (GI-aGvHD free screening biopsies and aGvHD clinical onset biopsies) and further re-grouped them again according to the use of Abx, our findings were confirmed in the serial biopsies. Pts who did not receive Abx showed significant increases in GPR at the onset of aGvHD (p=0.001 for GPR109A, p=0.014 for GPR43) whereas pts with Abx did not show GPR upregulation at the aGvHD onset (**Figures 3A, B**). Moreover, in the screening biopsies, GPR109A expression was significantly downregulated in the Abx group (p=0.028) whereas GPR43 only showed a trend of downregulation. At aGvHD onset, both GPR showed significant downregulation in the Abx group (p=0.004 for GPR109A, p=0.021 for GPR43) suggesting a detrimental effect of Abx in the course of protective GPR upregulation. When we performed binary logistic regression comparing aGvHD Lerner stage and Abx use, we identified antibiotic use but not aGvHD as an independent risk factor for the loss of GPR as well as FOXP3 (**Supplementary Table 2**).

## Association of GPR With FOXP3 and NLRP3 Expression

As SCFA have been reported to be involved in immunoregulation, we performed simultaneous PCR for FOXP3 expression in the serial gut biopsies. A highly significant correlation between GPR and FOXP3 was observed for both GPR (**Figures 4A, B**, p<0.0001). We dichotomized GPR expressions as "high" and "low" categories based on their median expression (median value: 2.57xE-002 for GPR109A and 1.5xE-003 for GPR43). Higher GPR expression was associated with higher FOXP3 expression and vice versa (p=0.000 for both GPR, data not shown). To confirm this association, we performed immunohistochemistry for FOXP3+ cellular infiltrates. We found that Tregs infiltration was significantly higher in GPR "high" category (p=0.001 for GPR109A, p=0.003 for GPR43) compared to GPR "low" category (**Figures 4C, D**). In addition, binary logistic regression confirmed that both GPR109A and GPR43 independently influence FOXP3 expression (GPR109A: odds ratio,0.74 [95% CI, 1.24-3.55]; p=0.005, GPR43: odds ratio, 0.61 [95% CI, 1.08-3.17]; p=0.024). We also observed a strong association of GPRs with inflammasome receptor NLRP3 in a serial biopsies (**Supplementary Figure 2A, B**). Patient biopsies with high GPR43 expression also had significantly higher NLRP3 expression (p=0.003). GPR109A, although not significant, showed a strong trend of upregulation with higher NLRP3 expression (p=0.087). Regression model revealed that GPR43, but not GPR109A, independently influence NLRP3 expression (GPR43: odds ratio,1.03 [95% CI, 1.1-6.7]; p=0.02, GPR109A: odds ratio, 0.54 [95% CI, 0.71-4.1]; p=0.2). The strong GPR-NLRP3 association was only seen in pts not receiving Abx. Pts on Abx did not show any GPR-NLRP3 association (**Supplementary Figure 2C, D**).

## Epithelial Cells and Immune Cells as a Cellular Source of GPR

To identify the cellular source of GPR, we next performed single and double immunofluorescence of GPR43 and CD68 on sigmoid colon biopsies of pts following transplantation. Within the non-hematopoietic compartment, epithelial cells seemed to be the major source of GPR expression (**Figure 5A**) labeled by GPR43 antibody (cytoplasmic domain, LS-A1578). The gut lumen bears

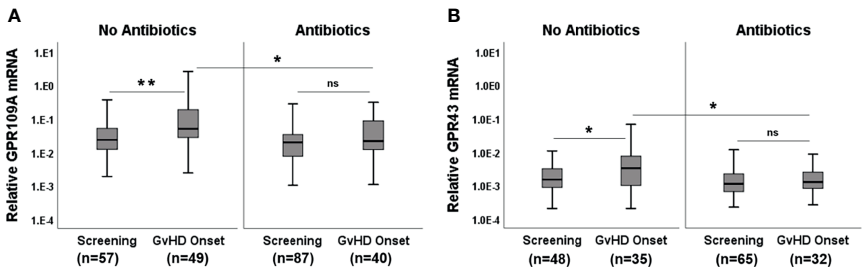



**FIGURE 3** | Effect of Abx at the onset of GvHD. **(A)** GPR109A expression at GvHD onset without or with Abx. **(B)** GPR43 expression at GvHD onset without or with Abx. *p < 0.05, **p < 0.01, Mann-Whitney U test. ns, not significant.

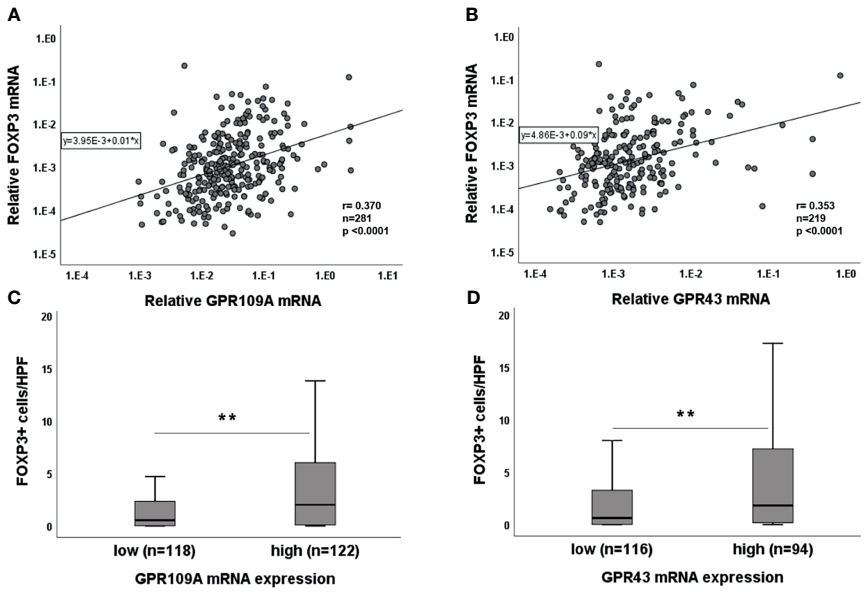



**FIGURE 4** | Association of GPR with FOXP3 expression. Correlation of **(A)** GPR109A and **(B)** GPR43 with FOXP3 mRNA. Association of **(C)** GPR109A and **(D)** GPR43 with FOXP3 cellular infiltrates. **p < 0.01, Mann-Whitney U test; r value, Spearman correlation.

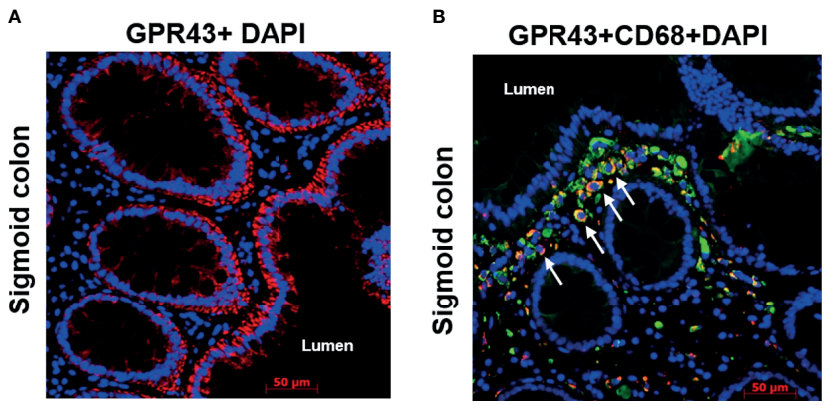



**FIGURE 5** | Immunofluorescence of GPR43 of a representative patient biopsy. Time from transplant to biopsy: 3.5 years, no GvHD at the time of biopsy. **(A)** GPR43 staining in the sigmoid colon of a patient. GPR43 is labelled with AlexaFlour (AF) 594 (red). **(B)** GPR43 and CD68 co-staining in the sigmoid colon of a patient. GPR43 is labelled with AF594 (red) and CD68 is labelled with AF488 (green). White arrow indicates colocalized signals. Nucleus is counterstained with DAPI (blue). Scale bar: 50 µm.

the highest concentration of SCFA and gut epithelium may express GPR in a positive feedback loop. In double immunofluorescence of CD68 and GPR43 (extracellular domain, LS-A6599), two signals co-localized suggesting macrophages as one of the cellular sources of GPR within the immune cell compartment (**Figure 5B**). These GPR43 positive macrophages seemed to accumulate close to the epithelium. The involvement of immune cells in GPR expression is also suggested by the localization dependent expression of GPR. When we compared GPR expression in serial biopsies obtained from different anatomical sections of the gastrointestinal tract, significant higher GPR (p=0.002 for GPR109A, p=0.001 GPR43, Kruskal-Wallis test) was observed in ileal biopsies (**Supplementary Figures 3A, B**). This might reflect a higher presence of immune cells in the ileum.

## Effect of SCFA on Immune Cells and Epithelial Cells *In Vitro*

### SCFA Upregulate GPR Expression and Alter Cytokine Production in mDCs

We next assessed the effect of SCFA in lipopolysaccharide (LPS) stimulated monocyte-derived dendritic cells (mDCs) from three healthy donors. 5mM acetate or 2.5 mM propionate or 0.5 mM

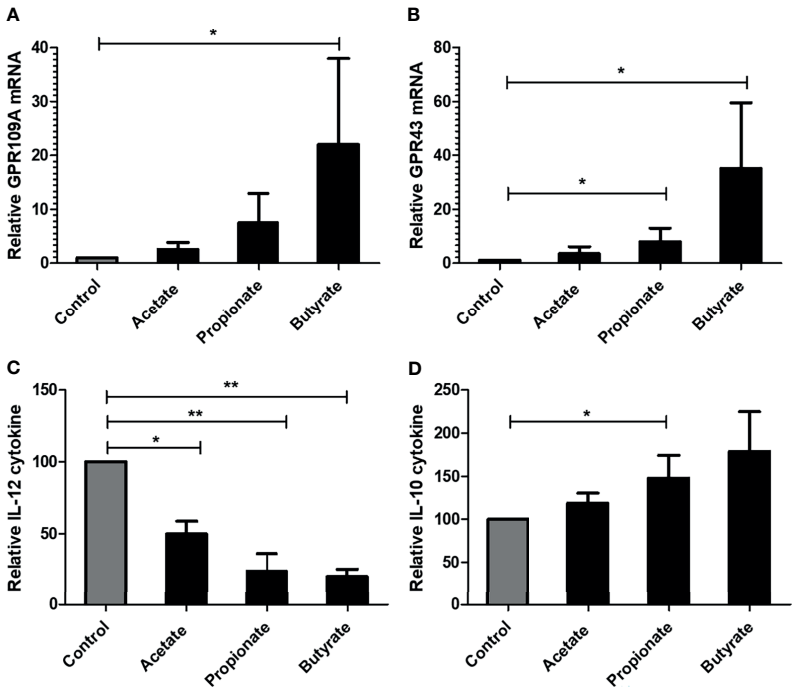



**FIGURE 6** | Effect of SCFA on in-vitro generated human monocyte derived DCs. DCs were cultured for 7 days and were stimulated with 100 ng/ml LPS for 48 hours. **(A, B)** GPR109A and GPR43 expression in mature DCs in presence of SCFA. **(C)** IL-12 cytokine release by DCs in presence of SCFA. **(D)** IL-10 cytokine release by DCs in presence of SCFA. n= 3 healthy donors. Bar represents mean +/- s.e.m. *p < 0.05, **p < 0.01, Mann-Whitney U test for A, B and D (non-normal distribution), one way ANOVA with Bonferroni correction for C (normal distribution).

butyrate was added together with LPS. The given concentration of SCFA did not induce cell death of mDCs when compared to control mDCs as observed by Annexin/7-AAD staining (data not shown). SCFA, especially butyrate, induced significantly higher expression of GPR109A and GPR43 in mDCs (**Figures 6A, B**). At the functional level, SCFAs were able to suppress the LPS induced activation of mDCs indicated by a reduction of pro-inflammatory cytokine IL-12 (**Figure 6C**) and an upregulation of anti-inflammatory cytokine IL-10 (**Figure 6D**).

### Butyrate Suppresses Pro-Inflammatory Cytokines and Induce GPR43 Expression in Caco-2 Cells

Following the immunomodulatory effect of butyrate on dendritic cells, we sought to investigate the effect of butyrate on epithelial cell line model Caco-2. In four individual experiments, fully differentiated Caco-2 cells on a transwell system were stimulated with 50 ng/ml IL-1β and 100 ng/ml TNF. 5 mM butyrate was added to the stimulated cells for 24 hours. Butyrate toxicity was excluded by MTT assay (data not shown). In absence of stimulation, Caco-2 (control) cells did not produce cytokines. IL-1 β stimulation was a pre- requisite for cytokine production by Caco-2 cells. The production of pro-inflammatory cytokine IL-8 and IL-6 by Caco-2 cells was significantly suppressed on both apical and basolateral side of the transwell system by the addition of butyrate (stim+butyrate) when compared to stimulated condition (stim) (**Figures 7A–D**). Stimulation also compromised barrier intergrity as shown by significant reduction of transepithelial electrical resistance (TEER) when compared to control (**Figure 7E**). The addition of butyrate showed rescue effect by significantly increasing TEER (**Figure 7E**). When we labelled Caco-2 cells with GPR43 antibody, we observed a stronger GPR43 signal in butyrate-treated epithelial cells compared to untreated control or stimulated control (**Figure 7F**).

## DISCUSSION

The human gut harbors a plethora of microorganisms that are crucial for development and normal physiological functions. An imbalance or maladaptations of these essential microorganisms, also termed dysbiosis, has been linked to numerous intestinal disorders including GvHD. Several studies have confirmed a strong association of microbiota damage with the occurrence of GvHD and associated transplant-related complications (7, 25, 26). Microbiota-derived SCFA such as acetate, propionate, and butyrate have been described in previous studies to be the key modulator of inflammation and GvHD by promoting anti-inflammatory myeloid cells and by maintaining epithelial barrier integrity (13, 19, 27, 28). These studies also revealed the involvement of G-protein coupled receptors GPR109A, GPR43, and GPR41 in the mitigation of GvHD. However, these studies

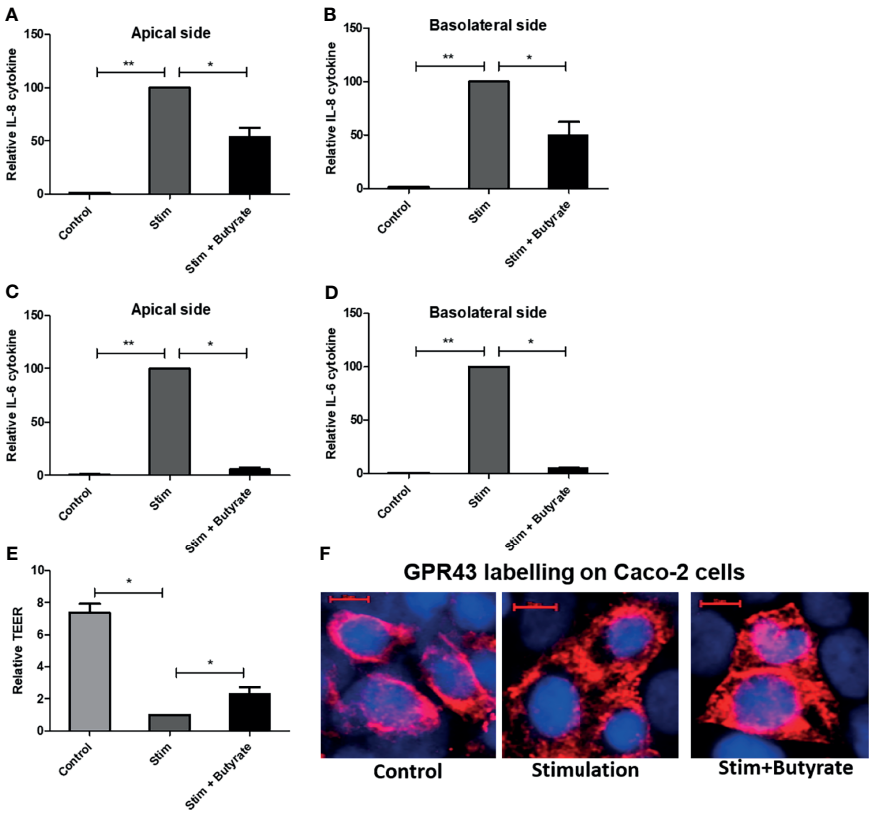



**FIGURE 7** | Effect of butyrate on human epithelial cell. Caco-2 cell were grown on collagen coated transwell for 21 days. Cell were treated with IL-1β and TNF for 24 hours without or with butyrate. **(A, B)** IL-8 cytokine release on apical and basolateral side of Caco-2 cells. **(C, D)** IL-6 cytokine release on apical and basolateral side of Caco-2 cells. **(E)**. Transepithelial electrical resistance (TEER) changes with stimulation (stim) alone or with butyrate. **(F)** GPR43 staining of Caco-2 cells in untreated control, stimulated control and butyrate treated condition. GPR43 is labelled with AF 594. Nucleus is counterstained with DAPI. Scale bar: 10μm. n = 4 independent experiments. Bar represents mean +/- s.e.m. *p < 0.05, **p < 0.01, Mann-Whitney U test.

were performed in mice and no data have previously been reported regarding the role of GPR expression in adult human GvHD.

Our clinical data show an increased expression of GPR43 and GPR109A in patients (pts) suffering from GvHD. Especially during GvHD onset or at higher grade GI-GvHD (Lerner II-IV), GPR expression was significantly enhanced. This might reflect a counter-regulatory mechanism of protective GPR signaling that is reactively induced to suppress T cell-mediated injury. There are only a few studies discussing counter-regulatory mechanisms in the gut of GvHD pts (29, 30). Landfried et al. showed an increase of IDO in the lower GI tract of GvHD pts (29) while Lord et al. showed an increase of FOXP3 Tregs in the gastric biopsies of GvHD pts (30). Takatsuka et al. showed significant increase of plasma IL-10 in GvHD patients (31). We speculate that the actual increase in regulatory parameters such as IDO, FOXP3, IL-10, and GPR in GvHD is a physiological counter-reaction to suppress the various inflammatory reactions going on in patients' system. In addition, it is known that inflammatory stimuli such as TNF, IL-1, LPS or GM-CSF can induce GPR expression on monocytes (32) and macrophages (33). Therefore, it is likely that the induction of GPR is, in part, the result of elevated inflammation in GvHD.

Commensal bacteria are the most prominent SCFA producers and have been reported to be suppressed after antibiotic treatment (9, 34). We recently demonstrated that Abx suppresses butyrogeneic bacteria that are responsible for SCFA production (35). Consistently, we found that (i) SCFAs induce GPR expression in human colon cell lines and mDCs and (ii) Abx significantly suppressed GPR expression in the intestinal biopsies of allo-SCT pts. Utilizing a regression model, Abx suppressed GPR expression independent of GvHD. Herein, we propose that the detrimental effect of Abx are confined not only to loss of commensals following reduction of SCFA but also to the loss of GPR expression. Cumulative and long-term antibiotic exposure revealed that GPR expression was highest in pts who did not receive Abx either before/at transplantation or before biopsy retrieval. On contrary, the lowest GPR expression was observed in pts who received Abx before transplantation and also at the time of biopsy indicating persistent long-term dysbiosis by cumulative ABX exposure. The fact that GPR upregulates in GvHD onset pts only in the absence of Abx but not in presence of

Abx implicates the potentially protective "commensal-SCFA-GPR" axis in GvHD patients which is clearly abrogated by Abx.

SCFAs have been reported to expand regulatory T cells (15, 36) and these cells prevent GvHD and promote immune reconstitution (37–40). We, therefore, addressed the interrelation of GPR and FOXP3 expression. We observed a high correlation between GPR and FOXP3 expression on mRNA level which was also confirmed in pts where immune cell infiltrates were directly stained for FOXP3 and the positive cells were counted in high power field (HPF). Strong association of GPR with Treg infiltrates point towards the GPR-FOXP3 axis that is again abrogated by the use of Abx. In addition, we saw a negative correlation between Abx use and FOXP3 expression suggesting a link between microbiota changes and immunoregulation although the exact pathways linking ABX use and FOXP3 suppression need to be further analyzed. As we used single antibody staining for our immunohistological analysis of FOXP3+ cells, we are thus far unable to further characterize the Treg subpopulations in more detail. In the clinical setting, it is still unclear whether both natural and induced Tregs are affected by SCFA and future studies using multiplex staining are required to address these questions.

Previous murine studies reported that the salutary effect of GPR in mitigating GvHD occurred *via* non-hematopoietic cells, namely intestinal epithelial cells in an NLRP3 dependent fashion (19, 20). In line with murine data, we also observed a strong association of NLRP3 with GPR expression in patient gut biopsies supporting the GPR regulation in epithelial cells. Immunofluorescence revealed intestinal epithelial cells as one of the cellular sources of GPR43 within the non-hematopoietic compartment which is in line with a previous study (41). In an intestinal epithelial cell line model, butyrate suppressed inflammatory cytokine release, rescued the damaged epithelial barrier and increased GPR43 expression indicating the positive feedback loop of ligand-receptor interaction. Within the hematopoietic compartment, CD68 positive macrophages coexpressed GPR43. Previous murine and human studies described leukocyte subpopulation as a source of GPR43 (42, 43). Immune cells like macrophages, dendritic cells, monocytes, and neutrophils likely play an inevitable role in GPR-mediated protection from GvHD and antibiotic treatment abrogates the necessary protective phenomenon due to dysbiosis, or, by inhibiting the bacterial translocation that would otherwise induce immune responses. Upon treatment with SCFA, in-vitro generated mDCs showed increased expression of GPR109A and GPR43 followed by reduced pro-inflammatory IL-12 and an increase in anti-inflammatory IL-10 cytokine release pointing towards the immunoregulatory phenomenon of SCFA and are in line with previous reports where bacterial metabolite exerted immune regulation by modulating antigen-presenting cells (17, 44). In our study, pts showed higher expression of both GPR in the ileum and there was a gradual recovery of GPR over the time after transplantation implicating the role of hematopoietic cells and recovering epithelial tissue. Our data is in line with previous murine studies that reported the involvement of immune cells in GPR-mediated protection against inflammation (27, 45).

Our study has some limitations. We were not able to directly assess microbiome status at the time of biopsy retrieval. This limited simultaneous analysis of GPR expression and microbial diversity and prompted us to use antibiotic treatment as a surrogate in the clinical settings of GvHD. Furthermore, epithelial interactions of SCFA with GPR are likely to be directly influenced by luminal metabolites of commensals, however, we do not know yet about the exact role of translocated bacteria and tissue metabolites which are likely to play an additional role due to the leakiness of epithelia in GvHD and tissue immune regulation (46). Nevertheless, our study is the first to address the interaction of microbiota and regulation of adaptive immune responses in human tissue biopsies of SCT pts. So far, only stimulation of peripheral blood Tregs has been reported in pts receiving fecal microbiota transplant (FMT) from healthy donors for treatment of refractory GvHD (11), thus both observations point to the fact that a diverse microbiota is needed to mount an adequate Treg cell response. Whether the observed association of GPR and FOXP3 expression is due to a direct effect of SCFA on Tregs induction, e.g. *via* HDAC inhibition as reported in an earlier study (15), or involves further mediators released by immune or epithelial cells, needs further investigation. The negative impact of antibiotic treatment on Treg cell response in tissues has so far been reported outside HSCT models. An association of early-life antibiotic exposure and the development of experimental asthma in murine models have been observed (47). In a murine model of pulmonary metastases, antibiotic treatment reduced T regs and increased the cytotoxic T cell response (48). Similarly, experimental FMT has been shown to increase Treg cell frequencies in the gut which were diminished after Abx exposure (49, 50). Overall, our observations are in line with the protective effects of high SCFA producing commensals in HSCT-associated complications and support the concept that microbiota restoration, e.g. by FMT may be beneficial in GvHD pts. So far, only a small and casuistic series of successful FMTs in clinical GvHD has been reported, but thoroughly designed clinical trials are now initiated to examine the exact contribution of microbiota reconstitution by FMT or more specific consortia of commensals to immunomodulation of GvHD.

To conclude, our data suggest so far neglected but deleterious effects of Abx on GPR expression and immunoregulation in clinical GvHD. We urge the need for microbiota preservation or restoration either by FMT, transfer of protective commensal consortia or by fiber-rich diet (51). In addition, our data strongly suggest restrictive use of Abx and support careful antibiotic stewardship to maintain microbiota, metabolites, receptors, and immunoregulation. This approach might be relevant for GvHD prophylaxis and treatment as well as several other diseases where dysbiosis is concerned.

## 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

The studies involving human participants were reviewed and approved by Aktive Ethikvoten der Ethik-Kommission an der Universität Regensburg Email: ethikkomission@ur.de. The patients/participants provided their written informed consent to participate in this study (approval no: 02/220, 09/059, 17-619-101).

## AUTHOR CONTRIBUTIONS

SakG performed the experiments, collected and analyzed the data, and wrote the manuscript. DWe designed the study, analyzed clinical data and discussed the manuscript. KH performed immunohistological analyses. EM, MH, and AS-K collected and analyzed clinical data in relation to biopsy results. AG, AH, CM, SarG, DWo, ME, PH, HP, and WH reviewed and discussed the manuscript. EH designed the study, performed data analysis and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.753287/full#supplementary-material

**Supplementary Table 1 |** Clinical characteristics of patients: MDS = myelodysplastic syndrome, MPS = myeloproliferative syndrome, BM, bone marrow; PBSC, peripheral blood stem cells; UCB, umbilical cord blood; CyA, cyclosporine; MTX, Methotrexate; Tacro, tacrolimus; MMF, mycophenolate mofetil; PTCy, post transplant cyclophosphamide.

**Supplementary Table 2 |** Analysis of factors influencing GPRs and FOXP3 expression. In a multivariable analysis, Abx use is an independent factor to suppress GPR expression as well as FOXP3 expression. OR, odds ratio; CI, confidence interval.

**Supplementary Figure 1 |** Cumulative effect of broad spectrum antibiotics on the expression of **(A)** GPR109A, **(B)** GPR43, **(C)** FOXP3+ cellular infiltrates and **(D)** FOXP3 mRNA. *p < 0.05, **p < 0.01, ***p < 0.001, Mann-Whitney U test.

**Supplementary Figure 2 |** Association of **(A)** GPR109A and **(B)** GPR43 with NLRP3 expression. Effect of broad-spectrum antibiotics on **(C)** GPR43-NLRP3 association and **(D)** GPR109A-NLRP3 association *p < 0.05, **p < 0.01, ***p < 0.001, Mann-Whitney U test.

**Supplementary Figure 3 |** Distribution of **(A)** GPR109A and **(B)** GPR43 mRNA expression within the GI tract of patients after allogeneic SCT. Stomach, duodenum, ileum, colon and sigmoid colon were evaluated in the serial biopsies of transplanted patients. *p < 0.05, **p < 0.01, ***p < 0.001, Mann-Whitney U test.

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# T-Cell Metabolism in Graft *Versus* Host Disease

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Allogeneic-hematopoietic stem cell transplantation (allo-HSCT) represents the only curative treatment option for numerous hematological malignancies. Elimination of malignant cells depends on the T-cells' Graft-*versus*-Tumor (GvT) effect. However, Graft-*versus*-Host-Disease (GvHD), often co-occurring with GvT, remains an obstacle for therapeutic efficacy. Hence, approaches, which selectively alleviate GvHD without compromising GvT activity, are needed. As already explored for autoimmune and inflammatory disorders, immuno-metabolic interventions pose a promising option to address this unmet challenge. Being embedded in a complex regulatory framework, immunological and metabolic pathways are closely intertwined, which is demonstrated by metabolic reprograming of T-cells upon activation or differentiation. In this review, current knowledge on the immuno-metabolic signature of GvHD-driving T-cells is summarized and approaches to metabolically interfere are outlined. Furthermore, we address the metabolic impact of standard medications for GvHD treatment and prophylaxis, which, in conjunction with the immuno-metabolic profile of alloreactive T-cells, could allow more targeted interventions in the future.

**Keywords: GvHD, T-cells, immunometabolism, GvT, allo-HSCT**

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## INTRODUCTION

Allogeneic-hematopoietic stem cell transplantation (allo-HSCT) is a well-established and potential curative option for numerous high-risk hematological malignancies. Its therapeutic success, which depends on a mainly T-cell-driven Graft-*versus*-Tumor (GvT) reaction, is limited by the occurrence of Graft-*versus*-Host-disease (GvHD). Primarily (and like GvT) driven by alloreactive donor T-cells, with immune responses directed against foreign (host) antigens, GvHD can result in severe damage of host tissue, accounting for the majority of allo-HSCT-related morbidity and mortality (1, 2).

In GvHD treatment, T-cell responses are mitigated by immunosuppressive agents. However, increased susceptibility to infections, high mortality rates in steroid-refractory GvHD, and tumor relapses, emphasize the need for a deeper understanding of the T-cell pathobiology (1). As of to date, it is still challenging to balance GvT and GvHD. Consequently, novel and, more selective

approaches that specifically target GvHD (but not GvT) while maintaining physiological immunity are required.

T-cell metabolism, function, and differentiation are tightly interconnected in health and disease (3). Interference with immune cell metabolism, a viable therapeutic approach in autoimmunity and inflammation, constitutes metabolic (T-cell) alterations as potential targets for disease control (4). In GvHD, chronic antigen stimulation results in robustly activated T-cells with a unique metabolic profile (5). A comprehensive characterization of such disease-specific metabolic (T-cell) signatures holds the potential for novel targeted therapeutic approaches counteracting GvHD.

Hence, this mini-review will provide an overview of known metabolic T-cell alterations in GvHD (summarized in **Figure 1**) and will illustrate potential intervention strategies (**Table 1**), which could ideally allow to selectively "turn-off" T-cell-mediated GvHD.

## DYSREGULATED T-CELL METABOLISM IN GVHD

In response to alterations of the tissue environment (e.g. nutrient fluctuations) and upon activation and differentiation, T-cells undergo metabolic reprogramming. This crosstalk between substantial metabolic- and immune-signaling pathways is regulated by metabolic checkpoints (e.g. Myc, HIF1-α, AMPK, mTOR) with immune-modulatory functions (26, 27). A detailed description of this regulatory framework is beyond the scope of this review and hence is covered elsewhere (28–33).

### Glucose Metabolism

Glycolysis is essential for T-cell activation, supporting T-cell growth and proliferation (5, 34). Importantly, besides that, glucose metabolism represents a key player in inflammation (35).

Several studies demonstrated increased glycolytic activity in T-cells when activated by allo-antigens (6, 36). In a murine bone marrow transplant (BMT) model, expression of the key glycolytic enzyme Hexokinase 1/2 and glucose transporter GLUT 1/3 were upregulated in the allogeneic in contrast to the syngeneic setup (6). A GLUT1-deficiency model further underscored the requirement of GLUT1 not only for T-cell proliferation and $CD4^+$ effector T-cell ($T_{eff}$) expansion but also for GvHD induction (8).

$CD4^+$ T-cell-differentiated $T_{helper}$ 1 (Th1), Th2, and Th17 T-cells are pathogenic in GvHD and preferentially utilize glycolysis (37). Glycolytic activity is crucial for Th1 and Th17-differentiation and blockade of Th17 induction is linked to decreased expression of glycolytic enzymes. Although $CD4^+$ and $CD8^+$ T-cells rely both on glycolysis, in the context of allo-HSCT there are subtle differences, with $CD4^+$ T-cells being even more dependent on glycolysis than $CD8^+$ T-cells. In fact, this increased (and potentially preferential) susceptibility of alloreactive $CD4^+$ T-cells towards glycolytic interference could be therapeutically exploited (8, 37). Moreover, we observe this glycolytic shift of the $CD4^+$ T-cell subset not only in models of acute but also of chronic GvHD, which has so far been very little investigated in terms of T-cell metabolism (38).

Targeting glycolysis has shown promising results in murine models: treatment with 2-deoxy-D-glucose (2-DG), a glucose-analog inhibiting initiation of glycolysis, diminished

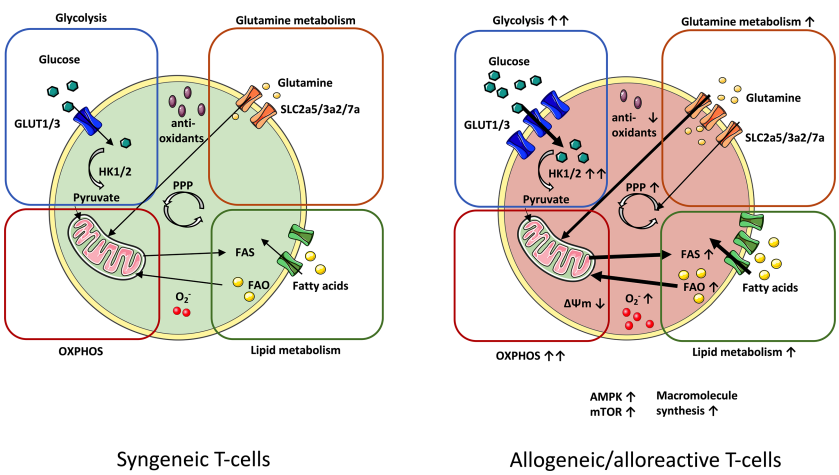


**FIGURE 1** | Metabolic profile of syngeneic *vs.* alloreactive T-cells. In order to meet their metabolic demands, GvHD-driving T-cells upregulate essential metabolic pathways. Glycolysis manifests as the principal source of energy in GvHD-causing T-cells. Fueling the TCA cycle with glycolysis-derived pyruvate reinforces increased OXPHOS activity in alloreactive T-cells. Enhanced OXPHOS potentiates production of ROS radicals ($O_2^-$), which is linked to lowered levels of antioxidants. Upregulation of glutamine metabolism further enhances OXPHOS by nourishing the TCA cycle with glutamine-derived a-ketoglutarate. Alloreactive/allogeneic T-cells display a superior lipid metabolism (FAS and FAO) and PPP-activity (fueled by glutamine as an anaplerotic source) as compared to syngeneic T-cells. Likewise, expression of the metabolic checkpoints AMPK and mTOR are elevated. Increased macromolecule synthesis complies with the demand of alloreactive T-cells for rapid cell growth and proliferation. AMPK, AMP-activated protein kinase; FAS, Fatty acid synthesis; FAO, Fatty acid oxidation; GvHD, Graft-*versus*-host-disease; mTOR, Mammalian target of rapamycin; OXPHOS, Oxidative phosphorylation; PPP, Pentose phosphate pathway; TCA, Tricarboxylic acid cycle.

**TABLE 1** | Selected *in vivo* studies investigating the effect of immuno-metabolic interventions and conventional GvHD therapy on T-cell metabolism and on outcome in allo-HSCT.

| Metabolic pathway | | Type of intervention | Mechanism of action | ROA | Effect on GvHD | Species | Ref. |
|---|---|---|---|---|---|---|---|
| **Metabolic inhibitors (or affected pathways)** | | | | | | | |
| Glycolysis | | 2-DG | HK2 inhibition | systemic (i.p.) | none | mouse | (6) |
| | | 3-PO | PFKFB3 inhibition | systemic (i.p.) | reduction | mouse | (6) |
| | | IL-1Ra antagonist | IL-1 receptor inhibition | *in vitro* treatment of donor T-cells | reduction | mouse | (7) |
| | | GLUT1 KO in donor T-cells | GLUT1 inhibition | genetic | reduction | mouse | (8) |
| OXPHOS | | BZ-423 | F1F0-ATPase inhibition | systemic (i.p.) | reduction | mouse | (9) |
| | | LYC-31138 | F1F0-ATPase inhibition | systemic (oral) | reduction | mouse | (10) |
| | | AMPK KO in donor T-cells | AMPK inhibition | genetic | reduction | mouse | (11) |
| | | Metformin | AMPK activation | systemic (i.p.) | reduction | mouse | (12) |
| Lipid metabolism | FAS | ACC1 KO in donor T-cells | ACC1 inhibition | genetic | reduction | mouse | (13) |
| | FAO | Etomoxir | CPT1 inhibition | systemic (i.p.) | reduction | mouse | (14) |
| | FAO | Orlistat | LAL inhibition | systemic (i.p.) | reduction | mouse | (15) |
| | FAO | 5-LO KO in donor leukocytes | 5-LO inhibition | genetic | reduction | mouse | (16) |
| | FAO | Zileuton | 5-LO | systemic (oral) | reduction | mouse | (16) |
| | SCFA signaling | GPR109a KO on donor T-cells | GPR109a inhibition | genetic | reduction | mouse | (17) |
| Glutamine metabolism | | Glutamine administration | Substrate substitution | systemic (i.p.) | reduction | mouse | (18) |
| | | Glutamine administration | Substrate substitution | systemic (oral) | reduction of GvHD related deaths | human | (19) |
| **Conventional GvHD therapy** | | | | | | | |
| N/A | | GCR KO in donor T-cells | GCR inhibition | genetic | increase | mouse | (20) |
| Glycolysis | | Rapamycin | mTORC1 inhibition | systemic (i.p.) | reduction | mouse | (6) |
| N/A | | BEZ235 | PI3K/mTOR inhibition | systemic (oral) | reduction | mouse | (21) |
| N/A | | CC214-2 | mTORC1/2 inhibition | systemic (oral) | reduction | mouse | (22) |
| Glycolysis | | Echinomycin | HIF-1α inhibition | systemic (i.p.) | reduction | mouse | (23) |
| N/A | | NFAT KO in donor T-cells | NFAT inhibition | genetic | reduction | mouse | (24, 25) |

*2-DG, 2-deoxy-D-glucose; 5-LO, 5-lipoxygenase; 3-PO, 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one; ACC1, acetyl-CoA-carboxylase-1; AMPK, AMP-activated protein kinase; CPT1, carnitine-palmitoyl-transferase; FAO, fatty acid oxidation; FAS, fatty acid synthesis; GCR, glucocorticoid receptor; GvHD, Graft-versus-Host-Disease; HIF1-α, hypoxia-inducible factor 1-alpha; HK2, Hexokinase 2; i.p., intraperitoneal; KO, knock out; LAL, lysosomal-acid-lipase; N/A, not available; NFAT, nuclear factor of activated T-cells; mTOR, mechanistic target of rapamycin; OS, overall survival; OXPHOS, oxidative phosphorylation; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-3; PI3K, phosphoinositide-3-kinase; Ref., reference; ROA, route of administration; SCFA, short-chain fatty acids.*

proliferation and activation of allogenic T-cells. However, that short-term *in vivo* treatment with 2-DG was not sufficient for GvHD-prevention and prolonged treatment resulted in severe toxicity. In contrast, 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3-PO), an inhibitor of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-3 (PFKFB3), which represents a glycolytic rate-limiting factor, efficiently controlled GvHD (6). Differences in efficacy might be due to the underlying mechanism of action: while 2-DG specifically interferes with glycolysis initiation, PFKFB3-inhibition has more dispersed effects: in addition to promoting glycolysis, PFKFB3 is involved in cell cycle regulation, T-cell survival, and function (6, 39).

Nonetheless, interpretation of those findings should be approached cautiously. A recent study revealed metabolic reprogramming of donor T-cells by AML blasts. AML-derived lactic acid (LA) was found to be responsible for diminished metabolic activity (including glycolysis) in T-cells of relapsing allo-HSCT patients (40). Thus, one needs to take into consideration that targeting the metabolism of alloreactive T-cells might promote disease relapse. However, the metabolic dysregulation mediated by AML-derived LA might differ from the metabolic status resulting from specific glycolysis inhibition (discussed above), complicating the comparison.

Beyond this, the increased glucose uptake might have diagnostic implications. Non-invasive, *in vivo* monitoring of glycolytic activity by FDG-PET was shown to allow GvHD detection in murine models, with current efforts for clinical translation being ongoing (38, 41–43).

## Oxidative Phosphorylation (OXPHOS)

By fueling the tricarboxylic acid (TCA) cycle with metabolic products and generating ATP in the mitochondrial electron transport chain (ETC), OXPHOS is an efficient provider of energy (5, 29).

Increased OXPHOS and oxygen consumption (as compared to non-alloreactive/syngeneic T-cells) was detected in murine

alloreactive T-cells. Enhanced superoxide production, decreased cellular antioxidant levels, and a hyperpolarized mitochondrial membrane potential suggest an abundant reactive oxygen species (ROS) production that is promoted by increased OXPHOS and an overall enhanced mitochondrial activity (9, 14, 44).

Targeting TCA- and ETC-activity with mitochondrial F1-F0-ATPase-inhibitors (BZ-423/LYC-31138), improved survival and limited GvHD in murine models (9, 10). As interference with the ETC by F1-F0-ATPase-inhibitors generates additional superoxide, cellular ROS burden further increases, leading to specific cell death of alloreactive T-cells. Thus, there is need to elucidate, whether the effects triggered by TCA/ETC blockade are ROS-dependent or the result of a rather bioenergetic deprivation (or both). Interestingly, PD-1-triggered ROS upregulation in T-cells is crucial for their subsequent metabolic modulation, as preceding PD-1-inhibition undermined the efficiency of F1-F0-ATPase-inhibition by LYC-31138. This effect might be explained by diminished ROS accumulation, which would (co-) facilitate apoptosis in alloreactive T-cells (45).

Noteworthy, the levels of TCA intermediates (including citrate, coenzyme-A) were found reduced in the allo- and syngeneic setup, which suggests pyruvate being predominantly converted to LA rather than TCA intermediates (6). However, OXPHOS-activity might change over time in reconstituting (T-) cells: in contrast to the general perception of increased OXPHOS activity in alloreactive T-cells at early timepoints after allo-HSCT (5-8 days), another study described similar OXPHOS levels in (murine) allogeneic and syngeneic T-cells at later timepoints (14 days post allo-HSCT) (6).

The key metabolic sensor AMP-activated protein kinase (AMPK) has been implicated as a driver of oxidative metabolism. Being reciprocally interconnected with the mechanistic target of rapamycin (mTOR), AMPK restricts anabolism (e.g. fat and protein synthesis) while improving catabolic pathways including OXPHOS and autophagy (5, 46). AMPK phosphorylation was found increased in murine alloreactive T-cells and genetic deletion of AMPK in donor T-cells showed protective effects against GvHD (11). In contrast, systemic administration of the AMPK-agonist metformin, promoted fatty acid oxidation (FAO) and alleviated GvHD (12, 47). Of note, metformin further inhibits complex I of the ETC, thereby interfering with T-cell metabolism also independent of AMPK activity (26).

Although the role of OXPHOS in alloreactive T-cells is still not conclusively clarified, ROS resulting from mitochondrial activity and required for proper T-cell activation as second messengers, may contribute to a continuous allo-activation upon BMT. In concurrence with this, targeting ROS pathways in preclinical models has reduced GvHD severity without impairing the GvT-effects (48).

## Lipid Metabolism

In addition to glucose metabolism, anabolism, and catabolism of fatty acids (FAs) regulate T-cell fate, proliferation, and differentiation of effector, memory, and Treg subsets (49).

Alloreactive T-cells exhibited increased FA-synthesis (FAS), with increased long-chain FA-transport and upregulated FAS-associated enzymes, alongside with enhanced FAO early after transplantation in a murine model (9, 14). Genetic interruption of *de novo* FAS *via* acetyl-CoA-carboxylase-1 (ACC1) inhibition in donor T-cells prevented acute GvHD and decreased glycolytic activity (13). This corroborates the notion that FAS is (amongst other functions) required for maintenance of glycolysis in allogeneic T-cells. Exemplary, the metabolic intermediate glycerol-3-phosphate (needed for FAS/triglyceride synthesis) also fuels glycolysis, which further underlines the complex interconnections between different metabolic pathways (50).

In addition, successful *in vitro* blockade of alloreactive T-cell expansion by sorafen A, a specific ACC1/2 inhibitor, might constitute a promising therapeutic strategy (13). Targeting FAO *via* etomoxir, which irreversibly inhibits carnitine-palmitoyl-transferase (CPT1), the enzyme responsible for shuttling FAs into the mitochondria, selectively affected alloreactive T-cells *in vitro* and *in vivo* (14). Moreover, etomoxir treatment inhibited PD-1-dependent increased respiration in murine alloreactive T-cells – a relevant consideration with respect to the emerging use of anti-PD1 therapies in the clinics, also in the context of allo-HSCT (45, 51).

Interfering with enzymatic FAO-regulation has additional implications in GvHD:

Lysosomal-acid-lipase (LAL) mediates intrinsic lipolysis by catalyzing the hydrolysis of cholesteryl esters and triglycerides in lysosomes and is required for physiological T-cell development and function (52). Pharmacological LAL-inhibition effectively controlled GvHD and preserved GvT-efficacy in a murine BMT model (15). Inhibiting 5-lipoxygenase (5-LO), an FAO-enzyme producing the proinflammatory leukotriene $B_4$, by zileuton (clinically used for asthma treatment) or by transplantation of 5-LO-deficient leukocytes, improved survival and alleviated GvHD (16).

In addition to the dysregulated FA-metabolism, data from clinical trials suggests that dysbiosis of gut microbiota leads to abundance of FAs that are linked to GvHD outcomes. A multicenter-study with adults found high levels of circulating short-chain FAs (SCFAs; e.g. butyrate) to be associated with protection from chronic GvHD (53). Further, SCFAs might be used as a predictor of therapeutic efficacy against acute GvHD: patients responding to acute GvHD treatment displayed higher plasma SCFA concentrations compared to non-responders (54). This is underlined by a study based on infants, which found antibiotic treatment and reduced SCFA production to be linked to an increased gut GvHD risk (55). The finding, that genetic depletion of the butyrate/niacin receptor GPR109a in donor T-cells alleviates GvHD severity while preserving GvT activity, further underlines the interconnection of (alloreactive) T-cell metabolism, the microbiome, and GvHD, opening up new avenues for therapeutic interventions (17).

## Glutamine Metabolism

Glutamine is an anabolic energy source for DNA/RNA synthesis and an alternative carbon source fueling the TCA-cycle in activated T-cells [leading to production of the citrate precursor α-ketoglutarate (α-KG)] (56).

Alloreactive T-cells upregulate their glutamine-dependent TCA anaplerosis, which is reflected by increased glutamine, decreased glutamate, and increased levels of aspartate and ornithine (products of glutamate conversion to α-KG) (6, 10). Increased expression of enzymes controlling the conversion to glutamate (i.e. Gfpt1, PPAT, GLS2) as well as enhanced glutamine transport in allogeneic T-cells, emphasize the bioenergetic demand during expansion of reconstituting donor T-cells (6).

Conversely, emerging evidence suggests glutamine supplementation to be beneficial in view of GvHD. In a murine acute GvHD model, systemic glutamine administration boosted Treg frequency, limited pro-inflammatory immune responses, protected from GvHD, and improved survival (18). In patients, a glutamine-enriched nutrition after transplantation increased overall survival with a lower incidence of GvHD-related deaths (19). Hence, while glutaminolysis is increased in alloreactive T-cells, systemic glutamine administration seems to have a rather GvHD-protective effect. Therefore, the context- and cell type-dependent role of glutamine remains to be further deciphered.

### Pentose Phosphate Pathway (PPP)

As a part of the anabolic metabolism, the PPP is critical for nucleotide synthesis and is implicated in maintenance of the cellular redox balance (via NADPH regeneration) (30, 37).

Murine allogeneic T-cells exhibited an overall increased PPP-activity and enhanced levels of PPP-regulating enzymes [e.g. glucose-6-phosphate-dehydrogenase (g6dp), phosphogluconate-dehydrogenase (pgd)]. Although inhibition of g6pd by dehydroepiandrosterone (DHEA), did not affect donor T-cell expansion, it decreased the frequency of IFN-γ-secreting T-cells (6). The oxidative arm of the PPP is crucial for antioxidant formation, including the ROS-buffer glutathione (GSH). GSH is implicated in the inflammatory T-cell response and promotes T-cell expansion by promoting metabolic skewing of activated T-cells towards glycolysis and glutaminolysis, thereby meeting the metabolic requirements of proliferating T-cells (57). Chronic allo-stimulation leads to sustained nucleotide biosynthesis to support anabolic cell growth, resulting in decreased pyrimidine catabolism and exhaustion of the intracellular GSH pool (4, 6, 9). The aforementioned strengthened glucose-uptake in allogeneic T-cells (6), subsequently can fuel both glycolysis and PPP, turning the PPP into an integrating interface between glycolysis and macromolecule synthesis. In addition, it was shown that alloreactive T-cells utilize glutamine as an anaplerotic source to fuel the PPP (10).

## CONVENTIONAL GVHD THERAPY AND ITS IMPACT ON T-CELL METABOLISM

In addition to specific metabolic targeting, immunosuppressive drugs, commonly utilized for GvHD prophylaxis and treatment, can affect T-cell metabolism:

### Glucocorticoids (GCs)

Immunosuppression by GCs, the first line treatment against GvHD, has profound effects on T-cell development, differentiation, and function (58). GCs are regulators of glucose homeostasis and were shown to inhibit glucose uptake and glycolysis in T-cells (58–60), which is consistent with reduced GvHD-activity following restricted glucose uptake by donor T-cells (8).

Further, GCs were shown to suppress FAO-activity and FAO-related mitochondrial function *in vitro* and *in vivo*, which was accompanied by impaired memory T-cell formation and decreased tumor clearance *in vivo* (61). As memory T-cells are important drivers of GvT (without causing GvHD) (62), this interference with memory T-cell differentiation is of clinical interest (due to the potential increased relapse risk following allo-HSCT).

Interestingly, GCs seem to have differential metabolic consequences in the long-term: in a murine model, perinatal GC-treatment resulted in diminished $CD8^+$ T-cell responses in adults, which was accompanied by increased OXPHOS-activity (63). Given the differentiation and reconstitution process of hematopoietic stem cells after allo-HSCT, in this scenario, those GC-triggered effects on T-cell immune-metabolism could be of significant relevance.

### Mammalian Target of Rapamycin (mTOR) Inhibitors

Inhibitors of the central metabolic checkpoint mTOR, are routinely used in transplantation medicine (26) (64). They have been introduced into the field of allo-HSCT and several clinical trials with the macrolide compound sirolimus revealed promising results for GvHD prophylaxis and treatment [reviewed in (65)]. Preclinical data evinced mTOR upregulation in alloreactive T-cells. In fact, mTOR can form two multiprotein complexes, mTORC1 and mTORC2 controlling its downstream effects including metabolic regulation, with mTORC1 being responsible for enhanced glycolysis in alloreactive T-cells and induction of GvHD (6). Blocking mTORC1 activity by sirolimus selectively attenuated glycolytic activity together with GvHD severity without affecting OXPHOS (6). Consequently, inhibiting phosphoinositide-3-kinases (PI3Ks) (upstream regulators of mTOR) simultaneously with mTOR, successfully prevented T-cell (allo-) activation and GvHD induction (21).

Novel dual mTORC1/2-inhibitors displayed stronger effects as compared to sirolimus in *in vitro* experiments leading to improved survival and reduced GvHD mortality *in vivo*. Importantly, T-cell responses against cytomegalovirus, an opportunistic virus that remains a major cause for morbidity in GvHD, were not affected (22).

The importance of glycolysis as an mTOR target in the GvHD context is further highlighted by the observation that direct blockade of hypoxia-inducible factor 1-alpha (HIF1α), an important regulator of aerobic glycolysis downstream of mTOR, with echinomycin, effectively reduced acute GvHD while preserving GvT by reducing glucose-dependent Th1 and Th17 cells and promoting Treg induction (23).

## Calcineurin Inhibitors (CNIs)

CNIs exert their immunomodulatory function by binding to immunophilins, resulting in calcineurin blockade. Upon activation, calcineurin, which is regulated by free cytosolic $Ca^{2+}$, dephosphorylates its prime target nuclear factor of activated T-cells (NFAT), enabling NFAT translocation into the nucleus and subsequent NFAT target gene induction [crucial for T-cell activation and cytokine production (66)]. Since NFAT transactivates IRF4, HIF1α and GLUT3, CNIs additionally interfere with T-cell metabolism (26) (67).

Although CNIs, such as ciclosporin A (CsA) or tacrolimus (FK506), are extensively and successfully used in GvHD prophylaxis, dose-dependent negative effects on GvT-efficacy have been reported (68). Interestingly, selective NFAT targeting in T-cells reduced GvHD with maintained GvT-activity, when only one NFAT-family member was ablated (24, 25). This suggests that CNIs have broader (off-)target effects than sole and individual NFAT-inhibition. Additionally, a recent study proposed an overall NFAT-independent amelioration of GvHD by CNIs. By means of a genetic mouse model, this work showed, that dephosphorylation inhibition of the tyrosine kinase Lck by CNIs is primarily mediating their GvHD-suppressive effects (69).

However, despite that CNIs affect metabolic checkpoints, data on their metabolic impact in GvHD still remain limited.

CNIs suppress glucose metabolism-dependent activation of T-cells, thus retaining T-cells in a quiescent metabolic state (70, 71). Metabolic profiling of CsA-treated T-cells further revealed amino acid metabolism and PPP as targets (71). By the use of STIM1/2 double-deficient mice, Vaeth *et al.* demonstrated store-operated $Ca^{2+}$-entry (SOCE) to regulate metabolic reprogramming *via* NFAT and the PI3K-AKT-kinase-mTOR pathway. Further, cell cycle entry of T-cells was found to be SOCE-dependent with SOCE-deficient T-cells being stuck in G0 phase, potentially explaining the regulation of T-cell proliferation by calcineurin blockade. These data propose a novel molecular mechanism by which SOCE, calcineurin, and NFAT control T-cell metabolism and function (70).

In clinical context, systemically administered CNIs additionally impact the glucose uptake in muscle and adipose tissue (crucial for glucose homeostasis), which may explain some of the CNI-mediated adverse systemic effects (26, 72). Moreover, novel cyclophilin-binding compounds bringing in new mechanisms of action might present an option for a more specific targeting with limited off-target effects (73).

## Inhibitors of *De Novo* Purine Synthesis

Similar to CNIs, data on the effect of the *de novo* purine synthesis inhibitors methotrexate (MTX) and mycophenolate-mofetil (MMF) on T-cell metabolism (in allo-HSCT) is rather limited. However, several *in vitro* studies implicate an effect of MMF on T-cell metabolism (i.e. *via* suppression of glycolysis), by interfering with AKT/mTOR signaling, thereby contributing to T-cell anergy and reduced T-cell proliferation (26, 72, 74). As of to date, no substantial data on potential MTX-elicited effects on T-cell metabolism are available [reviewed in (26, 72)].

Taken together, the vast evidence of studies on the immuno-metabolic impact of immunosuppressants address single agents. Combination therapies, as commonly utilized in clinical practice (2), that eventually lead to synergistic effects or even drive synthetic lethality (of alloreactive T-cells), remain largely unexplored. In one of the few studies, combination of sirolimus with CNIs has been shown to additively impact T-cell metabolism (71). Consequently, ideal (from the immuno-metabolic perspective) drug combinations for efficient therapeutic modulation have to be experimentally determined in the future.

# CONCLUDING REMARKS

In order to meet their metabolic demands, alloreactive T-cells upregulate essential metabolic pathways. With detailed knowledge on that metabolic signature of alloreactive T-cells in both acute and chronic GvHD, metabolic dissection of GvHD- and GvT-driving T-cells becomes more feasible. Via targeting alloreactive T-cells with customized metabolic inhibitors, this signature could be exploited therapeutically. Moreover, a bioenergetic profile, which specifically marks alloreactive T-cells, could be implemented as a novel GvHD-biomarker, consequently enabling intervention at early stages.

Emerging data on metabolic specificities of alloreactive T-cells, might also help to understand the underlying GvHD-pathobiology and complications frequently observed upon conventional GvHD treatments. In-depth bioenergetic characterization of the patients' alloreactive T-cells could impact treatment decisions. Selecting drugs based on the fit of their mechanism of action and the T-cells' actual metabolic profile, might lead to a more personalized approach, aiming at a secure and efficient treatment.

# AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and conceptual contribution to the work, and approved it for publication.

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REVIEW
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# Kinase Inhibition as Treatment for Acute and Chronic Graft-*Versus*-Host Disease

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Allogeneic hematopoietic stem cell transplantation (allo-HCT) is a potentially curative therapy for patients suffering from hematological malignancies *via* the donor immune system driven graft-*versus*-leukemia effect. However, the therapy is mainly limited by severe acute and chronic graft-*versus*-host disease (GvHD), both being life-threatening complications after allo-HCT. GvHD develops when donor T cells do not only recognize remaining tumor cells as foreign, but also the recipient's tissue, leading to a severe inflammatory disease. Typical GvHD target organs include the skin, liver and intestinal tract. Currently all approved strategies for GvHD treatment are immunosuppressive therapies, with the first-line therapy being glucocorticoids. However, therapeutic options for glucocorticoid-refractory patients are still limited. Novel therapeutic approaches, which reduce GvHD severity while preserving GvL activity, are urgently needed. Targeting kinase activity with small molecule inhibitors has shown promising results in preclinical animal models and clinical trials. Well-studied kinase targets in GvHD include Rho-associated coiled-coil-containing kinase 2 (ROCK2), spleen tyrosine kinase (SYK), Bruton's tyrosine kinase (BTK) and interleukin-2-inducible T-cell kinase (ITK) to control B- and T-cell activation in acute and chronic GvHD. Janus Kinase 1 (JAK1) and 2 (JAK2) are among the most intensively studied kinases in GvHD due to their importance in cytokine production and inflammatory cell activation and migration. Here, we discuss the role of kinase inhibition as novel treatment strategies for acute and chronic GvHD after allo-HCT.

**Keywords: GvHD, stem cell transplant (SCT), kinases, ruxolitinib, JAK1 and JAK2 inhibitors, BTK - Bruton's tyrosine kinase, ROCK**

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## INTRODUCTION

Patients suffering from hematological malignancies have only access to a very limited number of therapeutic interventions. Allogeneic hematopoietic stem cell transplantation (allo-HCT) is a potentially curative therapy for patients with hematological disorders (1, 2). Patients are pre-conditioned with chemotherapy or total-body irradiation to eradicate the underlying disease,

followed by transplantation of donor stem cells. The allogeneic cells elicit an anti-malignancy immune reaction (3). However, besides the beneficial anti-tumor immune response, a major limitation of allo-HCT is acute and chronic graft-*versus*-host-disease (aGvHD, cGvHD) (4–7). GvHD is a life-threatening complication of allo-HCT and establishes if the transplanted cells recognize the host's tissue as foreign. Host antigen-presenting cells (APCs) are activated and stimulate the donor cells, thereby causing cytokine release, strong immune cell activation and severe tissue damage (4, 5, 8–10). Acute GvHD is mainly based on T-cell activation and cytokine release, whereas B-cells are major players in cGvHD, which has features of autoimmune diseases and is often accompanied by organ fibrosis (5, 11). Acute and chronic GvHD are different diseases but share some similarities as both are inflammatory diseases initiated by APCs, followed by activation of alloreactive T- and B-cells, inflammation, tissue damage and organ failure (12). Acute GvHD is a risk factor for cGvHD development of (4, 11). Standard medications mainly rely on broad immunosuppression, which can on the one hand reduce GvHD activity, but on the other hand impair anti-malignancy immunity. There is an unmet need for specific and selective therapeutic strategies to control GvHD without disturbing beneficial immune responses after HCT. Kinase-mediated signaling pathways are among the most important signaling cascades to drive cytokine production and immune cell activation, thereby enhancing GvHD severity (13, 14). Many kinases share similarities, as e.g. JAK1/2, TAK1 and MAPK signaling are crucial for inflammatory cytokine signaling (15–17). TCR and BCR signaling, leading to cell survival, proliferation, migration and effector cytokine production, are mainly regulated by BTK and ITK, ROCK2, PI3K, mTOR, Syk and MEK (18–28). ROCK2, JAK1 and JAK2, as well as Syk, play a role in T-cell differentiation, including the induction of regulatory T-cells (27, 29–32). However, there is also a kinase involved in GvHD pathophysiology with a unique function, as ITPKB plays a pivotal role in regulating intracellular $Ca^{2+}$ levels and T-cell survival (33–35). Based on the various functions of kinases in GvHD pathophysiology, it was concluded that tyrosine kinase inhibitors (TKIs) could be a promising strategy to control B- and T-cell activation and GvHD after allo-HCT (36). In general, TKIs block substrate phosphorylation, thereby limiting cellular downstream effects and pathways. These signaling cascades also include effector functions, e.g. the production of pro-inflammatory cytokines by T-cells (36). Since GvHD is mainly characterized by increased pro-inflammatory cytokines, systemic sclerosis and organ damage, inhibition of activated TK signaling could be a promising strategy to reduce disease severity and progression (36–38). Many small molecules were evaluated or are currently investigated for the treatment of GvHD, with some compounds now being applied as standard therapy. **Table 1** summarizes clinical trials about kinase inhibitors in GvHD, which are cited in this article. In this review, we would like to focus on kinases as novel and known targets in acute and chronic GvHD.

## JANUS KINASES 1 AND 2 (JAK1/2)

One of the key players in mediating pro-inflammatory signaling is the Janus Kinase (JAK) 1/2. Signaling *via* JAK1/2 and signal transducer and activator of transcription (STAT) pathways are crucial for the stimulation of inflammatory cytokine production and the activation of a variety of immune cells during GvHD onset and progression (15).

**Acute GvHD:** The JAK/STAT signaling pathway has high importance in aGvHD onset and progression, as STAT1 and STAT3 are activated early after disease onset. The signaling plays a pivotal role in mediating T-cell activation and changes of the T-cell phenotype (39). Besides, JAK/STAT pathways are also important in the APC compartment in GvHD by influencing dendritic cell (DC) development, maturation, activation and migration into GvHD target organs (18, 40). JAK1 and JAK2 signaling can both be potently blocked with the selective inhibitor Ruxolitinib which has first been approved for the treatment of myelofibrosis by reducing pro-inflammatory signaling (41). Inhibition of JAK1/2 by Ruxolitinib does not only block DC activation and the common gamma chain downstream effects in T-cells (15), but also reduces the migration of neutrophil granulocytes into GvHD target organs (10, 42). Inhibition of JAK1/2 signaling by Ruxolitinib significantly reduced GvHD severity and increased the survival in a pre-clinical murine major-mismatch GvHD model (43). The findings could mainly be linked to a significant reduction of pro-inflammatory cytokine release *in vitro* and *in vivo*, reduction of donor T-cell infiltration into GvHD target organs and reduced allogeneic T-cell proliferation. The treatment also blocked allogeneic APC maturation and activation, thereby limiting T-cell proliferation. However, the blockade of JAK1/2 signaling also reduced T-cell proliferation stimulated by anti-CD3/CD28 activation beads. Direct effects on T-cells, including reduced activation and proliferation, could be linked to reduced STAT3 phosphorylation (**Figure 1**). Moreover, the number of regulatory T-cells (Tregs) was elevated in the intestine (43). Since Ruxolitinib application was seen to have cytopenia as side effects due to co-inhibition of JAK2, specific JAK1 inhibitors were designed to reduce cytokine signaling without side effects (44, 45). Itacitinib is a highly selective JAK1 inhibitor which has shown promising activity in inflammatory models, such as arthritis, inflammatory bowel disease and aGvHD (46). A preclinical study applied Itacitinib in a xenogeneic aGvHD humanized mouse model and found prolonged survival and reduced GvHD severity compared to control. Frequencies of CD4 and CD8 T-cells were lower on d21 and d28 after transplantation, whereas Treg frequencies increased. In a following GvL model, Itacitinib treatment reduced anti-leukemia immunity to some extent. However, more detailed analysis of JAK1 inhibition on T-cells would be necessary, including deeper phenotyping and effector function analysis (47). Baricitinib is another promising JAK inhibitor, blocking JAK1 and JAK2, and could prevent GvHD in a preclinical model (48). The investigators showed that single inhibition of JAK1 or JAK2 was not as effective as the double blockade with Ruxolitinib, hypothesizing that balanced blockade of both kinases

**TABLE 1** | Selected clinical trials of kinase inhibition in GvHD.

| Trial number* | Treatment and kinase target | Trial description | Status, Outcome Measures, Comments |
|---|---|---|---|
| NCT02953678 | Ruxolitinib; JAK1/2 | REACH-1; Ruxolitinib combined with steroids for SR aGvHD; Phase 2 | Completed; ORR at day 28; CR, VGPR, PR; 6-month/3-month DOR; RR; FFS; relapse-related mortality; incidence/severity of AEs; 71 participants; Single-cohort study |
| NCT02913261 | Ruxolitinib; JAK1/2 | REACH2; Safety/efficacy of Ruxolitinib vs. BAT in SR aGvHD; BAT selected by investigator; Phase 3 | Completed; ORR at day 28 and durable ORR at day 56; DOR; OS; cumulative steroid dose; event-free survival; FFS; NRM; MR; cGvHD incidence; PK; PROs; 310 participants; randomized open-label multi-center study |
| NCT03112603 | Ruxolitinib; JAK1/2 | REACH3; Ruxolitinib vs. BAT in SR aGvHD after allo-HCT; Phase 3 | Active; ORR of ruxolitinib vs. BAT in moderate to severe SR-cGvHD; FFS; change in modified Lee cGvHD symptom score; DOR; NRM; reduction in daily corticosteroid dose; MR; AEs; PK; 330 participants; randomized open-label multi-center study |
| NCT02614612 | Itacitinib; JAK1 | Itacitinib in combination with corticosteroids in aGvHD; Phase 1 | Completed; ORR; Itacitinib plasma concentrations; PK; 31 participants; open label study |
| NCT03320642 | Itacitinib; JAK1 | GRAVITAS-119; Itacitinib with calcineurin inhibitor-based intervention of GvHD prophylaxis; Phase 1 | Terminated by sponsor; Hematologic recovery; RFS; transplant-related mortality; immune reconstitution/engraftment; donor chimerism; OS; infections; 84 participants recruited; single group assignment |
| NCT03584516 | Itacitinib; JAK1 | GRAVITAS-309; Itacitinib and corticosteroids as initial treatment for cGvHD; Phase 2/3 | Recruiting; DLT; RR; Itacitinib plasma concentrations; time to response; OS; NRM; AEs; 431 participants; randomized, crossover assignment; ion part2, patients from placebo van cross over to experimental group after completion of primary analysis |
| NCT03846479 | Itacitinib; JAK1 | Itacitinib monotherapy for low risk GvHD; Phase 2 | Active; Minnesota standard risk clinical criteria; Ann Arbor Score 1; AEs; infectious complications; ORR; 70 participants; single group assignment |
| NCT04070781 | Itacitinib; JAK1 (plus Tocilizumab, IL6R) | Itacitinib and Tocilizumab for SR-aGvHD; Phase 1 | Recruiting; MTD of Tocilizumab given with Itacitinib; Safety and tolerability; ORR; time to response; DOR; Infections; PFS; OS; steroid discontinuation; 24 participants; single group assignment; multi-center trial |
| NCT04446182 | Itacitinib; JAK1 (with ECP) | Itacitinib and extracorporeal photopheresis for first-line therapy in cGvHD; Phase 2 | Recruiting; Assess recommended phase 2 dose of Itacitinib with ECP combination; DLT; ORR; AEs; FFS; withdrawal of immunosuppressants; organ-specific response; GvHD severity; RR; OS; 58 participants; single group assignment |
| NCT04200365 | Itacitinib; JAK1 | Itacitinib for SR-cGvHD; Phase 2 | Recruiting; Participants with SR-cGvHD after at least 6 months corticosteroids/other immunosuppressive therapies; combination therapies with Itacitinib; ORR; decrease or withdrawal of steroids; OS; AEs; quality of life; cGvHD progression/recurrence; RR; 40 participants; Single group assignment; multi-center study |
| NCT02759731 | Baricitinib; JAK1/2 | Baricitinib in SR- cGvHD after allo-HCT; Phase 1; Phase 2 | Recruiting; Safety, tolerability and efficacy of Baricitinib in patients refractory to steroids; 31 participants; non-randomized, open-label study |
| NCT04131738 | Baricitinib; JAK1/2 | Baricitinib for prophylaxis of GvHD; Phase 1 | Recruiting; Cumulative incidence of graft failure; cumulative incidence of grade III-IV aGvHD; TRM; 26 participants; non-randomized, open-label study |
| NCT02195869 | Ibrutinib; BTK | Safety and Efficacy of BTK ibrutinib in steroid dependent or refractory cGvHD; Phase 1b/2 | Completed; Safety and tolerability (phase 1b/2b); ORR, CR, PR (phase 2); sustained response rate; corticosteroid requirement; improvement in Lee cGvHD symptom score; 45 participants; non-randomized multi-center open-label |
| NCT02959944 | Ibrutinib; BTK | iNTEGRATE; ibrutinib/steroids vs placebo/ steroids in new onset cGvHD; Phase 3 | Completed; Response rate at 24 and 48 weeks; incidence of withdrawal of corticosteroids/all Immunosuppressants for GvHD treatment; improvement in Lee cGvHD symptom score; reduction of prednisolone dose; DOR; AEs; 193 participants; randomized double blind multi-center study |

*(Continued)*

**TABLE 1** | Continued

| Trial number* | Treatment and kinase target | Trial description | Status, Outcome Measures, Comments |
|---|---|---|---|
| NCT03474679 | Ibrutinib; BTK | Ibrutinib in participants with steroid refractory/dependent cGvHD; Phase 3 | Active; ORR; CR; PR; sustained response; DOR; change of corticosteroid requirement; improvement of Lee cGvHD symptom score; AEs; clinical laboratory abnormalities; PK; metabolism; drug half-life; Single group assignment |
| NCT04294641 | Ibrutinib; BTK | Front-line ibrutinib for newly diagnosed cGvHD; Phase 2 | Active/Recruiting; Efficacy of ibrutinib as first-line treatment for newly diagnosed cGvHD; ORR; safety; FFS; 24 month post-treatment survival; Pilot study; single group assignment |
| NCT02611063 | Fostamatinib; SYK | Fostamatinib in cGvHD after allo-HCT; Phase 1 | Recruiting; MTD at day 60; TRM; incidence of cGvHD; relapse of cGvHD; B-cell activation; B-cell death; absolute B-cell numbers; Single group assignment |
| NCT02701634 | Entospletinib; SYK | Entospletinib with systemic corticosteroids as first-line therapy in cGvHD; Phase 2 | Terminated; Best ORR; changes from baseline in Lee symptom scale (skin, mouth, eye, total); DOR; at least 50% reduction in systemic corticosteroid dose; FFS; AEs; study discontinuation; laboratory abnormalities; 66 participants; randomized double-blind placebo-controlled study |
| NCT03640481 | Belumosudil (KD025); ROCK2 | Belumosudil in cGvHD after at least 2 prior lines of systemic therapy; Phase 2 | Recruiting; ORR; change in Lee symptom score; response in individual target organs; PR; CR; change in corticosteroid and calcineurin inhibitor dose; FFS; OS; activity change; cGvHD severity change; drug half-life; time to response; PK; Randomized multi-center open label study |
| NCT04930562 | Belumosudil (KD025, BN101); ROCK2 | Efficacy/Safety of Belumosudil in cGvHD; Phase 2 | Recruiting; Individuals after at least first line of therapy; ORR; Single group assignment; open-label multicenter study |
| NCT02841995 | Belumosudil (KD025); ROCK2 | Safety, tolerability, activity of belumosudil in cGvHD; Phase 2 | Active; ORR; PR; CR; AEs as measure of safety and tolerability; Dose-escalation open-label study |
| NCT00803010 | Rapamycin; mTOR | GvHD prophylaxis after allo-HCT; Phase 2 | Completed; Comparison of tacrolimus/rapamycin as novel GvHD prophylaxis vs tacrolimus/methotrexate; Incidence of aGvHD; incidence of increased Treg numbers; OS (2 years post-transplant); 74 participants; parallel assignment |
| NCT00928018 | Sirolimus; mTOR | GvHD prophylaxis after reduced-intensity allo-HCT for lymphoma patients; Phase 3 | Completed; Comparison of group 1 (tacrolimus, sirolimus, methotrexate), group 2 (tacrolimus, methotrexate) and group 3 (cyclosporine, mycophenolate mofetil) as GvHD prophylaxis regimen; OS; PFS; disease progression; non-relapse mortality; incidence of GvHD; 139 participants; parallel assignment; multicenter randomized trial |
| NCT01231412 | Sirolimus; mTOR | GvHD prophylaxis after URD allo-HCT; Phase 3 | Completed; GvHD prophylaxis with or without sirolimus after allo-HCT; grade II-IV aGvHD; incidence of extensive cGvHD; grade III-IV aGvHD; NRM; OS; relapse/progression rate; 174 participants; randomized study; parallel assignment |
| NCT02806947 | Sirolimus; mTOR | BMT CTN 1501; Evaluation of steroid-free treatment of standard-risk aGvHD; Phase 2 | Completed; Evaluation of sirolimus as alternative to prednisolone as up-front treatment for patients with standard-risk aGvHD; ORR; PR; CR; treatment failure; aGvHD; disease-free survival; OS; NRM; malignancy relapse; cGvHD, incautious complications; 127 participants; randomized multicenter open label study |
| NCT01106833 | Sirolimus; mTOR | BMT CTN 0801; cGvHD treatment; Phase 2/3 | Completed; Comparative study of sirolimus and prednisolone vs sirolimus and calcineurin-inhibitor and prednisolone; Proportion of treatment success; OS; PFS; FFS; relapse rate; rate of discontinuation of systemic immunosuppressive therapy; prednisolone dose; cGvHD severity; 151 participants; randomized open-label multicenter trial |
| NCT02891603 | Pacritinib, Sirolimus; JAK2, mTOR | GvHD prevention combining pacritinib and sirolimus-based immunosuppression; Phase 1/2 | Recruiting; Combination of pacritinib, sirolimus and tacrolimus to prevent serious GvHD; STAT activity in circulating CD4 T-cells; incidence of aGvHD; Single arm study |

*(Continued)*

**TABLE 1** | Continued

| Trial number* | Treatment and kinase target | Trial description | Status, Outcome Measures, Comments |
|---|---|---|---|
| NCT00702689 | Imatinib | Imatinib Mesylate in cGvHD with skin involvement; Phase 2 | Completed; Change in range of motion (ROM); primary ROM response; AEs; ROM deficits; total skin score at baseline vs 6 months; cGvHD scores; lung function; change in immunosuppression; 20 participants; single group assignment |
| NCT01810718 | Nilotinib | Safety and efficacy of Nilotinib in steroid refractory/dependent cGvHD; Phase 1/2 | Completed; Phase 1: DLT; ORR; dose finding; phase 2: TTF; OS; biological evaluation (PDGF-R stimulating autoantibodies, fibroblast characteristics, changes of immune cell populations); 22 participants; prospective non-randomized open label multicenter study |
| NCT01155817 | Nilotinib | Nilotinib in steroid dependent/ refractory cGvHD; Phase 1 | Completed; Determination of safety/tolerability in steroid refractory/dependent cGvHD; AEs; clinical efficacy in cGvHD; physical changes; daily corticosteroid requirement; treatment failure; cGvHD symptom burden; 33 participants; single group assignment |

**All clinical trials are registered at https://clinicaltrials.gov; AEs, adverse events; aGvHD, acute GvHD; BAT, best available therapy; BTK, Bruton's tyrosine kinase; cGvHD, chronic GvHD; CR, complete response; DLT, dose-limiting toxicities; FFS, failure-free survival; GvHD, Graft-versus-Host Disease; HCT, hematopoietic stem cell transplantation; JAK, Janus kinase; MR, malignancy relapse/progression; MTD, maximum tolerated dose; mTOR, mammalian target of rapamycin; NRM, non-relapse mortality; ORR, overall response rate; OS, overall survival; PFS, progression-free survival; PK, pharmacokinetics; PR, partial response; PROs, patient reported outcomes; ROCK2, rho-associated coiled-coil containing protein kinase 2; ROM, range of motion; RR, relapse rate; SR, steroid-refractory; SYK, Spleen tyrosine kinase; Tregs, regulatory T-cells; TRM, treatment-related mortality; TTF, treatment to failure time; URD, unrelated donor; VGPR, very good partial response.*

is needed to optimally control GvHD. IFNγR and IL6R signaling, which is mainly regulated through JAK1/2, can be efficiently blocked with Baricitinib. Application of Baricitinib in murine GvHD resulted in 100 % survival, reduced early intestinal GvHD, and faster immune reconstitution with superior activity compared to Ruxolitinib. Mechanistically, Baricitinib treatment enhanced allogeneic Treg proliferation while blocking effector T-cell proliferation (48). GvHD-suppressive Tregs were increased by preservation of JAK3 signaling and increased STAT5 phosphorylation (49). Moreover, costimulatory molecule expression on allogeneic APCs was reduced. Most importantly, Baricitinib did preserve GvL activity and could control ongoing GvHD, making it a potential therapy for established GvHD and not only as prophylaxis treatment (48).

A first retrospective study included 95 patients receiving Ruxolitinib as a salvage therapy for steroid-refractory (SR-) GvHD and patients with severe intestinal or skin GvHD showed impressive response to JAK1/2 inhibition. Treatment with Ruxolitinib was liked to decreased pro-inflammatory serum cytokine levels and lower numbers of activated T-cells. The overall response rate (ORR) was 81.5 % and the overall survival (OS) of steroid-refractory aGvHD patients receiving Ruxolitinib was higher than ever reported for any other pharmacological therapy (50). This trial was followed by the REACH1 trial (NCT02953678), including patients with SR-aGvHD after HCT from any donor source. Comparable to the first results seen in patients, the time to response was 7 days and the ORR was 73.2 %. Also, the 6-month and 12-month OS was 51.0 % and 42.6 %, respectively, whereas the OS was lower in patients with grade III/IV aGvHD who received longer corticosteroid treatment before Ruxolitinib treatment. Mechanistically, biomarker analysis confirmed elevated hematopoiesis and a reduction of inflammatory cytokine release and signaling in patients receiving Ruxolitinib (51). The following REACH2 trial (NCT02913261) aimed to analyze the efficacy of Ruxolitinib in comparison to best available care in SR-aGvHD. Ruxolitinib significantly increased the median failure-free survival (FFS) and OS compared to control therapy, and the ORR at day 28 was significantly higher with Ruxolitinib treatment. The percentage of patients with complete response (CR) was at 34 % and 19 % under JAK1/2 inhibition or best available therapy, respectively. Comparable to side effects seen in the REACH1 trial, Ruxolitinib was again reported to cause thrombocytopenia (52). Besides JAK1 and 2 inhibition with Ruxolitinib, Itacitinib has shown promising preclinical efficacy in GvHD. Due to these findings and the hypothesis that selective JAK1 inhibition reduces side effects seen with Ruxolitinib, a phase I trial was initiated to determine if Itacitinib in combination with corticosteroids is safe and tolerable in patients with grade IIb-IVd aGvHD (NCT02614612). Treatment-naïve and SR aGvHD patients were included and distributed equally into two Itacitinib doses. Itacitinib was found safe to use; the most common nonhematologic AE was diarrhea, whereas hematologic AEs included anemia and thrombocytopenia. The d28 ORR was 75 % and 70.6 % of treatment naïve and SR aGvHD, respectively. Responses were seen across involved organs but median DOR was not reached in patients with treatment-naïve aGvHD. Upon Itacitinib treatment, corticosteroid doses could be reduced or discontinued in all patients. Overall, the study demonstrated that JAK1 inhibition with Itacitinib is effective and well tolerated in aGvHD. However, findings are limited due to small sample size and no comparator group (45). In another trial, Itacitinib was thought to be a promising prophylaxis treatment, as JAK/STAT blockade could limit T-cell migration into GvHD target organs; However, the study was terminated (NCT03320642). Itacitinib treatment is currently also investigated in more clinical trials as a therapy for low-risk GvHD (NCT03846479) or in combination

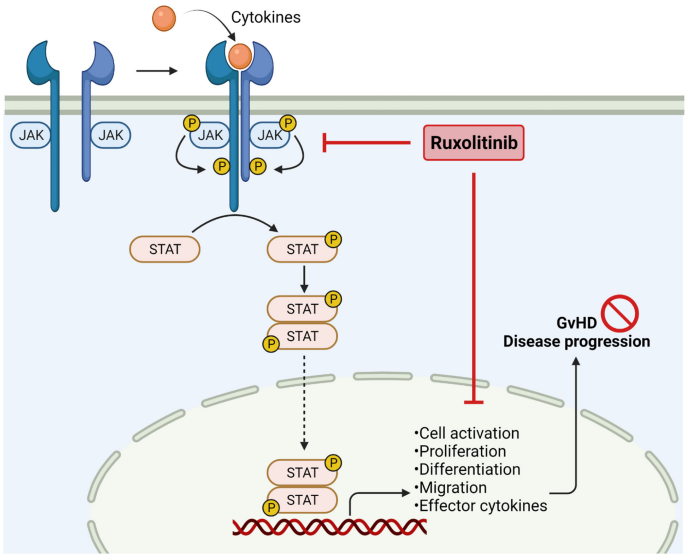


**FIGURE 1** | JAK2 inhibition in Graft-*versus*-Host Disease. Janus kinases are crucial to mediate extracellular signals. Binding of cytokines results in receptor dimerization and phosphorylation, subsequently phosphorylating STAT molecules by receptor tyrosine kinases. Phosphorylation of STAT leads to dimerization and translocation into the nucleus, followed by enhancing gene transcription. JAK/STAT signaling is important in regulating cell activation, proliferation, migration and effector cytokine production, thereby enhancing GvHD severity. JAK1/2 inhibition by ruxolitinib reduces pro-inflammatory signaling and cell migration, resulting in reduced GvHD disease progression. Created with Biorender.com.

with the anti-IL6R antibody Tocilizumab in aGvHD (NCT04070781).

**Chronic GvHD:** Besides application in aGvHD, Ruxolitinib treatment was also evaluated for the treatment of glucocorticoid-refractory cGvHD (53). Chronic GvHD occurs in 30-70 % of all patients undergoing allo-HCT and is treated with systemic glucocorticoids as first-line therapy (11, 54, 55). However, the disease becomes glucocorticoid-dependent or glucocorticoid-refractory in about 50 % of all patients, thereby significantly increasing the risk for poor outcomes (11, 53–55). Although the Bruton's tyrosine kinase inhibitor ibrutinib is approved in the US and Canada as second-line therapy, responses are limited. Moreover, the efficacy of ibrutinib has not been demonstrated in a randomized clinical trial (11, 56, 57). In pre-clinical analysis, inhibition of JAK1/2 was shown to be an effective treatment not only in aGvHD, but also in cGvHD (50, 53). JAK1/2 is crucial for the initiation and progression of inflammation and cytokine signaling, both being major regulators of acute and chronic GvHD (15, 42, 43, 50, 58). Based on the positive results of Ruxolitinib in aGvHD in the REACH1 and REACH2 trial, it was evaluated in the REACH3 trial (NCT03112603) for the treatment of glucocorticoid-refractory or -dependent cGvHD in comparison to best available therapy (BAT, control) (51–53). The REACH3 trial is a phase III randomized open-label multi-center study of Ruxolitinib in comparison to ten other therapeutic agents. At the primary study end point, the OR was higher with Ruxolitinib (49.7 %) compared to control (25.6 %). A higher OR was observed with Ruxolitinib than any other control treatment in most organs, except for lung and liver cGvHD where responses were similar. Moreover, patients receiving Ruxolitinib had a significantly longer FFS than the control group (>18.6 months vs. 5.7 months). Also, the response on the modified Lee Symptom Scale was higher with Ruxolitinib (24.2 %) at 24 weeks compared to BAT (11.0 %). The investigators reported decreased dose of glucocorticoids in both groups over time, whereas the decrease was slightly greater in the Ruxolitinib group. Overall, the DOR was higher in the Ruxolitinib group compared to control treatment. Regarding the safety profile, adverse events (AEs) of any grade were slightly more often seen in the Ruxolitinib group compared to control, whereas adverse events of grade 3 and 4 were comparable in both groups (57.0 % vs. 57.6 %). Most commonly, Ruxolitinib treated patients experienced thrombocytopenia (15.2 %), anemia (12.7 %), neutropenia (8.5 %) and pneumonia (8.5 %). The safety profile was comparable to results seen in patients with aGvHD (53, 59, 60). Bacterial, fungal and viral infections were seen in both groups at a comparable incidence. In summary, the REACH3 trial showed that Ruxolitinib is superior over common second-line therapies for SR-cGvHD. Ruxolitinib was found being an effective treatment options for patients with moderate and severe SR-cGvHD (53). The selective JAK1 inhibitor Itacitinib is currently investigated as first-line therapy in cGvHD (NCT03584516), but results are not available yet. Since preclinical evaluation of Baricitinib in GvHD were promising, clinical trials were initiated to evaluate Baricitinib treatment in patients with cGvHD (NCT02759731) or as a prophylaxis treatment for GvHD after allo-HCT (NCT04131738). However, both trials are still recruiting and did not publish any results yet. Novel approaches combine JAK inhibitors with other therapies to enhance treatment efficacy. One trial investigates the combination of Itacitinib with corticosteroids or other immunosuppressive therapies in cGvHD (NCT04200365), or as combination with extracorporeal photopheresis (ECP) as first-line therapy in cGvHD (NCT04446182). In a comparable attempt,

Ruxolitinib was combined with ECP in SR-cGvHD patients. Since both treatments alone did already show promising effects in GvHD, a combination therapy was thought to even elevate the therapeutic success. The 2-year survival rate was 75 % and the combination of both therapies was found safe to use with activity in at least a part of SR-cGvHD patients. However, this was only a single-center study and a detailed validation is needed in a prospective trial (61).

## RHO-ASSOCIATED COILED-COIL CONTAINING PROTEIN KINASE 2

Signaling pathways mediated by Rho GTPase are important regulatory mechanisms of the T-cell mediated immune response, including TCR signaling and effector cytokine production (62). The rho-associated coiled-coil protein kinases 1 and 2 (ROCK1 and ROCK2) are serine-threonine kinases activated by Rho GTPases. Activation of ROCK1 and ROCK2 leads to phosphorylation of downstream molecules, including STAT3 and STAT5, to enhance the transcription of target genes (63). Target molecules regulated by ROCK2 include pro-inflammatory cytokines like IL-21 and IL-17 (23, 64). The ROCK2 signaling pathway has been shown to be important to regulate the balance between Th17 cells and Tregs. ROCK2 activation causes STAT3 phosphorylation, subsequently enhancing the expression of Th17-specific transcription factors, including interferon regulatory factor 4 (IRF4), RAR-related orphan receptor (ROR) γt and RORα (30–32, 65). Blockade of ROCK2 using its selective inhibitor belumosudil shifts the Th17/Treg balance towards regulatory T-cells through a STAT5-dependent mechanism (30, 32, 66). Regarding the potency of Tregs to reduce GvHD severity (67), ROCK2 was hypothesized being a promising target in GvHD (**Figure 2**) (30, 32).

**Chronic GvHD:** A preclinical study evaluated the effects of the ROCK2 inhibitor KD025 in cGvHD (66). Efficacy was first assessed in a bronchiolitis obliterans syndrome (BOS) model, in which the mice develop organ fibrosis associated with increased B-cell activation in the GC, also seen in cGvHD patients (66, 68). Upon treatment, the mice had improved resistance, elastance and compliance, together with decreased histopathology scores in the major GvHD target organs. However, cGvHD mice still had higher pathology scores than BM-only non-cGvHD animals. Also, collagen and immunoglobulin (Ig) deposition, both typically being increased in cGvHD multiorgan and BOS models (66, 68), were significantly decreased upon ROCK2 inhibition (66). Increased numbers of TFH cells and GC B-cells was previously been reported during murine cGvHD in the BOS model (68–71), all effects being reversed upon ROCK2 inhibition (66). Mechanistically, ROCK2 inhibition with KD025 significantly decreased STAT3 phosphorylation, whereas STAT5 phosphorylation was increased (66), both also reported in patients (32). Moreover, ROCK2 inhibition led to decreased expression of IRF4 and RORγt, crucial regulators for Th17 development (30–32, 65, 66, 72). Interestingly, the treatment did also reduce B-cell lymphoma 6 (Bcl6) expression, described as a TFH transcriptional regulator (66). The effects of KD025 in cGvHD were confirmed using a second minor mismatch Scl-cGvHD model. Skin pathology and GvHD scores were reduced together with a reduction of epidermal hyperplasia, infiltration of nucleated cells into the dermis and hair follicle destruction. Consistent with the BOS model, STAT3 phosphorylation and IRF4 levels were lower in the spleens of ROCK2 inhibitor treated mice (66). In order to translate the effects into the human system, peripheral blood mononuclear cells (PBMCs) were isolated from cGvHD patients and cultured in Th17-skewing culture medium either in the presence of KD025 or vehicle treatment. Comparable to cells from healthy individuals, ROCK2 inhibition reduced the production of IL-21, IL-17 and IFNγ in cells from cGvHD patients (32, 66). Important to note, targeted ROCK2 inhibition did not interfere with anti-leukemia immunity (66). The preclinical data suggested that ROCK2 inhibition could reduce cGvHD severity by both, downregulation of cytokine production and reduction of TFH cells, which are important for disease progression (66).

Based on promising preclinical data, the safety and efficacy of the selective ROCK2 inhibitor belumosudil was evaluated in clinical trials. The results of the ROCKstar study (NCT03640481) were published recently (73). The trial included patients after allo-HCT with persistent cGvHD manifestations indicating systemic therapy. Patient who

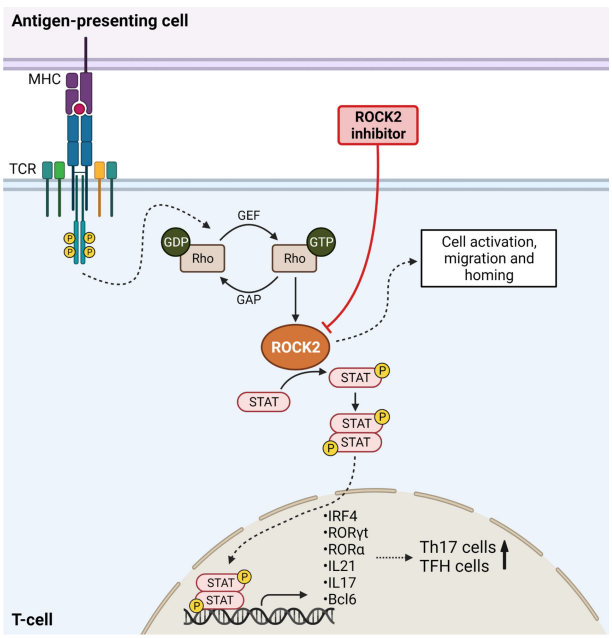


**FIGURE 2** | ROCK2 mediates Th17 differentiation in Graft-*versus*-Host Disease. TCR stimulation results in downstream ROCK2 activation, thereby phosphorylating STAT molecules. STAT is translocated into the nucleus to activate the transcription of Th17-specific transcription factors, thereby increasing the numbers of Th17 cells. ROCK2 activation further enhances the numbers of T follicular helper cells (TFH) and increases cell migration, activation and homing. Inhibition of ROCK2 blocks the differentiation of T-cells into TFH and Th17 cells and results in higher Treg numbers. Created with Biorender.com.

received two to five lines of therapy (LOT) were included. The ROCKstar study evaluated belumosudil at 200 mg once (QD) or twice (BID) per day. The best ORR was 74 % (200 mg QD) and 77 % (200 mg BID), high responses were seen in all groups, and all affected organs demonstrated a response. A symptom reduction was seen in 59 % (QD) and 62 % (BID) of all patients. AEs were reported but not unexpected for cGvHD patients treated with immunosuppressive therapies. Overall, ROCK2 inhibition was found safe and well tolerated in patients suffering from cGvHD (73). A similar study is evaluating safety and efficacy of BN101 (belumosudil) in patients with cGvHD at a daily dose of 200 mg (NCT04930562). Results of this trial have not been published yet. Following the promising safety studies, a phase IIa trial was conducted as dose-finding study and to further analyze safety and efficacy of belumosudil in cGvHD patients previously treated with one to three prior LOT (NCT02841995) (30). The study included 54 patients in three different treatment cohorts, 200 mg daily, 200 mg twice per day and 400 mg daily. The ORR was comparable between all cohorts, ranging from 62 % to 69 %. Detailed organ analyses revealed complete remission (CR) in all affected organs, except for the lungs where a partial response (PR) was the best response. In general, the responses were achieved rapidly, with more than 75 % of the responses seen at eight weeks. Later organ responses were mainly seen in the lung and the eyes. The percentage of patients achieving FFS with response at 12 months was 24 % (30). The 12- and 24-month OS rate was 91 % and 82 %, respectively. Upon ROCK2 inhibition, 35 % of all patients experienced clinical improvement and 86 % could reduce or discontinue corticosteroids. Overall, belumosudil was well-tolerated and found safe to use. The main AEs were upper respiratory infections, diarrhea, fatigue, headache and hypertension. Belumosudil treatment was discontinued in three patients due to potentially drug-related AEs and four patients died during the study due to disease relapse and cGvHD progression; none of the deaths was related to the treatment. Mechanistically, blood analysis revealed increased Treg numbers together with decreased numbers of Th17 cells (30). In summary, the selective ROCK2 inhibitor belumosudil is a promising treatment option in cGvHD, targeting both, fibrosis and inflammation. The mechanistic results were comparable to the observations from the preclinical study (66). ROCK2 inhibition with belumosudil was granted Breakthrough Therapy Designation by the US Food and Drug Administration (FDA) and received FDA approval for the use in SR-cGvHD.

## MAMMALIAN TARGET OF RAPAMYCIN (mTOR)

Donor T-cell activation and inflammatory cytokine secretion is a hallmark of GvHD after allo-HCT. Activation and effector functions of T-cells are tightly connected to the phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin (PI3K/AKT/mTOR) signaling cascade, which is crucial for the regulation of T-cell survival, proliferation, cell cycle progression, differentiation and metabolism (**Figure 3**) (7, 24–26, 74, 75). The major regulator of mTOR is a serine protein kinase formed of the mTORC1 and mTORC2 complexes (76, 77). The p70 ribosomal S6 Kinase (S6) is located downstream of mTOR and is the main signal transducer to enhance gene transcription and protein synthesis (7, 22). It is known that mTOR signaling is enhanced in GvHD, as T-cells isolated from allo-HCT recipients showed enhanced expression of Raptor and Rictor, both parts of the mTOR complex, and elevated S6 phosphorylation (78, 79).

**Acute GvHD:** In a preclinical study, transplantation of *Mtor*-deficient donor T-cells reduced aGvHD severity in mice, whereas more detailed analysis with *Raptor*-deficient allogeneic T-cells revealed that T-cell-mediated pathogenesis is dependent on mTORC1 but not on mTORC2 (79). Treatment with the mTORC1 inhibitor rapamycin (sirolimus) reduced GvHD severity in mice through reduction of pro-inflammatory cytokines and a blockade of T-cell proliferation and APC activity (79–81). Previous preclinical analysis revealed that rapamycin treatment is more effective in reducing murine GvHD mediated by $CD8^+$ or TCR $\gamma\delta^+$ T-cells than by $CD4^+$ T-cells. Proliferation of $CD8^+$ T-cells and production of Th1 and cytotoxic T-cell cytokines was inhibited, whereas Th2 cell differentiation was mainly unaffected. More detailed analysis was important for stratification of responsive patients, treatment time points and treatment combinations, as rapamycin did also reduce the GvL effect (80). Application of the immunosuppressant therapy cyclosporine A was shown to interfere with Treg activity, which is important to control GvHD. It was therefore crucial to clarify the effects of rapamycin on the different T-cell subsets, including Tregs. Preclinical and clinical studies revealed that rapamycin does not affect the expansion of Tregs (82–86). Treatment of mice suffering from aGvHD with rapamycin did not result in a survival benefit, however rapamycin enhanced survival of the mice when conventional T-cells (Tconv) and Tregs were transplanted together. The findings were confirmed by histopathological scoring. Rapamycin treatment and Treg transplantation reduced the proliferation of Tconv after allo-HCT. Mechanistically, rapamycin inhibited the proliferation of both, Tregs and Tconv, however Tregs were affected to a lesser extent and their immunosuppressive phenotype and FoxP3 expression was maintained. *In vivo* imaging confirmed that Treg proliferation is only slightly affected by rapamycin. In order to understand the differential impact of rapamycin on $CD4^+$ T-cells, downstream analysis of mTOR was conducted by S6 and 4-EBP1 phosphorylation analysis after IL-2 stimulation and revealed minimal usage of mTOR signaling by Tregs. The findings of this study help to better understand the synergistic activity of Tregs and rapamycin in protection from aGvHD (87). It was also shown that rapamycin treatment preserves the thymic reconstitution of Tregs after allo-HCT, important to reduce GvHD severity (86). An early preclinical analysis hypothesized that PI3K blockade could be a promising strategy to reduce GvHD as *in vitro* treatment of donor lymphocytes with

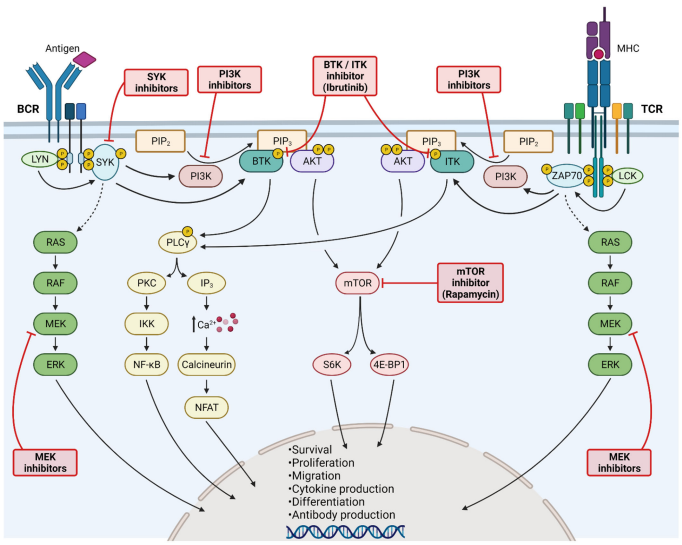


**FIGURE 3** | Kinase inhibition for Graft-*versus*-Host Disease treatment. Activation of B-cell (left side) and T-cell (right side) receptors leads to LYN/LCK phosphorylation, subsequently phosphorylating SYK and ZAP70. Both kinases further increase the downstream RAS/RAF/MEK/ERK signaling cascade. Activated SYK/ZAP70 stimulates also PI3K, as well as BTK and ITK. PI3K catalyzes the transformation of $PIP_2$ into $PIP_3$, being a binding site for BTK/ITK and AKT. BTK and ITK phosphorylate PLCγ, thereby enhancing PKC signaling and NF-κB translocation into the nucleus. Moreover, elevated calcium influx activates NFAT signaling. Activation of AKT stimulates mTOR signaling. All major kinase signaling pathways lead to cell survival, proliferation, differentiation and migration. Furthermore, cytokine and antibody production are enhanced. The signaling pathways can be blocked at various steps, including MEK, SYK, PI3K, BTK/ITK and mTOR inhibition. All kinase inhibitors have shown promising results in GvHD. Created with Biorender.com.

wortmannin reduced GvHD severity in mice (88). The role of the PI3K/AKT/mTOR pathway was further investigated in GvHD with an upstream blockade of PI3K using BKM120 or a novel dual PI3K/mTOR inhibitor BEZ235 (7). Both inhibitors blocked the signaling pathway as seen by decreased AKT and S6 phosphorylation. Both also reduced T-cell proliferation without affecting apoptosis, whereas the double inhibitor was more effective at lower concentrations. PI3K/mTOR pathway inhibition also reduced the secretion of Th1/Th2 effector cytokines, including IL-2, IL-6, IFNγ and TNFα. *In vitro* stimulation with anti-CD3/anti-CD28 increased early effector T-cells, which was reduced by BEZ235 treatment, but not by PI3K blockade alone. Based on promising *in vitro* data, major mismatch transplanted mice were treated with BEZ235; kinase inhibition significantly prolonged the survival of aGvHD mice and ameliorated GvHD (7). Castor et al. further investigated the role of PI3K in allo-HCT by transplanting PI3Kγ-deficient semi-allogeneic splenocytes in a murine GvHD model (89). Deficiency of PI3Kγ in the donor splenocyte compartment or inhibition of PI3Kγ with AS605240 significantly prolonged survival, increased body weight and reduced GvHD clinical scores in the recipient mice. Histological analysis revealed reduced damage of small intestine and liver and lower infiltration of $CD11c^+$ and T-cells into the small intestine in the absence of PI3Kγ in donor splenocytes. PI3Kγ-deficiency was also associated with reduced pro-inflammatory cytokine secretion. Intravital microscopy showed decreased numbers of rolling and adherent cells in GvHD mice treated with AS605240 compared to vehicle. Together with hints for maintained anti-leukemia immunity, inhibition of PI3Kγ could be a novel strategy to suppress GvHD severity, although further analysis is necessary to better understand its role after allo-HCT (89). A recent preclinical study applied rapamycin in a GvHD model with 50 % MHC disparity and reported increased splenic leukocyte counts, including Tregs and myeloid-derived suppressor cells (MDSCs) (90). Whereas T-cell activation, exhaustion and cytokine secretion remained unchanged, rapamycin treatment rendered the MDSC population more immunosuppressive, reported the first time for mTOR blockade. MDSCs re-isolated from rapamycin treated GvHD mice had an increased immunosuppressive capacity towards alloantigen-stimulated T-cells, confirmed by increased expression of iNOS, IDO and arginase-1. The importance of iNOS was underlined by application of a specific inhibitor, which abrogated the immunosuppressive phenotype of MDSCs. Since T-cell effector molecules remained unchanged with preserved GvL activity upon rapamycin treatment, the question how GvHD severity is reduced remains open. Nevertheless, the study described a novel and unknown role of mTOR kinase inhibition in GvHD on the myeloid compartment (90).

**Chronic GvHD:** The importance of mTOR signaling for GvHD pathogenesis is underlined by the finding of activating mTOR mutations in cGvHD patients which drive clonal $CD4^+$ T-cell expansion and cGvHD development (91). Consistent with these findings, Sugiyama et al. highlighted in preclinical cGvHD models that mTOR inhibition, in contrast to cyclosporine A,

does not increase the liability to cGvHD development. The investigators could see changed cGvHD scores in the skin and salivary glands upon rapamycin application compared to control (92).

Based on promising preclinical findings, a pilot clinical trial investigated the safety and efficacy of sirolimus as second-line therapy for GvHD treatment after allo-HCT (93). In total, 12 of 21 patients responded to the treatment, however, side effects were significant. AEs included thrombocytopenia, neutropenia and hemolytic uremic syndrome. Sirolimus had activity in patients with SR-GvHD, but dose optimizations were proposed due to severe toxicities (93). A combination of rapamycin with tacrolimus and low-dose methotrexate in GvHD patients was found feasible. In comparison to historical high-risk populations, the investigators reported lower rates of GvHD (94, 95). Following, a phase II trial combined rapamycin with tacrolimus as GvHD prophylaxis treatment after allo-HCT (NCT00803010). The combination treatment was superior over the control group and prevented high-grade aGvHD and moderate-severe cGvHD while promoting Treg reconstitution (96). A combination of sirolimus with calcineurin inhibitor could prevent GvHD in lymphoma patients after allo-HCT (97). Combining sirolimus/tacrolimus/methotrexate in lymphoma patients after allo-HCT did not affect OS, PFS and cGvHD, however, the addition of sirolimus prevented grade II-IV aGvHD (NCT00928018) (98). Recently, the efficacy of sirolimus was tested upon addition to standard GvHD prophylaxis (NCT01231412). Addition of sirolimus reduced grade II-IV aGvHD incidence, increased OS, but did not affect cGvHD (99). Whereas before-mentioned trials investigated sirolimus as prophylaxis treatment, Pidala et al. evaluated sirolimus for GvHD treatment (100). Sirolimus was tested vs prednisolone as initial treatment of patients with standard-risk aGvHD (NCT02806947). Day 28 CR/PR was comparable between both groups, however, CR/PR were significantly higher with sirolimus if compared to low-dose prednisolone. OS, disease-free survival, relapse and non-relapse mortality were comparable between both groups. Sirolimus reduced grade 2-3 infections, steroid exposure, hyperglycemia and enhanced patient-reported quality of life. Since sirolimus achieved comparable outcome at day 28 as prednisolone and spared steroid exposure, a confirming phase III trial is needed to also examine its efficacy in SR-aGvHD patients (100). Besides aGvHD, sirolimus was tested in combination with prednisolone or prednisolone/CNI in cGvHD (NCT01106833). CR/PR at 6 months, FFS and OS were the same at 2 years. Carpenter et al. concluded that sirolimus/prednisolone is an alternative, as a double-therapy is easier to administer and better tolerated than a triple-therapy (101). A first-in-human phase I/II clinical trial combines the JAK2 inhibitor pacritinib (PAC) with sirolimus and low-dose tacrolimus (PAC/SIR/TAC), aiming to reduce T-cell co-stimulation *via* mTOR and IL6 (NCT02891603). The effect of pacritinib/sirolimus was tested in human MLRs and a xenogeneic GvHD model and consistently suppressed allogeneic T-cell proliferation and GvHD severity (102, 103). STAT3 and S6 phosphorylation were reduced upon treatment, confirming JAK2/mTOR inhibition. In mice, the treatment reduced Th1 and Th17 cells while increasing Tregs. Anti-leukemia and anti-CMV immunity were preserved. Following, the PAC/SIR/TAC combination will be tested in the ongoing phase II trial (102). Overall, targeting mTOR signaling with sirolimus and blocking the PI3K pathway are both promising and established strategies to reduce acute and chronic GvHD either as prophylaxis or treatment of an established disease and may be preferred to other regimens for patients after allo-HCT.

## BRUTON'S TYROSINE KINASE (BTK) AND INTERLEUKIN-2 INDUCIBLE T-CELL KINASE (ITK)

Dysregulation of T- and B-cell activation and proliferation, enhanced antibody production, inflammation and organ damage are typically seen during GvHD development (104, 105). Stimulation of BCR and TCR and the subsequent activation of downstream pathways is crucial for GvHD induction after allo-HCT (106, 107). Bruton's tyrosine kinase (BTK) is part of the BCR signaling complex and kinase activation is necessary for survival, migration and proliferation of B-cells (19). Genetic BTK-deficiency results in a loss of peripheral B-cells and a blockade of immunoglobulin production (108). Activation of BTK subsequently phosphorylates phospholipase Cγ2 (PLCγ2), thereby facilitating further downstream effects like NF-κB and NFAT activation to enhance survival, proliferation and migration of B-cells (14, 19, 20). The interleukin-2 inducible T-cell kinase (ITK), another Tec family kinase, has functional similarities with BTK but is crucial for TCR signaling (20, 21). Comparable to BCR signaling, ITK is important for PLCγ2 activation downstream of the TCR, thereby facilitating signaling through NF-κB, NFAT and MAPK to activate T-cells, enhance proliferation and promote cytokine production (14, 20, 109, 110). ITK is important for driving the secretion of IL-2, IL-17 and Th2 cytokines, all being associated with cGvHD pathogenesis (11, 20, 111–114). Regarding the importance of T-cells and B-cells in both, acute and chronic GvHD, inhibition of BTK and ITK could be a promising strategy to inhibit GvHD development by blocking B- and T-cell activation, thereby hindering severe inflammation and fibrosis (14). Ibrutinib is an FDA-approved inhibitor, blocking both ITK and BTK, which was first approved for the use in lymphocytic leukemia (**Figure 3**) (21).

**Acute GvHD:** Since ibrutinib has inhibitory effects on both, BTK and ITK, a preclinical study determined the ability of ibrutinib to target donor-derived T-cells in an aGvHD model. For exclusion of donor B-cells, T-cell depleted BM was transplanted together with T-cells from B-cell KO donor mice. Treatment with ibrutinib improved aGvHD clinical scores and survival of mice, whereas the latter was not significantly changed. Although T-cell proliferation and activation was unaffected upon ibrutinib treatment, experiments with B-cell-deficient donor mice confirmed an effect of ibrutinib on donor T-cells after allo-HCT (14).

**Chronic GvHD:** Since application of ibrutinib led only to slight improvement in acute GvHD, ITK and BTK inhibition with ibrutinib was evaluated in a pre-clinical cGvHD model (104). Mice receiving the treatment survived significantly longer compared to vehicle, did not develop ascites and had delayed onset of proteinuria. Both was associated with cGvHD in mouse models. However, proteinuria was only prevented with longterm treatment. Notably, ibrutinib suppressed cGvHD development and prolonged survival if given after the disease was already established (14). Mechanistically, ibrutinib inhibited B-cell proliferation and co-stimulatory molecule expression, known to be crucial for GvHD pathogenesis (14, 115). T-cell proliferation was not affected. $CD4^+CD8^+$ thymocytes were increased, pointing towards enhanced immune reconstitution upon ibrutinib treatment. These findings were not confirmed by lineage staining after allo-HCT and need further detailed analysis to substantiate this hypothesis (14). In an additional scleroderma model (116) with prophylactic ibrutinib treatment prior to allo-HCT, the investigators found that inhibitor treated mice showed less cGvHD symptoms, reduced skin damage, less alopecia and lower GvHD scores after allo-HCT. Prophylactic effects were only seen with high-dose treatment. Ibrutinib treatment did also enhance the reconstitution of B-cells and reduced T follicular helper (TFH) cells after allo-HCT. Protective effects of ibrutinib were confirmed in an aGvHD model transitioning into cGvHD (14). In a second preclinical study, ibrutinib was applied in sclerodermatous cGvHD starting on day 25 after allo-HCT when the first symptoms became apparent (70, 117). The investigators found reduced clinical signs of cGvHD. These findings were accompanied by improved progression-free survival upon ibrutinib treatment. Moreover, ibrutinib application diminished B- and T-cell infiltration into lung and kidney and led to lower GvHD pathology scores in these cGvHD target organs. The investigators applied a second model, aiming to understand the effects of ibrutinib treatment on bronchiolitis obliterans (BO) in cGvHD (70). The treatment started on day 28 after allo-HCT and resulted in reduced pulmonary resistance and elastance, better compliance and lower lung fibrosis. Analysis from ibrutinib treated mice was comparable to non-GvHD mice. Withdrawal of therapy led to a loss of benefit, indicating that ibrutinib treatment need to be applied continuously. Contrary to the previously reported study, prophylactic ibrutinib treatment could not effectively combat cGvHD or BO (14, 70). To further clarify the role of BTK and ITK in cGvHD, the investigators transplanted WT bone marrow together with ITK-deficient T-cells into allogeneic recipients. Donor-derived T-cells are known to be important for cGvHD development. ITK deletion could reverse cGvHD signs in the lungs to values comparable with non-GvHD animals and ibrutinib treated cGvHD mice. Comparable results were seen when XID bone marrow, which lacks BTK, was used as allogeneic graft. B-cells driving cGvHD development rather arise from the transplanted bone marrow. These experiments highlight that both Tec kinases, ITK and BTK, play a role in cGvHD development. Analysis of *ex vivo* ibrutinib treated cGvHD patient-derived $CD4^+$ T-cells revealed reduced activation upon kinase inhibition. Reduced activation was also seen in patient-derived B-cells with lower BTK, ERK1/2 and PLCγ2 phosphorylation (70). Comparing both described preclinical studies, all models found that ibrutinib treatment affected B-cell activation and differentiation, whereas the effects on T-cells were variable. Clinical GvHD scores were improved in all models and effective in both, prophylactic treatment and treatment of established disease (20).

The promising preclinical data paved the way to further investigate ibrutinib treatment in cGvHD. A phase Ib/II study was conducted to determine safety and efficacy of ibrutinib in patients who failed at least one LOT for cGvHD (NCT02195869) (20, 118). Ibrutinib did not show dose-limiting toxicities. At a median follow-up of 13.9 months, 29 % of patients were still receiving the drug, whereas 71 % of patients discontinued due to adverse events (AEs), cGvHD progression and patient decision. Most AEs were low grade and well manageable and led to dose reductions. A total of 29 patients (69 %) developed infectious complications of any grade. The ORR was 76 % and 71 % of responders showed a response for more than 20 weeks. Even responses were seen in all cGvHD target organs. Corticosteroid therapy could be reduced with ibrutinib treatment. Detailed mechanistic analysis showed strong inhibition of BTK and ITK, reduced pro-inflammatory mediators in the serum, less germinal center (GC) B-cells and total B-cells and reduced numbers of Th17 and TFH cells (20, 118). Promising data from this trial led to the iNTEGRATE phase III clinical trial investigating ibrutinib in combination with prednisone in patients with newly diagnosed moderate to severe cGvHD after allo-HCT (NCT02959944). Response rate was slightly higher in the ibrutinib group and corticosteroids could be withdrawn at 21 and 24 months in the ibrutinib arm. Patients receiving ibrutinib had improved Lee symptom scores. Another phase III trial evaluates the efficacy of ibrutinib in patients with SR-cGvHD (NCT03474679) and an additional phase II trial is currently recruiting to investigate ibrutinib as first-line therapy for newly diagnosed cGvHD who did not receive any systemic treatment for cGvHD (NCT04294641). So far, both of the last mentioned trials did not publish any results yet.

Based on very promising preclinical and clinical trial data, ITK and BTK inhibition with ibrutinib could be a very potent therapy in chronic GvHD. However, additional detailed analysis of the underlying mechanism is necessary to improve the therapeutic success. Since clinical trials focus on cGvHD, further analysis and preclinical models are needed to investigate the role of ITK blockade in aGvHD.

## SPLEEN TYROSINE KINASE (SYK)

The non-receptor cytoplasmic spleen tyrosine kinase (Syk) was hypothesized being an important regulator of GvHD as it has functions in transmitting signals from surface receptors, including Toll-like receptors (TLRs) (119), Fc receptors (120),

as well as chemokine receptors (27, 121, 122). Moreover, Syk activation is known to be crucial for TCR signaling upon peptide binding, as well as playing an important role in T-cell lineage commitment, mainly for Th17 responses which are known to be involved in GvHD pathophysiology (27, 29, 123). Based on the knowledge that Syk inhibition, e.g. using Fostamatinib, has beneficial effects in inflammatory diseases, the relevance of Syk in GvHD was further evaluated (**Figure 3**) (27, 124–126).

**Acute GvHD:** In a preclinical murine aGvHD model, daily treatment with Fostamatinib led to significantly improved survival, reduced histopathology scores and reduced pro-inflammatory serum cytokine concentrations. Fostamatinib treatment did not interfere with donor lineage engraftment and immune reconstitution. Syk phosphorylation is rapidly increased upon CD3/CD28 stimulation of T-cells and higher pSyk 525/526 levels were also seen in T-cells isolated from aGvHD mice. Using luciferase transgenic T-cells, Syk inhibitor treatment was found to reduce alloreactive donor T-cell expansion *in vivo*. The findings were confirmed by CFSE staining, indicating reduced T-cell proliferation *in vivo* upon Syk inhibition. Besides blockade of T-cell proliferation, Syk inhibition also reduced T-cell migration towards CXCL12. Contrary to previous findings about the importance of Syk in T-cell lineage commitment, Fostamatinib treatment did not change the percentage of Th2 and Th17 cells after allo-HCT. Effects on T-cells could be further affected by APCs as Syk inhibition was also connected to diminished costimulatory molecule expression and reduced DC migration *in vivo* and *in vitro*. Although proliferation and effector cytokine secretion of allogeneic donor T-cells was significantly reduced by Fostamatinib, the GvL effect was preserved, as confirmed by *in vivo* bioluminescence imaging using luciferase transgenic leukemia cells and *ex vivo* killing assays. Overall, pharmacological inhibition of Syk was found being a novel treatment strategy in aGvHD by reducing T-cell expansion and costimulation while preserving anti-leukemia immunity (27).

**Chronic GvHD:** Besides its role in TCR signaling, Syk is also involved in BCR signaling and controlling cell migration and adhesion (36). Knowing the importance of B-cells in cGvHD, Syk inhibition was also hypothesized being a major regulator of cGvHD pathophysiology (8, 11, 36, 69, 127). In a cGvHD model with multiorgan involvement, Syk-mediated BCR signaling in allogeneic B-cells was validated being crucial for cGvHD development (69). The investigators isolated B-cells from cGvHD animals at day 60 after allo-HCT and showed increased Syk phosphorylation at Y348 (69). Comparable results were found in B-cells from cGvHD patients (107). The importance of Syk signaling was further evaluated using Syk-deficient allogeneic BM donors for a model of cGvHD with multiorgan involvement. The mice did not develop pulmonary dysfunctions after transplantation with Syk KO BM, whereas Syk-deficient T-cells did not attenuate cGvHD severity. Additionally, Syk was not only important in the initiation of cGvHD, but also in disease progression as pulmonary dysfunction was reversed upon tamoxifen-induced Syk depletion. The Syk inhibitor Fostamatinib (126, 128), was applied during active disease and reduced cGvHD severity in the lung and improved pulmonary dysfunctions (69). Contrary, improvement of skin inflammation was not seen in three Scl-cGvHD models upon Fostamatinib treatment. However, one model showed attenuated skin GvHD and clinical GvHD scores upon Syk inhibition (69). In an additional study of Scl-cGvHD, the investigators proved that Syk phosphorylation is increased in T- and B-cells, as well as in $CD11b^+$ cells, after allo-HCT (129). Early treatment with Fostamatinib reduced the severity and fibrosis of Scl-cGvHD and the expression of pro-inflammatory molecules in the skin. Moreover, the migration of antigen-specific memory $CD4^+$ T-cells and the proliferation and activation of allogeneic $CD4^+$ and $CD11b^+$ cells was suppressed, comparable to results seen in the aGvHD setting (27, 129). Since this data is contrary to a before-mentioned study, it is important to note that effects of Fostamatinib were mainly seen if the treatment was applied early after cGvHD induction (69, 129). When B-cells isolated from patients were treated with Fostamatinib *in vitro*, the drug preferentially killed cGvHD B-cells seen by increased apoptosis if compared to non-cGvHD control B-cells (69, 107). Additionally, Syk inhibition blocked the differentiation of CD4 T-cells into Th2 and Th17 phenotypes (8, 69, 129). This was different if compared to Syk inhibition in aGvHD described above (27). Using the second-generation highly selective Sky inhibitor entospletinib, Poe et al. demonstrated that inhibitor treatment blocked the development of eye GvHD and also significantly reduced hair loss in GvHD animals. Besides reducing GvHD severity, entospletinib treatment led to improved reconstitution of monocytes, B-cells and T-cells at 28 and 42 days after allo-HCT. Moreover, pathogenic activated $GL7^+$ B-cells and Th2 cells were diminished upon Syk inhibition, both playing a role in acute and chronic GvHD (130–132). T-cell differentiation was changed upon entospletinib treatment with increased numbers of Tregs and a reduction of Th17 cells. Overall, the treatment significantly prolonged the survival of the mice after allo-HCT and reduced skin inflammation and GvHD severity (130).

Taken together, the data derived from genetic and pharmacological approaches in pre-clinical murine GvHD models clearly indicate that Syk plays an important role in GvHD pathophysiology. Pharmacological targeting of Syk could be a novel attempt to treat GvHD. Based on these promising findings of Syk inhibition with Fostamatinib *in vitro* and from preclinical *in vivo* models, the efficacy of Fostamatinib to prevent and treat cGvHD after allo-HCT is currently evaluated in a phase I trial (NCT02611063). Another phase II trial was investigating the efficacy and tolerability of entospletinib in combination with systemic corticosteroids cGvHD as first-line therapy, however, the study was terminated (NCT02701634). Based on pre-clinical studies, it would also be interesting to evaluate effects of Syk inhibition in patients with aGvHD. However, ongoing clinical trials first focused on cGvHD, which might be due to the importance of Syk in BCR downstream signaling, whereas it is not essential for TCR downstream events.

## PLATELET-DERIVED GROWTH FACTOR RECEPTOR

**Chronic GvHD:** cGvHD is often presented with dermal fibrosis and sclerosis, associated with the presence of stimulatory anti-platelet-derived growth factor receptor (PDGFR) antibodies, suggesting a direct link between skin fibrosis and PDGF signaling (133). PDGFR stimulation causes enhanced collagen production, which could contribute to organ damage (134). Besides PDGFR stimulation, TGFβ is known as an important mediator of fibrosis in cGvHD (135, 136) and inhibition of both reduced pro-fibrotic activity and pulmonary fibrosis in experimental models (137, 138). Imatinib was first developed as a treatment for BCR-ABL positive CML, but also inhibits PDGFR and was therefore hypothesized as a novel therapeutic intervention in cGvHD by reducing fibrosis (133, 139). In preclinical analyses of bleomycin-induced fibrosis, imatinib inhibited the development of dermal fibrosis by reducing COL1A1, COL1A2, and fibronectin 1 transcription. Moreover, the induction of extracellular matrix proteins, stimulated by PDGF and TGFβ, was reduced upon imatinib application (139, 140). Additionally, imatinib could also reduce fibrosis in kidney and liver, target organs of cGvHD (140). Based on this, Belle et al. applied imatinib in a murine model of Scl-cGvHD but found limited impact besides reduced PDGFR phosphorylation. T-cell proliferation was slightly inhibited, but GvHD scores were unchanged (141). Contrary to these findings, another study reported that both, imatinib and nilotinib prevent the development of Scl-GvHD in mice (142). Nilotinib is a second-generation TKI targeting BCR-ABL and PDGFR with a higher affinity than imatinib (36). Both TKIs inhibited dermal fibrosis and reduced dermal thickness if given as prophylaxis treatment. Additional to these findings, GvHD was also significantly reduced when imatinib or nilotinib was given after onset of clinical disease (142). Serum analysis of patients treated with nilotinib showed reduced inflammatory cytokine secretion, including TNFα, IFNγ and IL-2 (143). Using *ex vivo* cultures, GvHD-derived fibroblasts expressed higher levels of collagen genes, which was significantly reduced upon nilotinib application. Confirming *in vitro* data, skin analysis from cGvHD patients showed decrease of COL1α1 and COL1α2 protein levels, TGFβ inhibition and p-Smad2 reduction upon treatment with nilotinib (144). Both, imatinib and nilotinib showed efficacy in clinical trials. Treatment of SR-cGvHD with skin involvement led to improved joint range of motion and better skin scores (NCT00702689), proposing imatinib as possible salvage therapy for SR-cGvHD (133). Olivieri et al. reported high OR of imatinib treatment in patients with refractory cGvHD who previously failed at least two LOTs (145). Comparable, scleroderma symptoms disappeared upon imatinib application and the treatment was well-tolerated (146). A retrospective study confirmed a beneficial activity of imatinib as a salvage therapy in Scl-cGvHD (147). After introduction of the second-generation TKI nilotinib, the compound was tested in SR-cGvHD (NCT01810718). The 2-year OS was 75 % with FFS of 30 %. Based on promising long-term outcomes and well-manageable side effects, nilotinib was hypothesized as a promising treatment in SR-cGvHD (148). Another trial investigating safety and efficacy of nilotinib in SR-cGvHD did not post any results yet (NCT01155817). Taken together, pre-clinical and clinical data confirm the TKIs imatinib and nilotinib as promising therapeutic interventions for cGvHD with organ fibrosis.

## INOSITOL 1,4,5-TRIPHOSPHATE 3-KINASE B (ITPKB)

TCR stimulation and its ligation, mediated through the contact of T-cells with APCs results in a dramatic increase of intracellular calcium ($Ca^{2+}$) levels. Calcium influx is essential for activation, maturation and effector functions of T-cells. TCR engagement leads to the activation of PLC-γ, thereby increasing the intracellular levels of inositol 1,4,5-triphosphate ($IP_3$). Binding of $IP_3$ to its specific receptors in turn stimulates the release of calcium from intracellular storage compartments. Continuous depletion of intracellular calcium storage stimulates the opening of cell membrane based calcium channels to enhance the influx of calcium from the extracellular compartment. The intracellular increase of $Ca^{2+}$ is required to activate calcium-dependent kinases and the transcription factor calcineurin, leading to activation of nuclear factor of activated T-cells (NFAT), thereby enhancing the transcription of a variety of different genes necessary for T-cell activation and effector functions (33, 149). However, a very strong increase of the intracellular cytoplasmic $Ca^{2+}$ concentration leads to the transcription of pro-apoptotic signaling pathways and activation-induced cell death (AICD). The modulation of intracellular calcium levels was therefore hypothesized to be a potential therapeutic strategy for autoimmune diseases (149, 150). A major regulator of intracellular $Ca^{2+}$ levels is the inositol 1,4,5-triphosphate 3-kinase (Itpk) family, comprising Itpka, Itpkb, Itpkc and inositol polyphosphate multikinase. The Itpk family acts as a negative regulator of $Ca^{2+}$ influx through conversion of $IP_3$ to inositol 1,3,4,5-tetrakisphosphate ($IP_4$) and this regulatory mechanism is known to be highly important for T-cell development and survival (33–35, 151). Among all kinases in the Itpk family, Itpkb is most abundant in hematopoietic cells and genetic deficiencies of Itpkb lead to impaired T-cell development in the thymus, mainly based on AICD of immature $CD4^+CD8^+$ T-cells (34, 35, 152). Deletion of Itpkb in mature activated T-cells was shown to be a novel strategy to prevent T-cell driven autoimmunity through increase of intracellular calcium levels (**Figure 4**) (150). A recent study highlighted the therapeutic potential of Itpkb deletion and inhibition to control acute and chronic GvHD (149).

**Acute GvHD:** In major mismatch allo-HCT models, mice receiving allogeneic Itpkb-deleted T-cells survived significantly longer and experienced less weight loss compared to mice receiving T-cells with functional Itpkb. Complementary, histopathological analysis on day 7 after transplantation showed

lower aGvHD pathology scores if mice received T-cells deficient for Itpkb compared to mice receiving wildtype T-cells. Consistent with these findings using a genetic approach, inhibition of Itpkb with GNF362 (150) prolonged the survival of mice receiving high doses of allogeneic T-cells (149). Itpkb deficiency caused reduced donor T-cell survival but no reduced inflammatory cytokine production (153). Itpkb$^{-/-}$ T-cells had comparable or even higher intracellular tumor necrosis factor α (TNFα) and interferon γ (IFNγ) levels compared to Itpkb wildtype T-cells. However, quantitative T-cell analysis in the spleen, mesenteric lymph nodes and the small intestine and liver revealed lower numbers of Itpkb deficient T-cells compared to the WT control, whereas the proliferative capacity remained unchanged. Reduced numbers of donor Itpkb$^{-/-}$ T-cells could be linked to lower T-cell survival, indicated by higher abundance of active caspase 8 in CD4$^{+}$ and CD8$^{+}$ T-cells from Itpkb$^{-/-}$ donors after HCT (149, 154, 155). Since the survival of Itpkb deficient T-cells is impaired, GvL activity had to be investigated. Interestingly, mice receiving A20 leukemia cells and Itpkb$^{-/-}$ T-cells survived significantly longer compared to mice receiving leukemia cells only or leukemia in combination with WT T-cells. Leukemia expansion was only seen in mice transplanted with leukemia cells only. The results indicate that Itpkb deficiency maintains anti-leukemia immunity while reducing aGvHD severity. Comparable results were achieved using the Itpkb inhibitor GNF362 (149).

**Chronic GvHD:** In comparison to T-cell mediated aGvHD, cGvHD establishes as an autoimmune-like disease (8, 11). Mice receiving Itpkb deficient T-cells had significantly improved pulmonary resistance, elastance and compliance compared to mice receiving WT T-cells. Comparable results were seen for lung and liver collagen, as well as for cGvHD histopathology scores. Mice with established cGvHD were also treated with the Itpkb inhibitor GNF362 and the investigators found improved pulmonary function, most probably based on reduced lung macrophage infiltration. Using a Scl-cGvHD model, Itpkb inhibition did result in reduced skin and liver histopathology scores, lower infiltration of proinflammatory macrophages and a reduction of IFNγ-producing T-cells.

In summary, the study could highlight the importance of Itpkb in regulating acute and chronic GvHD and delivered promising data indicating that pharmacological inhibition of Itpkb could be a potential novel therapeutic approach to control GvHD without impairing anti-leukemia immunity (149).

## TGFβ-ACTIVATED KINASE 1 (TAK1)

Dysregulated innate immune cells are key players in GvHD pathogenesis by activating APCs in the pro-inflammatory milieu (16) and inhibition of inflammatory cytokine signaling could ameliorate GvHD severity in mice and humans (15). Aiming to further understand the role and contribution of innate immune cells in GvHD, Kobayashi et al. performed gene expression profiling on monocytes from patients who experienced GvHD after allo-HCT (156). The investigators found increased expression of TGFβ-activated kinase 1 (TAK1) and downstream signaling molecules, including TNFα, IL-6 and IL-1β, in monocytes from patients with GvHD compared to patients who did not experience GvHD after allo-HCT. TAK1 is a member of the mitogen-activated kinase (MAPK) family and is a key regulating factor upstream of nuclear factor κB (NF-κB), c-Jun N-terminal kinase (JNK),

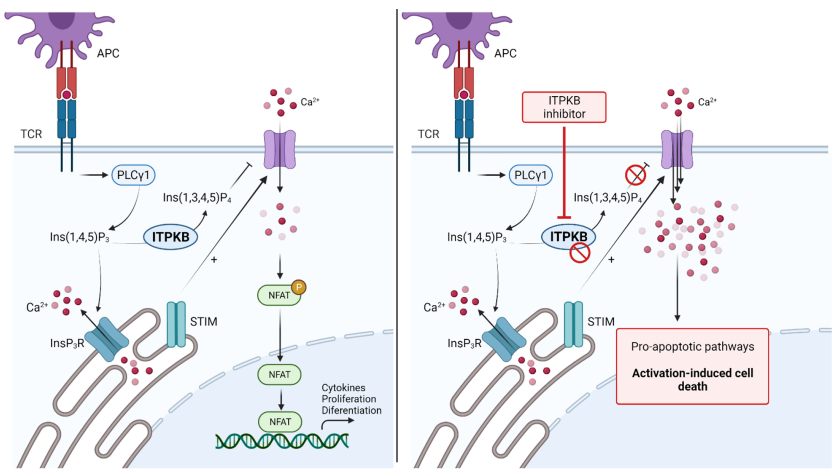


**FIGURE 4** | Blockade of Inositol 1,4,5-triphosphate 3-kinase B (ITPKB) as a novel treatment for Graft-*versus*-Host Disease. TCR stimulation activates downstream PLCγ, thereby increasing intracellular $IP_3$ levels. Binding of $IP_3$ to $IP_3R$ activates the release of $Ca^{2+}$ from intracellular storage compartments. Furthermore, STIM stimulate the influx of extracellular $Ca^{2+}$ to activate NFAT signaling and gene transcription. ITPKB is a rate-limiting step as it catalyzes the formation of $IP_4$ from $IP_3$. $IP_4$ acts as a control mechanism for calcium signaling by blocking the respective channels in the extracellular membrane. Genetic deletion or inhibition of ITPKB disturbs this control mechanism and leads to increased calcium influx, which stimulates pro-apoptotic signaling pathways, leading to activation-induced cell death. Since ITPKB is predominantly found in hematopoietic cells, this kinase is thought to be a novel target molecule for the treatment of GvHD. Adapted from "NFAT Signaling Pathway", by Biorender.com (2021). Retrieved from https://app.biorender.com/biorender-templates.

extracellular signal-regulated kinase (ERK) and p38 in toll-like receptor (TLR) signaling (16). Based on its role in mediating inflammatory signaling, inhibition of TAK1 was hypothesized being a novel strategy to ameliorate GvHD severity by reducing pro-inflammatory signaling and T-cell alloreactivity (156).

**Acute GvHD:** Patient-derived monocytes were LPS-activated and treated with the TAK1 inhibitor 5Z-7-oxozeaenol (OZ), followed by analysis of cytokine production. Pro-inflammatory cytokine secretion was suppressed in a concentration-dependent manner. Application of OZ in a major mismatch murine aGvHD model prolonged the survival of GvHD mice, reduced GvHD scores and did result in lower serum levels of pro-inflammatory cytokines (156). In a different study, Mathew and Vinnakota et al. investigated the role of microglia in central nervous system (CNS) aGvHD and also found TAK1 being an important mediator of aGvHD-induced neurotoxicity (157). It could be shown for the first time that CNS-GvHD in mice is connected to an activation and expansion of microglia. Comparable, the numbers of microglia were higher in the grey and white matter of patients suffering from GvHD after allo-HCT compared to non-GvHD and non-HCT controls. Moreover, costimulatory molecules were increased on microglia from mice after allo-HCT compared to syngeneic (syn-) HCT and untreated controls. CNS-GvHD was connected to enhanced microglia-derived TNF production in mice and humans, driven by TAK1. Also, other inflammatory cytokines connected to TAK1 signaling were seen elevated in microglia after allo-HCT. Genetic depletion of TAK1 in microglia did alleviate CNS-GvHD associated pathology, as well as memory and cognitive deficits in mice after allo-HCT. Comparable results were seen using the TAK1 inhibitors Takinib and OZ. Takinib did even reduce IFNγ and IL-17 production of T-cells infiltrated into the brain. Based on these two studies and the finding that TAK1 inhibition does not interfere with anti-leukemia immunity after allo-HCT (157), TAK1 could be a novel and promising target to ameliorate aGvHD and CNS-GvHD (157).

## MITOGEN-ACTIVATED PROTEIN KINASE (MEK) INHIBITION

The onset and pathogenesis of GvHD is correlated with strong TCR activation and stimulation of TCR downstream signaling pathways to enhance alloreactivity and cytokine production (158). Signaling through the rat sarcoma/mitogen-activated protein kinase kinase/extracellular-signal regulated kinase (RAS/MEK/ERK) cascade is also crucial to translocate transcription factors and to enable target gene transcription, cell proliferation, migration, survival and differentiation (**Figure 3**) (28, 159).

**Acute GvHD:** One pre-clinical study investigated activated signaling molecules in alloreactive T-cells isolated from mice suffering from aGvHD and identified significantly increased phosphorylation of ERK1/2 and STAT3 (160). Inhibition of ERK1/2 and STAT3 phosphorylation was thought to be a novel method to reduce donor T-cell alloreactivity (160). ERK1/2 phosphorylation was inhibited using the selective MEK1/2 inhibitor SL327. MEK1/2 is located upstream of ERK1/2 (161–163). SL327 could dose-dependently reduce the proliferation of T-cells upon CD3/CD28 stimulation and in a mixed lymphocyte reaction (MLR) (160, 164). A second preclinical study applied flow cytometry-based pERK1/2 analysis of human T-cells activated with PMA and Ionomycin and a preferential increase of ERK1/2 phosphorylation in naïve and central memory T-cells was seen (165). Application of U0126, a classical MEK inhibitor, and selumetinib, a second-generation MEK inhibitor, reduced ERK1/2 phosphorylation dose-dependently. The latter inhibitor is tested in various clinical trials for different cancer entities and was found safe to use with little to no hematologic toxicity (166–169). Besides proliferation, MEK inhibition reduced effector cytokine production by memory T-cells. Based on these results, the investigators aimed to elucidate the effect of MEK inhibition in an alloreactive setting. The proliferation of T-cells activated with allogeneic HLA-mismatched DCs was significantly suppressed by MEK inhibition while virus-specific T-cell responses were not affected (165). Comparable results were seen using the MEK inhibitor trametinib (170). Selumetinib suppressed cell division even stronger than the calcineurin inhibitor tacrolimus (165, 171). MEK inhibition was further evaluated in experimental major-mismatch aGvHD mouse models, where selumetinib significantly prolonged the survival of GvHD mice (165). The MEK inhibitor trametinib could also suppress GvHD in a xenogeneic model and enhanced the engraftment of diverse T-cell clones. In this model, MEK inhibition suppressed T-cell activity responsible for GvHD while promoting human T-cell reconstitution (172).

**Chronic GvHD:** In an additional preclinical analysis, trametinib treatment enhanced survival and reduced GvHD scores in a MHC-haploidentical GvHD model (173). MEK inhibition suppressed $CD8^+$ T-cells and elevated naïve T-cells after allo-HCT. Trametinib also reduced target organ damage and lymphocyte infiltration. A second model confirmed the potency of MEK inhibition, as the development of cutaneous GvHD, skin sclerosis and alopecia was reduced upon trametinib application. Although trametinib was reported to be well-tolerated without toxicities *in vivo* (174), donor cell engraftment and myeloid immune reconstitution were suppressed. Since MEK inhibition was shown to suppress T-cell effector functions and proliferation, it is of high importance to investigate the effect on anti-tumor immunity. Surprisingly, MEK inhibition did not affect T-cell mediated anti-tumor immunity against mastocytoma cells, as T-cell transplanted mice survived longer compared to vehicle. Mice receiving tumor only without T-cells did not benefit from MEK inhibition, implicating that trametinib does not directly affect tumor cells. In comparison, the GvHD prophylaxis treatment tacrolimus shortened the survival of leukemia-bearing mice as it suppressed both, GvHD and GvL (173). Although preclinical results for MEK/ERK inhibition are promising in aGvHD and GvL models and MEK inhibitors were not reported having limiting toxicities in mice, to the best of our knowledge no

clinical trial was initiated yet to evaluate the efficacy of MEK inhibition GvHD patients.

## AMP KINASE (AMPK)

GvHD following allo-HCT is predominantly driven by alloreactive donor T-cells which cause severe tissue damage (4). Novel therapeutic options aim to impair T-cell functions to reduce life-threatening GvHD without affecting GvL activity, including the idea to metabolically re-program T-cells after allo-HCT (175). Following transplantation, T-cells increase oxidative phosphorylation and fatty acid oxidation (176, 177). Since it is known that allogeneic effector T-cells require fatty acid oxidation (FAO) during GvHD, it was hypothesized that AMP kinase (AMPK) activation plays a major role in regulating T-cell activity after allo-HCT (175, 177).

**Acute GvHD:** Monlish and colleagues recently identified that alloreactive donor T-cells selectively increase AMPK activation during aGvHD after allo-HCT as they found elevated phosphorylation of AMPKα and the downstream molecule ACC in $CD4^+$ and $CD8^+$ T-cells isolated from mice on day 7 after allo-HCT (175). The investigators established AMPK double knockout (AMPK-dKO) mice lacking AMPKα1 and AMPKα2 in all peripheral T-cells. AMPK is a heterotrimeric molecule with the α subunit as kinase domain and the β/γ subunits being important for stability and substrate specificity (175, 178–180). Although T-cell development and *in vitro* proliferation in a MLR was not different between AMPK-dKO and WT T-cells, AMPK-deficient T-cells caused less severe GvHD after transplantation into lethally irradiated recipient mice. In two different GvHD models, the survival was prolonged and clinical GvHD scores were lower if mice received AMPK-dKO T-cells compared to WT T-cells (175). Comparable findings were described in a second study (181). In addition, the infiltration of AMPK-dKO T-cells into GvHD target organs was reduced. The anti-leukemia response was not impaired upon depletion of AMPK. Mechanistically, reduced GvHD was connected to reduced recovery and decreased expansion of AMPK-depleted T-cells after allo-HCT (175, 181). The fewer recovery was linked with increased apoptosis in mainly $CD8^+$ T-cells, whereas the results are highly variable and the interpretation is therefore questionable. Although AMPK is a metabolic enzyme, depletion did not affect any investigated metabolic pathway, but rather affected other cell populations. Co-transplantation experiments revealed that AMPK-dKO T-cells stimulated an increase of WT Tregs. Given the importance of Tregs to reduce GvHD severity, increased Treg numbers due to accompanying AMPK-dKO cells were named as a major mechanism to suppress GvHD severity (175, 182–184). Although there are no AMPK inhibitors available yet, inhibition of AMPK in T-cells could serve as novel target for GvHD treatment; however, more detailed analysis is needed to better understand the role of AMPK after allo-HCT (175).

## P38 MITOGEN-ACTIVATED PROTEIN KINASE (MAPK)

The p38 mitogen-activated kinase (MAPK) is a major control mechanism for cellular responses, proliferation and cytokine production (17, 185, 186). Different p38 MAPK isoforms are expressed in most tissues and cell types and are activated by extracellular stimulatory signals, including inflammatory cytokines, growth signals and stress signals (17, 185, 187, 188). Activation of the p38 MAPK signaling cascade results target gene expression, including inflammatory mediators and cytokines (17, 188–190). Of all isoforms, p38α was reported as major regulator in inflammatory responses and therapeutic blockade with the p38α-specific inhibitor VX-702 was applied in rheumatoid arthritis (RA) to reduce inflammatory signals (191).

**Chronic GvHD:** Since enhanced p38 MAPK phosphorylation was found in fibroblasts from systemic sclerosis patients and p38 MAPK blockade reduced elevated type I collagen expression, the signaling pathway was hypothesized playing a role in sclerosis pathogenesis (17, 192, 193). Systemic sclerosis has various clinical similarities with Scl-cGvHD and therapeutic blockade of p38 MAPK signaling was thought to reduce cGvHD severity (17, 116, 194–196). Phosphorylation of p38 MAPK (Thr180/Tyr182) was increased in the skin of Scl-cGvHD mice compared to syngeneic BMT mice (17). Application of VX-702 delayed the onset of skin fibrosis and alopecia development and improved skin GvHD scores. The dermal thickness, collagen area and levels of procollagen I αI were reduced upon VX-702 treatment. More detailed analysis by histology and flow cytometry revealed reduced infiltration of $CD4^+$ and $CD8^+$ T-cells, as well as lower numbers of myeloid cells and macrophages into the skin of Scl-cGvHD mice upon p38 MAPK inhibition. Of all analyzed cytokines, only IL-6 and IL-13 were significantly reduced upon treatment, whereas major drivers of Scl-cGvHD, like TGFβ and IFNγ, were unchanged (17). However, complete tissue transcription profile analysis might mask some minor changes in immune cells and it would have therefore been better to analyze effector and inflammatory cytokine production on single cells levels and to screen for cytokines in the serum. Nevertheless, consistent with histology data, tissue RNA analysis revealed decreased expression of COL1A2 and fibronectin 1 upon p38 MAPK inhibition (17). In summary, the study showed that p38 MAPK is activated in cGvHD and therapeutic blockade could be a novel therapeutic intervention.

**Acute GvHD:** Although p38 MAPK inhibition seems promising in cGvHD, the treatment is questionable since reduced p38α MAPK levels (heterozygous p38α-KO) in donor grafts were found to accelerate acute intestinal GvHD in mice (197). Surprisingly, and contrary to the previously described cGvHD study, loss of donor p38 reinforced GvHD severity and reduced the survival of the mice. Cytokine analysis confirmed higher TNFα levels in the gut in allo-$p38\alpha^{+/-}$ grafted mice compared to the WT setting. Although p38α loss prolonged the survival of donor-derived intestinal intraepithelial lymphocytes *in vitro* and *in vivo*, donor lymphocyte expansion was decreased in the mesenteric lymph nodes upon p38α deficiency. Although the

role of p38α-loss in the recipient compartment was not investigated, the study revealed a dichotomous effect of p38α in regulating inflammatory responses, cytokine expression, lymphocyte proliferation and intestinal GvHD after allo-HCT (197). Taken together with the before-mentioned study, the role of p38 MAPK in GvHD is still unclear and needs more detailed investigation, also comparing effects in chronic and acute GvHD as these could always be different (17, 197). Differences could be due to the models as the study on cGvHD applied an inhibitor, whereas the aGvHD study investigated the role of p38 MAPK using a genetic approach with p38-deficient donor cells.

## AURORA KINASE A

**Acute GvHD:** TCR activation and co-stimulation *via* CD28 stimulates mTOR and aurora kinase family signaling in T-cells, thereby activating substrates needed for T-cell proliferation (198). Transcriptomic analysis found increased expression of aurora kinase A in aGvHD patients and mice after allo-HCT. Inhibition of aurora kinase A prolonged survival and reduced GvHD scores in mice, however the animals could not be fully rescued (199). It was hypothesized that inhibition of aurora kinase A and JAK2 could be combined, as JAK2 activation by inflammatory stimuli leads to STAT3 activation and effector cytokine production (200). Betts et al. treated human MLRs with the JAK2 inhibitor TG101348, aurora kinase A inhibitor alisertib or a combination. T-cell proliferation was synergistically suppressed by the combination. Compounds targeting both kinases, had similar effects. Both kinase inhibitors reduced $CD4^+$ and $CD8^+$ T-cell activation *in vitro*, whereas the combination had the strongest impact. Also, T-cells produced less IL-17 and IFNγ upon kinase blockade. Interestingly, the induction of Tregs was significantly reduced upon kinase inhibition, but Tregs had potent inhibitory functions, mainly based on upregulation of surface CD39. Dual kinase inhibition caused higher ATP consumption, confirming the functionality of CD39 upregulation. Based on *in vitro* findings, the investigators applied JAK2 and aurora kinase A inhibitors in a xenogeneic aGvHD model. The survival of the recipients was significantly increased upon combination treatment, accompanied by lower GvHD scores. A novel dual inhibitor showed even stronger effects without impairing GvL activity. Based on these findings, more research is needed to further elucidate the role of aurora kinases in GvHD and their potency as novel targetable molecule (103).

## CONCLUSIONS AND OUTLOOK

In summary, the presented pre-clinical and clinical investigations reveal that kinase inhibition offers a huge variety of novel approaches to target both, acute and chronic GvHD. Since GvHD involves a vast number of pathways and signaling cascades for immune cell activation, proliferation and effector cytokine production, as well as in inflammatory signaling and fibrosis, the disease is hard to treat with only a single compound. Moreover, acute and chronic GvHD are completely different diseases and involve distinct pathways, making the treatment even more complicated. However, the involvement of different pathways is also a chance, presenting a variety of kinases as potentially targetable candidates. The great number of studies indicates how intensively researched kinases, the major signal transducers in immune cell signaling, are in the context of GvHD after allo-HCT. Although some compounds are already far in clinical trials, many questions remain unanswered, making deeper research necessary to unravel the potential of kinase inhibition in GvHD. Preclinical and clinical analyses revealed that treatment with single compounds has therapeutic limitations, as GvHD is mediated by a variety of pathways and not only by a single activated molecule. Novel therapeutic strategies should involve the combination of kinase inhibitors with other therapeutic interventions, as it is already investigated for JAK1 and JAK2 inhibitors with ECP. Moreover, different kinase inhibitors could be combined to potentiate the efficacy of the individual kinase inhibitors to enhance treatment success. JAK1 and/or JAK2 inhibitors, leading to reduced inflammatory cytokine production and decreased APC activation, could be combined with ROCK2 inhibition which potently blocks fibrosis and TFH formation in cGvHD. Also, ROCK2 blockade results in higher Treg numbers. Combinations of Syk, PI3K/mTOR and ITK/BTK could be beneficial as these kinases mediate early B- and T-cell activation. It might also be possible to apply kinase inhibitors sequentially to first hit a target being activated in aGvHD and target a second kinase to reduce the risk of aGvHD transforming into chronic GvHD. However, these strategies are still speculative and combination therapies should be carefully tested in preclinical models. Since many kinases are not only involved in disease but also in physiological signaling processes, the application and combination of inhibitors has the risk of potential side effects. Kinases such as MEK and MAPK are active in most cells and tissues and severe side effects are likely upon inhibition. Treatment-related adverse events have to be considered and highly specific molecules need to be designed to reduce off-target effects. So far, some kinases are only investigated pre-clinically, but should be tested clinically if effects are seen. After dose-finding studies, the inhibitors might first be investigated in SR-GvHD. Based on performance, the inhibitors might also be evaluated as first-line or prophylaxis therapies for patients after allo-HCT. However, transformation of these compounds into clinical trials is speculative as they first need to be critically evaluated in pre-clinical model. Taken together, the approval and clinical application of some kinase inhibitors, including the JAK1/2 inhibitor Ruxolitinib and the ROCK2 inhibitor belumosudil, is promising to better control acute and chronic GvHD after allo-HCT, thereby making allo-HCT available for more patients with severe hematological malignancies.

## AUTHOR CONTRIBUTIONS

LMB and RZ developed the overall concept of this article. LMB collected and reviewed literature, discussed the studies and wrote the manuscript. RZ helped to write the manuscript and critically

revised the manuscript. All authors contributed to the article and approved the submitted version.

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# Anti-Thymocyte Globulin Treatment Augments 1,25-Dihydroxyvitamin D3 Serum Levels in Patients Undergoing Hematopoietic Stem Cell Transplantation

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Application of anti-thymocyte globulin (ATG) is a widely used strategy for the prevention of graft-versus-host disease (GvHD). As vitamin D3 serum levels are also discussed to affect hematopoietic stem cell transplantation (HSCT) outcome and GvHD development, we analysed a possible interplay between ATG treatment and serum levels of 25-hydroxyvitamin D3 and 1,25-dihydroxyvitamin D3 in 4 HSCT cohorts with different vitamin D3 supplementation. ATG is significantly associated with higher serum level of 1,25-dihydroxyvitamin D3 around HSCT (day -2 to 7, peri-transplant), however only in patients with adequate levels of its precursor 25-hydroxyvitamin D3. ATG exposure had no impact on overall survival in patients supplemented with high dose vitamin D3, but was associated with higher risk of one-year treatment-related mortality (log rank test p=0.041) in patients with no/low vitamin D3 supplementation. However, the difference failed to reach significance applying a Cox-model regression without and with adjustment for baseline risk factors (unadjusted P=0,058, adjusted p=0,139). To shed some light on underlying mechanisms, we investigated the impact of ATG on 1,25-Dihydroxyvitamin D3 production by human dendritic cells (DCs) *in vitro*. ATG increased gene expression of *CYP27B1*, the enzyme responsible for the conversion of 25-hydroxyvitamin D3 into 1,25-dihydroxyvitamin D3, which was accompanied by higher 1,25-dihydroxyvitamin D3 levels in ATG-treated DC culture supernatants. Our data demonstrate a cooperative effect of 25-hydroxyvitamin D3 and ATG in the regulation of 1,25-dihydroxyvitamin D3 production. This finding may be of importance in the context of HSCT, where early high levels of 1,25-dihydroxyvitamin D3 levels have been shown to be predictive for lower transplant related mortality and suggest that vitamin D3 supplementation may especially be important in patients receiving ATG for GvHD prophylaxis.

**Keywords: HSCT, vitamin D3, ATG, GvHD, dendritic cells**

## INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (aHSCT) is a potentially curative treatment modality for different haematological malignancies, however its success is limited due to associated complications such as infections or graft- versus-host disease (GvHD) which still remain the major cause of non-relapse mortality in aHSCT (1).

Anti-thymocyte globulin (ATG), a polyclonal antibody-mixture raised in rabbits against the human lymphoblastic T cell line Jurkat, is an immunosuppressive drug used for GvHD prophylaxis during conditioning. A meta-analysis reported that ATG use is associated with reduced risk of acute and chronic GVHD. However, the efficacy of ATG in the prevention of GvHD in HSCT patients may depend on many confounding variables such as dose, type and timing of its administration and transplantation characteristics (2). Administration of ATG results in T-cell depletion, which is presumed to represent the main mechanism by which ATG reduces the incidence of GvHD (3).

Vitamin D3 insufficiency, usually defined as serum levels of its stable metabolite 25-hydroxyvitamin D3, is a common finding (not only) in HSCT patients, however, the clinical impact of vitamin D3 deficiency is controversially discussed. A recent meta-analysis described no statistically significant association between 25-hydroxyvitamin D3 deficiency and neither acute nor chronic GVHD (4). In contrast, Radujkovic and colleagues showed in two cohorts (n=890 patients) that pre-transplant 25-hydroxyvitamin D3 deficiency (<20 ng/mL) was associated with a higher risk of relapse in patients allografted for myeloid malignancies (5).

Although the impact of 25-hydroxyvitamin D3 is extensively studied in the context of HSCT and GvHD, little is known about 1,25-dihydroxyvitamin D3, the active form of vitamin D3. It is textbook knowledge that 1,25-dihydroxyvitamin D3 is produced in the kidney, while its precursor vitamin D3 is present in high amounts in the skin or in the gut, where it is taken up from the diet. Both organs represent immunological "barriers" and target organs for GvHD development and local extrarenal production of 1,25-dihydroxyvitamin D3 might be of importance for immune regulation. We previously showed that myeloid cells such as dendritic cells (DC) express the vitamin D3-1–hydroxylase CYP27B1 and thus are able to convert 25-hydroxyvitamin D3 to bioactive 1,25-dihydroxyvitamin D3 (6–8) which may support local immunosuppression in skin and gut (9).

Analysing a discovery cohort consisting of 143 HSCT patients, our data highlight peri-transplant (day −2 to 7), 1,25-dihydroxyvitamin D3 levels, but not the commonly monitored 25-hydroxyvitamin D3 levels, as potent predictor of 1-year transplant-related mortality (TRM). This finding was further confirmed by analysing three additional cohorts, consisting altogether of 365 patients and suggest to monitor both vitamin D3 metabolites in HSCT patients (10).

Cyclosporine, Dexamethasone and ATG are known immunosuppressive treatments used for GvHD prophylaxis. Here we analysed whether these typical transplant-related drugs influence 1,25-dihydroxyvitamin D3 production by monocyte- derived dendritic cells (DCs) *in vitro*. Our data indicate that besides its classical role for T cell depletion, ATG may also impact the immune response in patients *via* modulation of the vitamin D3 metabolism.

## MATERIAL AND METHODS

### Patient Characteristics

Four cohorts with a total of n=508 patients were included in our analyses. The discovery cohort consisted of n=143 patients at the Regensburg University Medical Center with HSCT between May 2012 and February 2015. All HSCT recipients in the discovery cohort received oral high dose vitamin D3 supplementation (Vigantol oil, 20.000 IU/ml, Merck) consisting of a 50,000 IU-dose upon admission to hospital (d-16 to d-6) followed by daily administration of 10,000 IU. To monitor 25-hydroxyvitamin D3 and 1,25-dihydroxyvitamin D3 serum levels, blood was drawn repeatedly during inpatient stay, and thereafter during routine outpatient visits. Measurements were performed at least once during the indicated time intervals. When multiple measurements were available for the same time interval, the median value was used. Serum calcium levels were assessed twice a week. The described supplementation dose was maintained until patients reached 25-hydroxyvitamin-D3 serum levels of 150–200 nmol/L with subsequent dose adjustment to avoid 25-hydroxyvitamin-D3 levels >150–200 nmol/L

Our replication stage consisted of three patient cohorts from various clinical settings to replicate our initial findings and to generalize for other clinical settings: (I) HSCT patients from Regensburg transplanted between March 2015 and May 2017 receiving the same high-dose vitamin D3 supplementation as the discovery cohort, (II) HSCT patients from Regensburg transplanted between March 2011 and February 2013 receiving vitamin D3 supplementation at lower dose (ranging from 1000 to 5000 IU/d, Vigantoletten, 1000 IU/tablet, Merck), (III) HSCT patients from the University Medical Center Hamburg-Eppendorf transplanted between February 2012 and August 2014 receiving no vitamin D3 supplementation. Eligibility and exclusion criteria for all three replication groups were the same as in the discovery cohort, yielding n=115, n=107 and n=143 patients in replication cohort I, II, and III, respectively. All cohorts analysed in the present study were already described in detail in (10).

### Isolation of Monocytes

Monocytes were isolated with the approval of local ethic committee, from healthy donors as described previously (11). All human participants gave written informed consent.

### Culture of Monocyte-Derived DCs

For DC differentiation, 0.5 to 1.0 × $10^6$ monocytes/mL were cultured for five days in RPMI medium supplemented with 10% fetal calf serum (PAN Biotech), IL-4 (144 U/mL), and granulocyte macrophage colony-stimulating factor (GM-CSF,

225 U/mL; both from PeproTech, Hamburg, Germany). iDCs were then stimulated with 100 ng/mL LPS (from Salmonella abortus equi S-form, Enzo Life Sciences, Lörrach, Germany), 25-hydroxyvitamin D3 (Sigma-Aldrich) (25 nM to 100 nM) and or ATG (Fresenius, Bad Homburg, Germany) (now named Grafalon®, distributed by Neovii Biotech, Gräfelfing, Germany) (100 µg/mL), Cyclosporine A (Sandimmun, Novartis), Dexamethasone (Jenapharm, mibe GmbH), IgG isotype control (polyclonal, rabbit, Molecular Innovations, Novi, MI, USA) (100 µg/mL) for 48 hours.

## Preparation of RNA, Reverse Transcription, and Quantitative Real-Time PCR

Total cellular RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA concentration was measured using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Schwerte, Germany). Reverse transcription was performed with 500 ng RNA in a total volume of 20 µl using an M-MLV Reverse Transcriptase from Promega (Mannheim, Germany). For reverse transcription-quantitative real-time PCR, 1 µl cDNA, 0.5 µl of primers (10 µM), and 5 µl QuantiFast SYBR Green PCR Kit (Qiagen) in a total of 10 µl were applied, using the Mastercycler Ep Realplex (Eppendorf). Primer sequences (all from Eurofins MWG Operon, Ebersberg, Germany) were as follows (-5′-3′); (F-Forward; R- Reverse): CYP27A1_F: GTCTGGCTACCTGCACT TCTTACTG CYP27A1_R: TCAGGGTCCTTTGAGAGGTGGT CYP27B1_F: TGGCAGAGCTTGAATTGCAAATGG; CYP27B1_R: ACTGTAGGTTGATGCTCCTTTCAGGT; 18S_F: ACCGATTGGATGGTTTAGTGAG; 18S_R: CCTACGGAAACCTTGTTACGAC

## Preparation of Whole Cell Lysates and Western Blotting

Whole cell lysates were prepared using RIPA buffer (Sigma-Aldrich) and quantified with the Qubit Protein Assay Kit (Thermo Fisher Scientific). Samples were separated by 12% SDS-PAGE and transferred to PVDF membranes, blocked with 5% milk (Sucofin) in TBS buffer with 0.1% Tween for 1 h, and incubated with primary antibodies overnight: anti-VDR ((D2K6W) Cell Signaling Technology, Danvers, MA, USA, clone or anti-actin (Sigma Aldrich). Membranes were incubated with secondary antibodies for 1 h at RT and analyzed using the chemiluminescence system Fusion Pulse 6 (Vilber Lourmat).

## Vitamin D Measurement

Vitamin D levels were measured directly after serum withdrawal or from sera stored at -80°C by the Department of Clinical Chemistry, University Medical Center of Regensburg. From May 2012 to October 2014, 25-hydroxyvitamin D3 serum levels were analysed by a chemiluminescence immunoassay according to the manufacturer's instructions (Immunodiagnostic systems, Frankfurt am Main, Germany). After attesting for comparability, from November 2014 on, 25-hydroxyvitamin D3 serum levels were analyzed by liquid chromatography high-resolution tandem mass spectrometry as described in (12). 1,25-dihydroxyvitamin D3concentrations were measured using a radioimmunoassay according to the manufacturer's instructions (Immunodiagnostic systems, Frankfurt am Main, Germany) by the Department of Clinical Chemistry, University Medical Center of Regensburg. For the replication cohorts and DC supernatants, 1,25-dihydroxyvitamin D3levels were measured by the MVZ Laborzentrum Ettlingen, Germany, using the same method as described above.

## Statistical Analysis

Statistics were calculated using GraphPad Prism, Version 8 (La Jolla, CA, USA) or using SPSS Statistics version 26 (IBM, Armonk, USA). Comparisons between groups were performed using the appropriate statistical methods depending on Gaussian distributions, number of groups and variables. A value of $p < 0.05$ was considered statistically significant. To examine one-year-survival, a Kaplan-Meier curve was generated to visualize differences between patients receiving ATG with patients that did not receive ATG. The log rank test and an unadjusted Cox-model were used to test for difference in survival of patients.

# RESULTS

## Higher 1,25-Dihydroxyvitamin D3 but Not 25-Hydroxyvitamin D3 Serum Levels in Patients With ATG Therapy

Vitamin D3 metabolites were already shown to be implicated in the outcome of HSCT. Although the impact of 25-hydroxyvitamin D3 was debated in several studies (13, 14), the active metabolite 1,25-dihydroxyvitamin D3 did not gather the same attention. In a recent study, our group demonstrated that high 1,25-dihydroxyvitamin D3 levels are predictive for treatment related mortality. Using the same cohorts of patients and in order to understand the factors that influence vitamin D3 levels, we evaluated the influence of typical transplant-related factors on 1,25-dihydroxyvitamin D3 and 25-hydroxyvitamin D3 levels. The discovery cohort consisted of n=143 patients at the Regensburg University Medical Center and received oral high-dose vitamin D3 supplementation. The replication cohort I consisted of patients with oral high-dose vitamin D3 supplementation similar to the discovery cohort. The other two replication cohorts received either vitamin D3 supplementation at lower dose, or no vitamin D3 supplementation. 25-hydroxyvitamin D3 levels were followed over time and are presented in association with ATG treatment (**Figure 1A**). 25-hydroxyvitamin D3 levels increased over time but no impact of ATG administration was observed. When analyzing 1,25-dihydroxyvitamin D3 levels (**Figure 1B**) we observed that ATG administration led to higher 1,25-dihydroxyvitamin D3 in the time frame around transplantation (days -2 to 7). We subsequently analyzed the effect of ATG administration in our three additional cohorts (Replication cohorts I, II and III) for the time interval around transplantation (days -2 to 7).

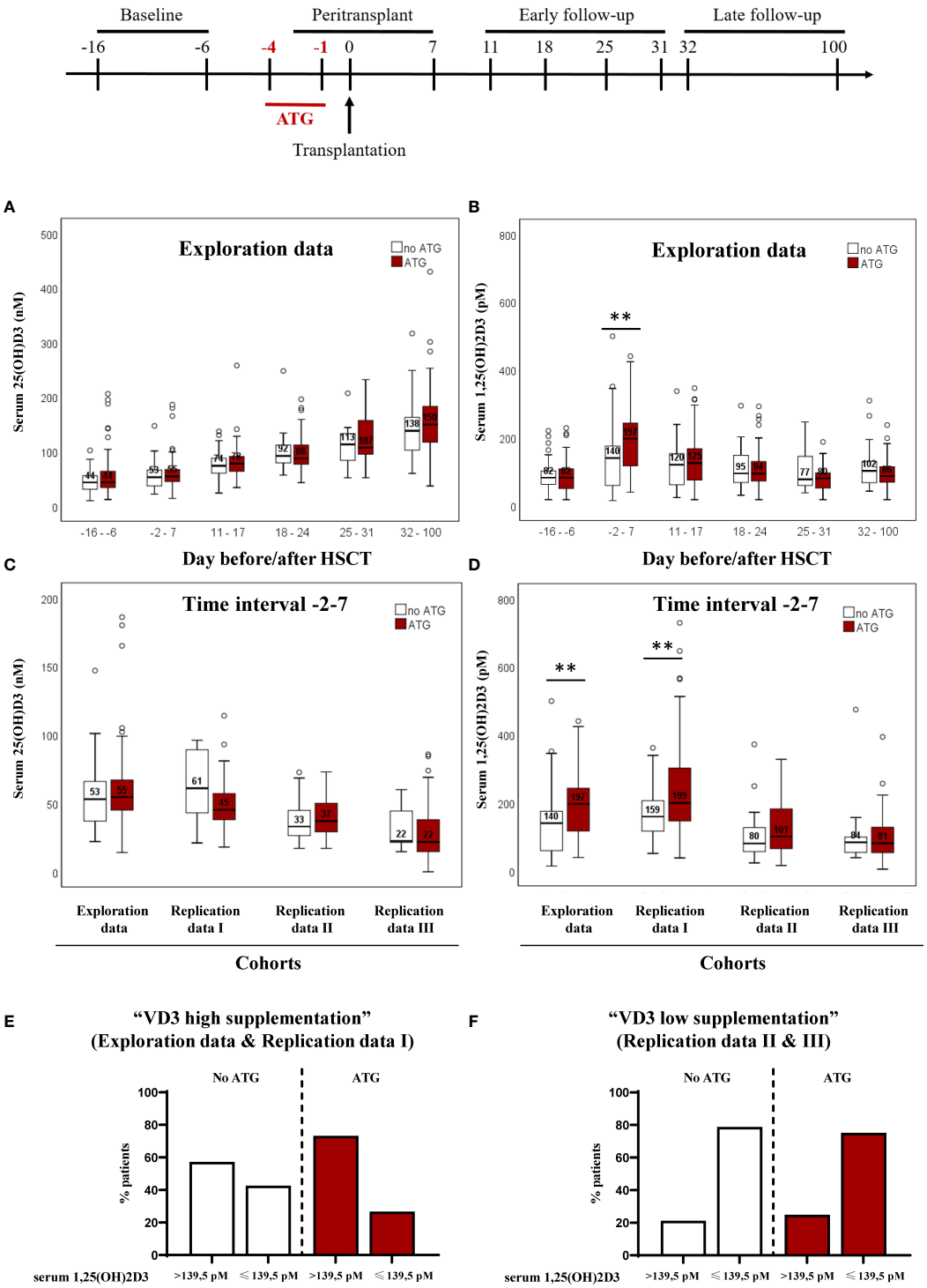


**FIGURE 1** | Impact of ATG treatment on 25(OH)D3 and 1,25(OH)2D3 circulating serum levels of patients undergoing hematopoietic stem cell transplantation Panel **(A)** depicts the median serum 25-hydroxyvitamin D3 levels followed over time in association with ATG treatment for the discovery cohort. In panel **(B)**, the median serum 1,25(OH)2D3 level is presented. Panel **(C)** depicts the median serum 25(OH)D3 levels from days -2 to 7 around HSCT of patients receiving ATG and patients that did not received ATG. In panel **(D)**, the median serum 1,25(OH)2D3 level is presented. Numbers indicate median serum 25(OH)D3 and 1,25(OH)2D3 values, error bars indicate 95% confidence interval. Statistical analysis was performed with Mann-Whitney-U test, two-tailed (**p < 0.01). In **(E, F)** the distribution of patients below and above the calculated cut-off is shown, in relation to ATG treatment for the cohorts with high and low/no vitamin D3 supplementation, respectively.

ATG application showed no association with 25-hydroxyvitamin D3 levels measured at peri-transplant time (**Figure 1C**) for all of the 4 cohorts. Although no impact of ATG administration was observed, basal and ATG-stimulated 25-hydroxyvitamin D3 levels differed between the cohorts and only the exploration cohort and the replication cohort I presented 25-hydroxyvitamin D3 levels higher than 50 nM, a concentration usually considered adequate. In contrast to 25-hydroxyvitamin D3, patients receiving ATG therapy revealed higher 1,25-dihydroxyvitamin D3 compared with patients that did not receive ATG (**Figure 1D**) in the exploration cohort and the replication cohort I, where basal 25-hydroxyvitamin D3 levels met the criteria for vitamin D sufficiency [see Peter et al. (10)].

Our previous study highlighted peritransplant 1,25-dihydroxyvitamin D3levels, but not the commonly monitored 25-hydroxyvitamin D3 levels, as potent predictor of 1-year transplant-related mortality (TRM) independent of severe aGvHD. The optimal threshold for 1,25-dihydroxyvitamin D3 to identify patients at risk was 139.5 pM (10). Next we analyzed patient distribution below and above this cut-off in relation to ATG administration (**Figures 1E, F**). In the two cohorts (exploration combined with replication I) with high vitamin D3 supplementation, 73,3% of ATG-treated patients had 1,25-dihydroxyvitamin D3 levels above the cut-off. Without ATG treatment 57,3% showed 1,25 levels above the threshold (**Figure 1E**). In contrast, patients with low/no supplementation (replication II combined with replication III), only 24,9% with ATG and 21,2% without ATG treatment reached higher levels than the defined cut-off (**Figure 1F**). These results demonstrate the importance of sufficient 25-hydroxyvitamin D3 serum levels for the described positive effect of ATG on 1,25-dihydroxyvitamin D3 production.

## ATG Treatment Increases 1,25-Dihydroxyvitamin D3 Production by Human Monocyte-Derived Dendritic Cells (DCs) *In Vitro*

To shed some light on the interplay between ATG administration and 1,25-dihydroxyvitamin D3 production and to confirm a dependence on sufficient 25-hydroxyvitamin D3 levels, we incubated human monocyte-derived dendritic cells with ATG in the presence of different concentrations of the vitamin D3 precursor 25-hydroxyvitamin D3. As observed in **Figure 2A**, spontaneous conversion of 25-hydroxyvitamin D3 into 1,25-dihydroxyvitamin D3 was very low and not dependent on the provided 25-hydroxyvitamin D3 level in the culture medium. However, ATG treatment of monocyte-derived DCs

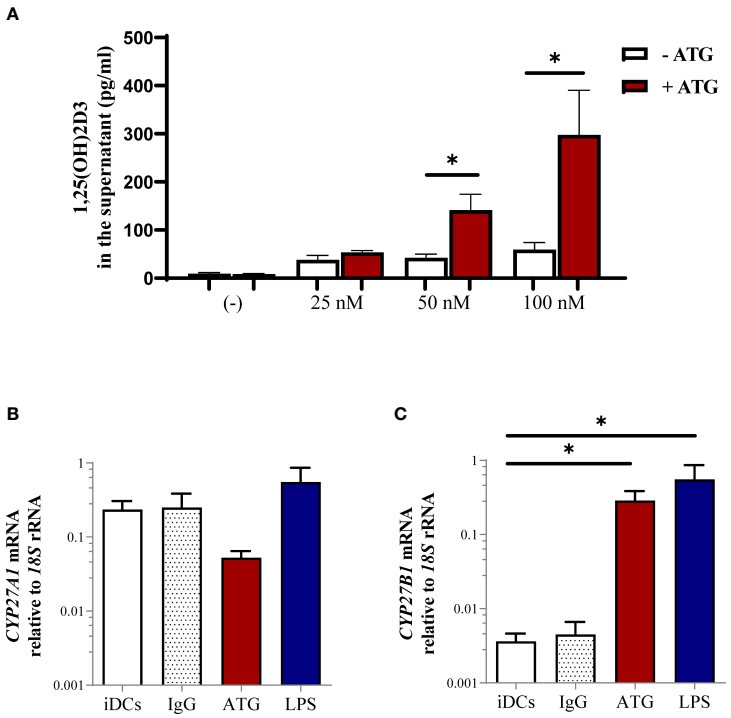


**FIGURE 2** | Impact of ATG treatment on production of 1,25(OH)2D3 by human monocyte-derived dendritic cells (DCs). **(A)** Human monocyte-derived DCs were stimulated for 24 h in the presence of different 25(OH)D3 concentrations in the presence or absence of ATG (100 µg/ml). After 24 h the production of 1,25(OH)2D3 was analyzed by means of chemiluminescence immunoassay. Data are means ± SEM (n≥ 3). Statistical analysis was performed using Mann-Whitney-U test, two-tailed. Panel **(B)** depicts CYP27A1 and **(C)** shows CYP27B1 mRNA expression of DCs analyzed by means of quantitative real-time PCR relative to 18S rRNA expression. Data are means ± SEM (n = 4). Statistical analysis was performed using Kruskal-Wallis and Dunn's posthoc test [*p ≤ 0.05, tested versus immature DC (control)].

increased the conversion of 25-hydroxyvitamin D3 to 1,25-dihydroxyvitamin D3 but only when DCs were cultured in the presence of 25-hydroxyvitamin D3 precursor levels in concentrations superior to 50 nM.

To investigate the possible mechanism by which ATG leads to the enhanced production of the active metabolite 1,25-dihydroxyvitamin D3, we incubated human monocyte-derived DCs with ATG-Fresenius and analyzed enzymes related to vitamin D3 metabolism. CYP27A1, the enzyme involved in the production of 25-hydroxyvitamin D3 from vitamin D3 and CYP27B1, the enzyme converting 25-hydroxyvitamin D3 to the active 1,25-dihydroxyvitamin D3 metabolite. We detected a trend towards a downregulation of *CYP27A1* mRNA upon treatment with ATG, indicating that ATG could potentially also modulate the first step of vitamin D3 metabolism (**Figure 2B**). In contrast, the expression of *CYP27B1* (**Figure 2C**) was significantly upregulated by ATG treatment. Lipopolysaccharide was used as a positive control and led to a comparable induction of *CYP27B1* mRNA (**Figure 2C**). Accordingly, LPS and ATG incubation induced comparable amounts of 1,25-dihydroxyvitamin D3 in supernatants of DC cultures (data not shown). This would be in line with a causal model that ATG therapy stimulates directly 1,25-dihydroxyvitamin D3 production in (myeloid) cells and in turn leads to increased 1,25-dihydroxyvitamin D3 serum levels after ATG administration.

## ATG but Not Other Immunosuppressive Drugs Such as Cyclosporine or Dexamethasone Induce 1,25-Dihydroxyvitamin D3 Production and VDR Expression in DCs

Due to our observation that ATG treatment upregulates the expression of *CYP27B1* resulting in higher 1,25-dihydroxyvitamin D3 production, we sought to investigate whether other immunosuppressive drugs such as cyclosporine and dexamethasone would have the same effect. Cyclosporine A, a calcineurin inhibitor, is a key immunosuppressive drug administered in the transplantation setting and it has been demonstrated that cyclosporine increases 1,25-dihydroxyvitamin D3 levels in rats (15). The interplay between Dexamethasone, and 1,25-dihydroxyvitamin D3 was already demonstrated in different studies (16–18). Hidalgo et al. (16) demonstrated the synergism between 1,25-dihydroxyvitamin D3 and dexamethasone in inhibiting cell growth and increasing vitamin D receptor (VDR) expression. Therefore, we incubated human monocyte-derived DCs with 25-hydroxyvitamin D3combined with either ATG, dexamethasone or cyclosporine. As observed in **Figure 3A**, only ATG treatment led to a significant increase in 1,25-dihydroxyvitamin D3 production by DCs. Interestingly, the expression of the VDR (**Figure 3B**) was also upregulated in myeloid cells after ATG treatment which could probably lead to a paracrine immune suppression of DCs by the produced 1,25-dihydroxyvitamin D3.

## ATG Induces a Tolerogenic Phenotype in DCs

Tolerogenic dendritic cells are characterized by the expression of co-inhibitory molecules such as Ig-like transcripts (ILTs), low expression of costimulatory molecules (e.g. CD83, CD86) and no or little production of proinflammatory cytokines such as IL-12 or IL-6 (19, 20). As 1,25-dihydroxyvitamin D3 is known to induce tolerogenic DCs (21), we next analyzed co-inhibitory molecules on DCs after exposure to ATG. Surface expression of ILT-3 but not ILT-2 or PD-L2 was significantly upregulated in the presence of ATG indicating that paracrine or autocrine 1,25-dihydroxyvitamin D3 production may support a more tolerogenic phenotype of DC (**Figures 4A, B**). In line with our previous work (22), ATG also induced a semi-mature phenotype with reduced expression of CD80, CD83 and CD86 in comparison to LPS and did not induce IL-12 nor IL-6 secretion (data not shown) (22). Combined application of 25-hydroxyvitamin D3 and ATG or did not alter cytokine secretion. In addition, no significant amounts of IL-12 or IL-6 were detected after cyclosporine and dexamethasone treatment (data not shown).

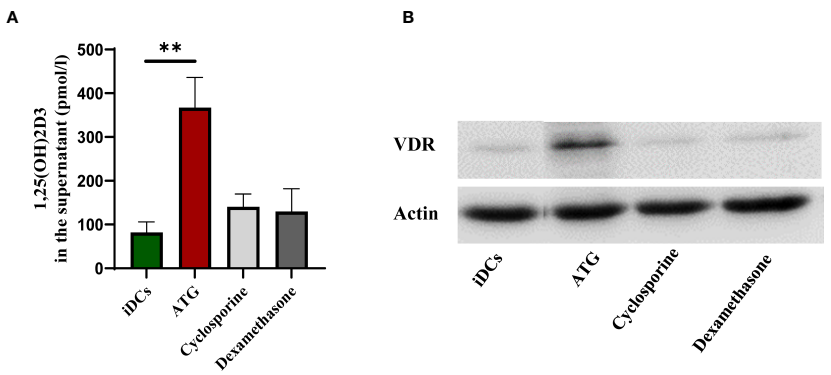


**FIGURE 3** | Comparative effect of ATG, Cyclosporine and Dexamethasone on 1,25(OH)2D3 production and VDR expression. Human monocyte-derived DCs were stimulated for 24 h in the presence 100 nM 25(OH)D3 with or without ATG (100 µg/ml), Cyclosporine A (1,7 µM) and Dexamethasone (100 nM). Panel **(A)** After 24 h the production of 1,25(OH)2D3 was analyzed by means of chemiluminescence immunoassay. Data are means ± SEM (n = 3). Statistical analysis was performed using Kruskal-Wallis and Dunn's posthoc test [**p ≤ 0.01, tested versus immature DC (control)]. In **(B)**, VDR expression was analysed by western blot. A representative donor is shown.

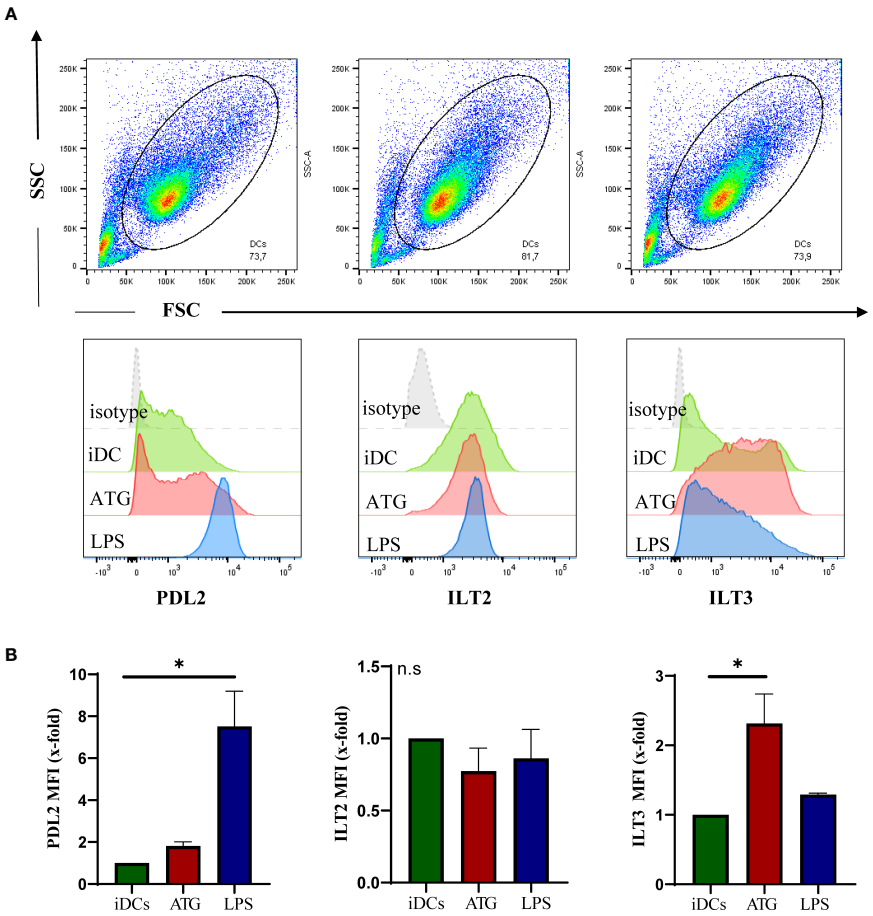


**FIGURE 4** | ATG effect on DC phenotype. Human monocyte-derived DCs were stimulated for 48 h with or without ATG (100 µg/ml) or LPS. Afterwards, cells were harvested, washed and stained by means of flow cytometry. Shown is a representative dot blot of the respective cell populations and overlaid histograms of the isotype (grey) **(A)**. Median fluorescence intensities are summarized in **(B)**. Data are means ± SEM (n = 3). Statistical analysis was performed using Kruskal-Wallis posthoc test (*p ≤ 0.05, tested versus immature DC (control); n.s. not significant).

## ATG Has No Impact on Patients Survival Supplemented With High Dose Vitamin D3

To clarify a potential impact of interplay between vitamin D3 supplementation and ATG we performed survival analyses. In a combined analysis of patients from our discovery cohort and replication cohort I (n= 255, all with high dose vitamin D3 supplementation) ATG had no impact on patient survival (**Figure 5A**). Surprisingly, in patients with low/no vitamin D3 supplementation (n= 250, combined replication cohort II and III), the ATG exposure group had a diminished survival compared to patients without ATG treatment (Log Rank analysis p=0.041) (**Figure 5B**). Conversely, after applying a Cox-model regression without and with adjustment for baseline risk factors such as sex and age, the difference failed to reach significance (unadjusted p=0.058, adjusted p=0.139). As shown in **Table 1**, the only factor that remained significant after adjustment was the age of the patients. Based on these data, we suggest that especially patients treated with ATG should be supplemented with high dose vitamin D3.

## DISCUSSION

Although several studies addressed the importance of 25-hydroxyvitamin D3 serum levels in the context of HSCT, little is known about the active 1,25-dihydroxyvitamin D3 metabolite. We previously demonstrated that peri-transplant 1,25-dihydroxyvitamin D3 levels were the only significant independent predictor for one-year-survival besides severe aGvHD (10).

Nevertheless, why patients with high 1,25-dihydroxyvitamin-D3 levels are at less risk for TRM than those with low levels remains unknown. It is possible that high 1,25-dihydroxyvitamin D3 levels around transplantation result in an immunosuppressive environment as 1,25-dihydroxyvitamin D3 has been shown e.g. to tolerize dendritic cells and induce regulatory T cells (23–25). Similar results have been reported for ATG (22). In light of our results, one could speculate that part of ATG immune regulatory effect is based on the induction of 1,25-dihydroxyvitamin D3, as patients with ATG had a peak of 1,25-dihydroxyvitamin-D3 around transplantation.

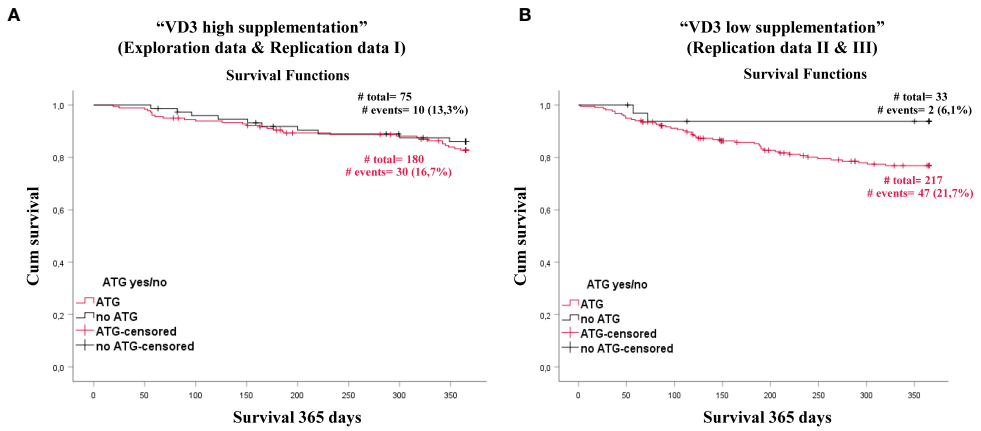


**FIGURE 5** | ATG treatment impact on treatment related survival on vitamin D high or vitamin D low supplemented patients. Shown is a Kaplan- Meier curve comparing patients that received ATG (red) with patients that did not received ATG treatment (black) for patients receiving high vitamin D3 supplementation **(A)** and patients receiving no or low vitamin D3 supplementation **(B)**.

Our *in vitro* experiments demonstrate the importance of adequate 25-hydroxyvitamin-D3 levels for the ATG effect. In concentrations below 50 nM, no positive effect on 1,25-dihydroxyvitamin D3 production was observed upon ATG treatment. This result goes in line with the observation made in patients: 1,25-dihydroxyvitamin D3 levels were only enhanced by ATG treatment in the discovery and replication cohort 1. These were the cohorts where the 25-hydroxyvitamin D3 serum levels met the sufficiency criteria (above 50 nM).

A more detailed *in vitro* analysis demonstrated that ATG treatment of human monocyte-derived dendritic cells lead to an upregulation of *CYP27B1* at mRNA level and resulted in a higher 1,25-dihydroxyvitamin D3 amount in culture supernatants. Since this enzyme is responsible for the conversion of 25-hydroxyvitamin-D3 to 1,25-dihydroxyvitamin D3, this result provides a possible mechanism by which ATG could be involved in 1,25-dihydroxyvitamin-D3 synthesis. As other immunosuppressive drugs such as cyclosporine and dexamethasone had no effect on 1,25-dihydroxyvitamin D3 production, the ATG effect seems to be specific and not related to its immunosuppressive activity. However, a study by Lee et al. described that cyclosporine treatment can also result in increased 1,25-dihydroxyvitamin D3 serum levels in mice (26), accompanied by higher renal CYP27B1 expression and decreased VDR expression. These results are not contradictory to ours as 1,25-dihydroxyvitamin-D3 levels seem to depend on classical renal production of 1,25-dihydroxyvitamin D3 in this study, whereas our *in vitro* data with human cyclosporine-treated DCs indicate that extrarenal production of 1,25-dihydroxyvitamin

**TABLE 1** | Association between ATG treatment, high/low vitamin D3 supplementation and TRM.

| Model | Cox Regression | | | | | |
|---|---|---|---|---|---|---|
| | High vitamin D supplementation n = 255 | | | Low vitamin D supplementation n = 250 | | |
| | #at risk/TRM | Exp(B)/HR (95% CI) | P value | #at risk/TRM | Exp(B)/HR (95% CI) | P value |
| **Unadjusted** | 255/40 | | | 250/49 | | |
| ATG yes/no | | 1.253 (0.612; 2.563) | 0.537 | | 3.929 (0.954; 16.181) | 0.058 |
| **Adjusted I** | 255/40 | | | 250/49 | | |
| ATG yes/no | | 1.136 (0.555; 2.325) | 0.728 | | 2.953 (0.703; 12.412) | 0.139 |
| age | | 1.096 (1.047; 1.147) | **0.000078** | | 1.033 (1.007; 1.059) | **0.012** |
| **Adjusted II** | 255/40 | | | 250/49 | | |
| ATG yes/no | | 1.074 (0.522; 2.212) | 0.846 | | 2.956 (0.705; 12.398) | 0.138 |
| age | | 1.096 (1.048; 1.147) | **0.000064** | | 1.032 (1.007; 1.059) | **0.013** |
| sex | | 1.531 (0.819; 2.860) | 0.182 | | 0.734 (0.389; 1.385) | 0.340 |
| **Adjusted III** | 235/38 | | | 250/49 | | |
| ATG yes/no | | 1.032 (0.496; 2.148) | 0.932 | | 3.012 (0.718; 12.635) | 0.132 |
| age | | 1.099 (1.047; 1.153) | **0.000120** | | 1.034 (1.006; 1.062) | **0.015** |
| sex | | 1.640 (0.863; 3.120) | 0.131 | | 0.718 (0.379; 1.359) | 0.309 |
| tumor stage | | 0.959 (0.500; 1.841) | 0.900 | | 0.831 (0.461; 1.496) | 0.537 |
| conditioning | | 2.091 (0.499; 8.769) | 0.313 | | 0.815 (0.364; 1.824) | 0.619 |

*Shown are the results from Cox proportional hazard models for the association between ATG treatment in patients with high or low vitamin D supplementation with TRM without and with adjustment for risk factors. P values ≤0,05 are marked in bold.*

D3 by myeloid cells is not regulated by cyclosporine. Further studies in patients should clarify the role of cyclosporine on 1,25-dihydroxyvitamin D3 production *in vivo*.

Dexamethasone is a glucocorticoid drug used to treat a number of conditions such as rheumatic problems, allergies and asthma (27, 28). The synergistic effect between dexamethasone and Vitamin D3 was already demonstrated in several studies (16, 29–31). In our hands, dexamethasone did not increase 1,25-dihydroxyvitamin D3 levels nor modify the VDR expression in human DCs. In a study conducted by Akeno et al. (32) the authors investigated the effects of dexamethasone on CYP27B1 and CYP24A1. In line with our data, they did not detect an increase in 1,25-dihydroxyvitamin D3 levels in mice fed either a normal diet nor in mice fed a calcium and vitamin D3- deficient diet.

Hidalgo et al. (16) also studied the interaction between 1,25-dihydroxyvitamin D3 and dexamethasone. The authors found that treatment of murine squamous cell carcinoma cells with dexamethasone in combination with 1,25-dihydroxyvitamin D3 lead to the upregulation of VDR. In contrast, dexamethasone alone was not able to upregulate VDR expression. In our setting, dexamethasone was used in combination with 25-hydroxyvitamin D3, the precursor of 1,25-dihydroxyvitamin D3. Although human DCs express CYP27B1 and have the capacity to produce 1,25-dihydroxyvitamin D3 in a autocrine fashion, it can well be that the amount of 1,25-dihydroxyvitamin-D3 produced by the cells was not enough to generate a sufficient level to synergize with dexamethasone and upregulate the VDR. Taken together, our data provide a possible mechanism by which ATG leads to an increased 1,25-dihydroxyvitamin D3 serum levels in patients with sufficient 25-hydroxyvitamin D3. Together, both compounds can synergize in creating a "tolerogenic" environment that may help to maintain the immune balance.

ATG is normally used in patients receiving a graft from unrelated donors (33) but Kröger et al. (34) demonstrated that ATG-treatment can also significantly lower the incidence of chronic GvHD (cGvHD) after allogeneic transplantation from HLA-identical siblings. Admiraal et al. showed that optimum ATG dosing is associated with higher survival probability (35).

There are different ATG preparations, raised either in horses or rabbit by immunization with human thymocytes or with Jurkat T-cell line (36). The antigens targeted by the different preparations have been well described and differ in specificity. While rabbit ATG (thymoglobulin, (THG) also known as ATG-Genzyme, Sanofi Genzyme) is derived from rabbits immunized with human thymocytes, ATG Fresenius is produced in rabbits immunized with the Jurkat T-cell line (37). It has been demonstrated that although THG contains antibodies against several T- and B- cell antigens such as CD2, CD3, CD4, CD8, CD11, CD20, CD25, human leukocyte antigen DR (HLA-DR) and HLA class I, ATG-Fresenius is infrequently active against CD3, CD4 and HLA-DR but instead targets CD28, CD29, CD45, CD98 and CD147. Furthermore, competitive binding assays have demonstrated that THG has stronger reactivity than ATG-Fresenius to activated peripheral blood mononuclear cells. Based on this, the doses of ATG given depend on the chosen ATG preparation and are typically higher for ATG-Fresenius than for THG. Consequently, is seems safe to assume that ATG-Fresenius and THG differ in their immunosuppressive activity. Whether the impact on vitamin D3 metabolism depends on the given ATG preparation remains unclear and more studies should be conducted to clarify this interesting aspect.

In our analysis, we did not find any significant association of ATG with survival after applying Cox analyses. This could be due to the fact that our analysis incorporated the use of ATG in a dichotomous fashion and not in a concentration-dependent manner. However, it has to be emphasized that not all patients receiving ATG had high 1,25-dihydroxyvitamin D3 levels and that high levels also occurred in patients without ATG treatment. Therefore, other factors are obviously involved in the regulation of peri-transplant 1,25-dihydroxyvitamin D3 levels besides ATG and sufficient serum 25-hydroxyvitamin D3 levels that allow conversion to 1,25-dihydroxyvitamin D3.

Further studies should identify other factors that determine early 1,25-dihydroxyvitamin D3 serum levels in HSCT patients. Alternatively, supplementation with 1,25-dihydroxyvitamin D3 analogs could be an option to increase early 1,25-dihydroxyvitamin D3 levels to support patient survival.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee University Hospital Regensburg. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

Conceptualization, KP and MK. Methodology, CM, AP, GS, and LW. Investigation, CM, NB, SD, SG, and KH. Writing—original draft preparation, CM. Writing—review and editing, MK, KR, IH, KS, WH, and EH. Supervision, MK. Funding acquisition, MK and KP. All authors have read and agreed to the published version of the manuscript.

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REVIEW
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# T Helper Cell Lineage-Defining Transcription Factors: Potent Targets for Specific GVHD Therapy?

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Allogenic hematopoietic stem cell transplantation (allo-HSCT) represents a potent and potentially curative treatment for many hematopoietic malignancies and hematologic disorders in adults and children. The donor-derived immunity, elicited by the stem cell transplant, can prevent disease relapse but is also responsible for the induction of graft-versus-host disease (GVHD). The pathophysiology of acute GVHD is not completely understood yet. In general, acute GVHD is driven by the inflammatory and cytotoxic effect of alloreactive donor T cells. Since several experimental approaches indicate that CD4 T cells play an important role in initiation and progression of acute GVHD, the contribution of the different CD4 T helper (Th) cell subtypes in the pathomechanism and regulation of the disease is a central point of current research. Th lineages derive from naïve CD4 T cell progenitors and lineage commitment is initiated by the surrounding cytokine milieu and subsequent changes in the transcription factor (TF) profile. Each T cell subtype has its own effector characteristics, immunologic function, and lineage specific cytokine profile, leading to the association with different immune responses and diseases. Acute GVHD is thought to be mainly driven by the Th1/Th17 axis, whereas Treg cells are attributed to attenuate GVHD effects. As the differentiation of each Th subset highly depends on the specific composition of activating and repressing TFs, these present a potent target to alter the Th cell landscape towards a GVHD-ameliorating direction, e.g. by inhibiting Th1 and Th17 differentiation. The finding, that targeting of Th1 and Th17 differentiation appears more effective for GVHD-prevention than a strategy to inhibit Th1 and Th17 cytokines supports this concept. In this review, we shed light on the current advances of potent TF inhibitors to alter Th cell differentiation and consecutively attenuate GVHD. We will focus especially on preclinical studies and outcomes of TF inhibition in murine GVHD models. Finally, we will point out the possible impact of a Th cell subset-specific immune modulation in context of GVHD.

**Keywords: aGVHD, GvL, CD4+ T cells, T helper cell differentiation, transcription factors**

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## INTRODUCTION

Allogenic hematopoietic stem cell transplantation (allo-HSCT) represents a potent and potentially curative treatment for many hematopoietic malignancies and hematologic disorders in adults and children. Its success is based on a complete replacement of the patients' immune system by a myeloablative conditioning regimen and reconstitution from a healthy donor stem cell graft. The donor derived immunity can prevent disease relapse but is also responsible for the main complication of allo-HSCT, the graft-versus-host disease (GVHD).

Acute GVHD pathophysiology is not completely understood yet. In general, acute GVHD is driven by the inflammatory effect of donor T cells upon antigen-recognition of allo-antigens presented by host antigen-presenting cells (APCs). The subsequent alloreactive cytotoxicity of activated T cells effects the GVHD target organs (gastrointestinal tract, skin, and liver) and leads to an amplification loop of inflammation there.

Since several experimental approaches indicate that CD4 T cells play a key role in initiation and progression of acute GVHD, the contribution of the different CD4 T helper (Th) cell subtypes in the pathomechanism and regulation of the disease is a central point of current research. Acute GVHD is thought to be driven by a Th1/Th17/Th22 axis whereas Treg cells are attributed to attenuate GVHD effects. As the differentiation of each Th subset highly depends on the specific composition of activating and repressing transcription factors (TFs), these present a potent target to alter the Th cell landscape towards a GVHD-ameliorating direction by the inhibition of Th1 and Th17 differentiation. In this review, we discuss the current advances of potent of potent TF inhibitors in order to alter Th cell differentiation and attenuate GVHD in murine models.

## T HELPER CELL SUBSETS AND DIFFERENTIATION

To date, eight different T helper cell types are known: Th1, Th2, Th9, Th10, Th17, Th22, follicular T helper cells (Tfh) and regulatory T cells (Treg). Th cell lineages derive from naïve CD4 T cell progenitors and lineage commitment is initiated by the surrounding cytokine milieu and subsequent changes in the TF profile. Each T cell subtype has its own effector characteristics, immunologic function, and lineage specific cytokine profile, leading to the association with different immune responses (**Figure 1**). In this review we will focus on the Th1, Th2, Th17 and Treg subsets, the involved TFs in their differentiation as well as their impact on GVHD.

### Th1 and Th2 Cells

In 1986, Mosmann and colleagues identified two distinct classes of CD4 helper T cells, which exhibited a different cytokine profile. The differentiation in these two classes, later called Th1 and Th2, was found to be stable and deterministic (1). Th1 cells differentiate in the presence of interferon (IFN)-γ and interleukin

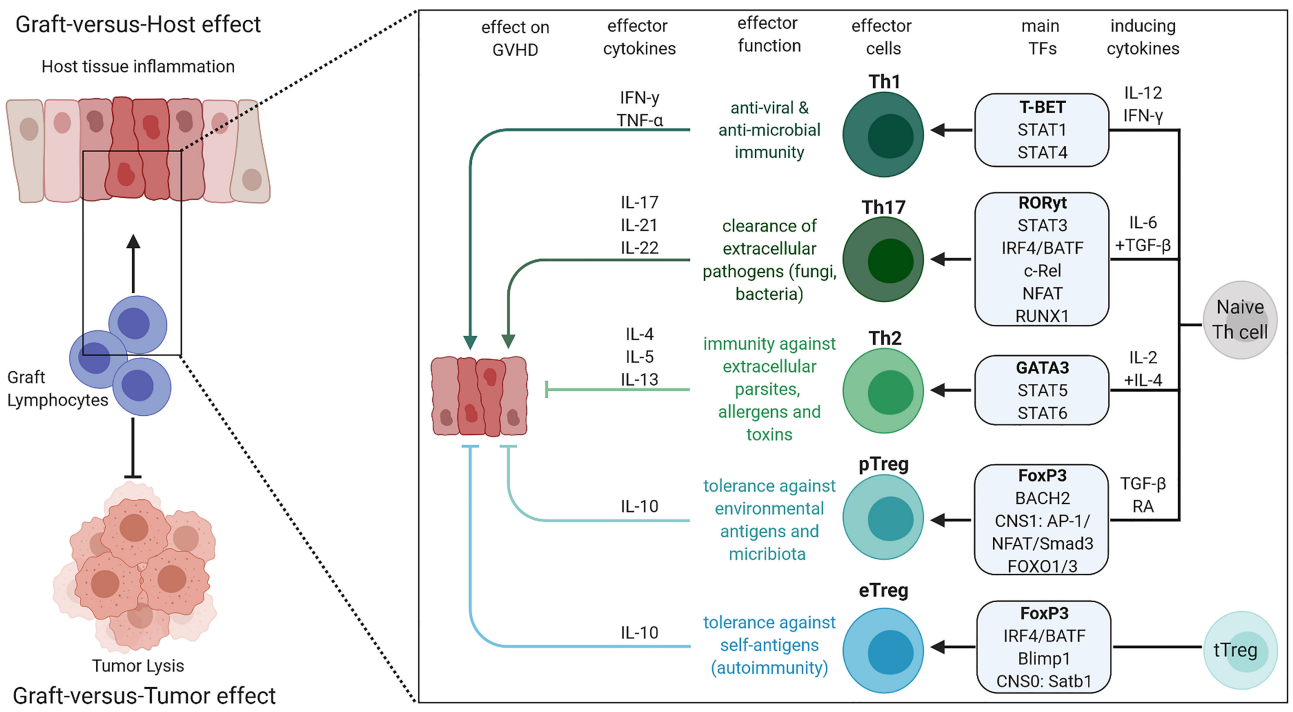


**FIGURE 1** | Overview of Th1, Th2, Th17, eTreg and pTreg differentiation, their effector cytokines, roles in the immune system and impact on GVHD. The figure was created with BioRender.com.

(IL)-12 to produce their effector cytokine IFN-γ, which has high relevance for anti-viral and anti-microbial immunity (1–3). In contrast, Th2 cells differentiate in the presence of IL-2 and IL-4 and produce the effector cytokines IL-4, IL-5 and IL-13, which play an important role in the immune response against extracellular parasites, bacteria, allergens, and toxins (1, 4–7).

In the early 2000s, Szabo et al. discovered that the underlying mechanisms of the Th1/Th2 paradigm was the initiation or repression of distinct genetic programs upon activation, directed by Th lineage specific master transcription factors (8). With this regard, T-bet was described as a master regulator of Th1 cells, which induces IFN-γ production by activating Th1 genetic programs while repressing Th2 responses (8–12). A few years earlier, GATA3 was characterized as a master transcriptional regulator for Th2 cell differentiation (13, 14).

Further studies on the mechanisms, how T-bet and GATA3 mediate Th1 and Th2 differentiation respectively, revealed the mutual inhibition of the two master TFs and the involvement of many more interacting molecules and relevant signaling cascades (15–17). T–bet was found to be induced by Signal Transducers and Activators of Transcription Protein 1 (STAT1) and IFN-γ during T cell activation and to induce STAT1 dependent processes as the induction of Interleukin-12 receptor subunit beta-2 (IL-12Rβ2) (12). Additionally, STAT4 which is activated by IL-12, and the downstream acting TF c-Rel were identified as crucial transcriptional regulators for Th1 differentiation (15, 18–22). In contrast, Th2 differentiation was associated with IL-2 dependent STAT5 signaling and IL-4 dependent STAT6 signaling pathways, which induce the expression of GATA3, IL-2 receptor (R)a and IL-4Ra as well as IL-2 and IL-4 effector cytokines (23–25).

## Th17 Cells

Th17 cells were first described as an independent and distinct Th subset from Th1 and Th2 cells, producing IL-17a, IL-17f, IL-22 and IL-21 as effector cytokines in the early 2000s (26, 27). First thought that IL-23 was the inducing cytokine for Th17 cells, three groups simultaneously discovered that TGF-β and IL-6 induced Th17 differentiation (28–30), while IL-1β and tumor necrosis factor (TNF)-α can potentiate Th17 differentiation in presence of IL-6 and transforming growth factor beta TGF-β (31–33). The leading role of Th17 cells is the clearance of extracellular pathogens as fungi and bacteria but dysregulation of Th17 effects is associated with various autoimmune diseases like inflammatory bowel disease, rheumatic arthritis, experimental autoimmune encephalomyelitis (EAE), and multiple sclerosis [reviewed by Tesmer at al. (34)].

In 2006, the transcription factor retinoid acid-related orphan receptor (ROR)γt (*Rorc*) was identified to be uniquely expressed in mouse Th17 cells and necessary for Th17 differentiation (35). Besides, RORγt as master transcription factor, several other TFs were described to be crucial for Th17 differentiation and function. STAT3 was found to drive the transcription of Th17 specific genes like *Il17a, Il17f and Il23r* (36, 37) and to suppress TGF–β-induced forkhead box protein 3 (FoxP3) expression and hence regulatory T cell differentiation (28). Interferon Regulatory Factor 4 (IRF4) and Basic Leucin Zipper ATF-Like Transcription Factor (BATF) also play a significant role in Th17 differentiation by initiating the transcription of Th17 defining genes as *Il17, Il21, Il23r* and *Rorc* (38–40). IRF4 was also shown to physically interact with RORγt (38) and STAT3 (36). The transcriptional regulators c-Rel, p65, nuclear factor of activated T cells (NFAT) c2 and Runt-related transcription factor 1 (RUNX1) were found to directly regulate RORγt by binding to the *Rorc* promotor (41–43). Additionally, RUNX1 and hypoxia-inducible factor 1-alpha (HIF1α) physically interact with RORγt to potentiate or co-activate IL-17a expression (44, 45). Importantly, T-bet and GATA3 can inhibit RUNX1 expression or binding to DNA respectively which inhibits Th17 differentiation.

## Regulatory T Cells

In contrast to the immune effector function of Th1, Th2 and Th17 cells, regulatory T cells (Tregs) are characterized by their immunosuppressive capacity and are essential mediators of self-tolerance. Already in the 1960's it was found that a thymus-derived cell population was mediating immunologic tolerance. Later on, Sakaguchi and colleagues characterized these cells further as CD4 T cells expressing the IL-2 receptor alpha chain (CD25) (46). However, it was unclear if Tregs represent a distinct cell line until the Treg master transcription factor FoxP3 was discovered (47, 48). The importance of FoxP3 for Treg differentiation is well displayed by scurfy mice which lack FoxP3 expression and suffer from inflammatory autoimmune syndrome (47, 49). Additionally, the maintenance of FoxP3 expression after differentiation is essential for Treg immunosuppressive function (50, 51). Besides the expression of FoxP3, the development, maintenance, and function of Tregs also highly depends on TGF-β (52–55).

In contrast to other effector T helper cells, regulatory T cells differentiate in the thymus [thymus-derived Tregs (tTregs)], dependent on high affinity interaction with complexes of MHC-II and tissue-restricted self-antigens and IL-2 receptor signaling (56). However, Tregs can also differentiate from naïve T cells in the periphery (pTregs), sometimes also referred to as induced Tregs (iTregs). These cells are induced by non-self-antigens and are most likely mediating immunologic tolerance of environmental antigens and commensal microbiota [reviewed by Lee et al. (57)].

pTreg and tTreg differentiation are implemented on a transcriptional level by different involvement of regulatory elements, four conserved non-coding sequences (CNSs) of the *Foxp3* locus (58). CNS1, regulated by the transcription factors Activator protein 1 (AP-1), NFAT, Small mothers against decapentaplegic homolog 3 (Smad3) and Forkhead box O (FOXO) (57, 59–62), was found to be necessary for pTreg but not for tTreg development, while CNS0, regulated by special AT-rich sequence-binding protein-1 (Satb1) is essential for tTreg generation (63). CNS2, which is regulated by the TF Protein C-ets-1 (Ets-1), cAMP response element-binding protein (CREB), RUNX, STAT5, NFAT and c-Rel is important for stable FoxP3 expression during differentiation and functionality of Tregs (58, 64–69). In contrast, CNS3 which is regulated by c-Rel and FOXO TFs influences Treg cell numbers (57, 58, 62). Additionally, gaining the full suppressor function of tTregs as effector Tregs

(eTregs) depends on the transcription factors IRF4 and B lymphocyte-induced maturation protein-1 (Blimp-1), which drive the expression of the immunosuppressive cytokine IL-10 (70), while BACH2, a transcriptional repressor, inhibits the genomic binding of IRF4, and mediates pTreg differentiation and maintenance (71).

## Cross Regulation of T Helper Cell Differentiation

In general, Th differentiation fates are tightly connected and regulated. For example, Th1 and Th2 cells inhibit the development of each other by their lineage specific transcription factors (72, 73) and by the cytokines IFN-γ and IL-4 (74, 75). The differentiation of Th17 cells can also be inhibited by these cytokines and by the expression of the TF T-bet (26, 72, 76). However, fully differentiated Th17 cells are resistant to IFN-γ and IL-4 inhibiting effects *in vitro* (27).

The T cell fate of Th17 and Tregs is connected especially tightly, as many factors were shown to have a reciprocal role in Th17 and Treg development. One reason for that is the response of both cell types to TGF-β signaling. However, IL-6 regulates the TGF-β response between both subsets, since it is necessary for Th17 induction, while it inhibits TGF-β induced Treg differentiation (77, 78). On the contrary, Tregs can lose their FoxP3 expression and reprogram to IL-17 secreting cells in the absence of TGF-β (79). Many more regulatory pathways also show that contradictive role in Th17 and Treg development. The activation of mammalian target of rapamycin (mTOR) *via* HIF1α promotes Th17 differentiation, whereas the lack of HIF1α and mTOR drives Treg development (80). As another example, inhibiting protein kinase CK2 was shown to block Th17 development and promotes Treg cell differentiation in mice due to a defect in STAT3 phosphorylation (81). FoxP3 itself, can also associate with RORγt and inhibit RORγt activity (82). GATA3 was shown to play a vital role in Treg differentiation as it binds to CNS2 elements and represses the development of a Th17 phenotype (83). A similar effect was reported on IL-2 which promotes Treg development and inhibits Th17 differentiation dependent on STAT5 (84). In general the opposing regulation of genes like *Il17* through STAT3 and STAT5 seems to be a crucial mediator of reciprocal Th17/Treg differentiation (85).

# THE IMPACT OF TH CELLS IN GVHD

The role of different Th-subsets in GVHD-induction and progression has been investigated with various approaches and GVHD-mouse models for quite a long time. First focusing on Th-subset specific cytokines, these studies mostly provided paradoxical results regarding the role of Th1, Th2 and Th17 cells in GVHD. However, following experiments with Th-defining TF knockout T cells improved the understanding of Th-subset involvement in GVHD. Overall, Th2 and Tregs are subsets with a protective effect on GVHD while Th1 and Th17 cells promote GVHD induction and progression. The following paragraph will give more detailed information on the various approaches revealing the role of the different Th subsets in aGVHD.

## Protective T Helper Cell Subsets in GVHD

First studies examined the effect of Th2-associated cytokines in GVHD in the 1990's. Injection of the Th2 inducing cytokines IL-2 and IL-4 led to Th2 polarization and protected recipient mice from GVHD-associated mortality (86, 87). Comparable results were observed after the administration of Granulocyte-macrophage colony-stimulating factor (GM-CSF) to recipient mice, which induced IL-4 production and inhibited GVHD-development (88). Another study confirmed the GVHD-attenuating effect of IL-4 produced by Th2 cells, also having a skewing effect on Th2 cytokines (89). On the contrary, other studies showed that the absence or neutralization of IL-4 ameliorated GVHD, implying a detrimental role of Th2 cells (90, 91). However, these contradicting results regarding the role of IL-4 in GVHD may be based on different mouse models and experimental settings (92). Despite the overall protective role of IL-4 secreting Th2 cells in GVHD, the location of these cells might define their pathogenic relevance, as they were associated with pathophysiological changes in the lung, but not in colon, liver, and skin during GVHD (93). IL-13, another Th2 effector cytokine, was also shown to have an ameliorating effect on GVHD. Although one study correlated IL-13 levels with GVHD severity in patients (94), transplantation experiments of IL-13$^{-/-}$ cells in an established mouse GVHD model resulted in increased mortality and decreased Th2 cytokine levels but elevated serum levels of TNF-α, a critical mediator of GVHD, in these mice (95). Further studies showed the counteracting role of IL-13 to TNF-α production and its augmenting role in IL-4 and IL-5 secretion following allo-bone marrow transplantation (96), supporting the notion that IL-13 has a protective function in GVHD. In general, the transplantation of Th2 cells to recipient mice showed beneficial effects on GVHD-survival (97, 98) and an alteration of the Th1/Th2 balance towards the Th2 cells leading to increased IL-4 levels and attenuated GVHD (98–100). Ultimately, a study investigating IL-4, IL-5, IL-9, and IL-13 quadruple cytokine-deficient T cells in a well-established mouse model demonstrated that combined Th2 cytokine deficiency resulted in enhanced T cell proliferation, higher TNF-α, IL-2, IFN-γ and IL-17a serum levels and overall aggravated GVHD (101).

A few further experiments on Th2 defining TFs gained similar results in GVHD models. Atorvastatin (AT) treatment was shown to modulate Th1/Th2 differentiation by inhibiting the production of the isoprenoid derivates farnesly-pyrophosphate (PP) and geranylgranyl-PP, of the mevalonate pathway. Inhibition of these isoprenoid derivates combined by AT or individually by a farnesyltransferase inhibitor (FTI) or a geranylgeranyltransferase inhibitor (GGTI) respectively, resulted in an upregulation of GATA3, and in case of AT and FTI treatment also an downregulation of T-bet expression in antigen-primed T cells (102). GGTI and FTIs were also shown to have ameliorating CD4 T cell specific effects on GVHD while sparing CD8 T cells in their capacity to mediate GVL and protect from viral infections (103). AT treatment also induced Th2 polarization and cytokine secretion and

inhibited GVHD development by partially acting through STAT6, a transcription factor essential for Th2 differentiation in response to IL-4 and IL-13 (23, 104, 105). STAT6 was shown to be required for Th2 involved NKT-cell mediated GVHD prophylaxis (106). Additionally, transplanted STAT6$^{-/-}$ T cells, unable to differentiate to Th2 cells skewed towards Th1 cells and mediated aGVHD with major involvement of the colon. On the contrary, STAT4$^{-/-}$ T cells, which predominantly differentiated to Th2 cells, showed less severe signs of GVHD but later involvement in skin pathology (107). STAT5, another critical TF in Th2 differentiation, was found to have a dual role in Th2 and Treg differentiation in GVHD, as overexpression of STAT5 led to increased Treg numbers and attenuated GVHD, while in the absence of Tregs, anti-inflammatory Th2-cytokines increased (108).

Tregs are the second CD4 T cell subset which play a protective role in GVHD. In general, responsible for immune homeostasis and balanced immune responses, Tregs have an outstanding role in controlling GVHD development. First experiments on CD4+ CD25 + Tregs in GVHD showed that depletion of these cells aggravated GVHD, while supplementation with Tregs had the contrary effect (109, 110). The capacity of Tregs to attenuate GVHD was associated with their expansion-inhibiting effect on allogeneic T cells in the early phase of GVHD (111). The beneficial effect of Tregs in GVHD prevention was demonstrated in fully allogeneic, haploidentical and xenograft mouse models (111–116). FoxP3 expression was additionally found to negatively correlate with GVHD severity in patients (117). Importantly, murine, and human Tregs attenuate allogeneic T cell reactions, without impeding the graft-versus-leukemia (GVL) effect (111, 118–121). The use of *in vitro* induced Tregs (iTregs) as a GVHD therapeutic revealed effective protection in the early phase after transplantation but unstable FoxP3 expression over time led to aggravation of GVHD, making this approach less promising as initially thought (122, 123). However, additional combinatory induction with IL-2 and rapamycin was shown to stabilize FoxP3 expression in these cells (113, 124), which enabled the first successful application of iTregs as GVHD-prophylactic therapy to humans (125).

Despite this broad outline of Treg research in GVHD, many recent publications have already summarized the role of Tregs in GVHD in a detailed way (126–128), for which reason we will not go into further details at this point.

Overall, Th2 and Tregs were shown to have an attenuating and protective role in GVHD. While Th2 cells can still mediate local GVHD-associated pathophysiological changes in the lung, Tregs are an overall protective cell population in GVHD having crucial homeostatic functions, which are tightly regulated in balance with other Th-subsets.

## Detrimental Th Subsets in GVHD

Contradicting first studies on Th1 cytokines in the 1990's led to unconclusive results regarding the role of Th1 cells in GVHD. The main Th1-inducing and -associated cytokines IL-2, IFN-γ and IL-12 were found to ameliorate GVHD in several early studies which indicated a protective function of Th1 in GVHD (129–132). However, other groups showed, that increased IFN-γ levels in serum correlated with GVHD severity (133, 134) and that IFN-γ was critical for tissue pathology during GVHD (97). Besides the beneficial role of IFN-γ in the induction of GVHD-associated effects in the lung (135), it was shown to have adverse effect in acute GVHD pathology in the GI tract (93, 136–138). Additionally, the effect of IFN-γ in GVHD was found to be dependent on the irradiation regimen used (139). Overall, the reciprocal effect of IFN-γ in GVHD seems to be highly dependent on conditioning, location, timing, and the stage of allo-immune response [reviewed by Lu and Waller, (140)].

Similar to IFN-γ, contradicting findings were made, when Th17-associated cytokines were assessed in GVHD mouse models. One study suggested a protective role of IL-17a in GVHD, as IL-17$^{-/-}$ T cells accelerated GVHD while the systemic administration of IL-17a and the neutralization of IFN-γ prevented this effect (141). Other studies reported improved transplantation outcomes when IL-17a$^{-/-}$ T cells were used (142) and severe GVHD induction when *in vitro* generated IL-17+ cells were infused (143). Altogether, these studies indicated that, similar to IFN-γ, the role of IL-17 in GVHD is dependent on timing and conditioning regimen. IL-17 probably contributes to early development of GVHD but is dispensable for overall GVHD induction (142). Neutralization of the IL-17 inducing cytokine TGF-β was shown to increase aGVHD severity indicating an ameliorating effect of Th17 cells in GVHD (144). However, TGF-β is also relevant for the differentiation of Tregs which are GVHD protective, and its absence resulted in enhanced Th1 cell proliferation indicating Th17-independent mechanisms that lead to enhanced GVHD (144). IL-6, which induces TGF-β dependent differentiation of Th17 but not Treg cells, was found to play a relevant role in GVHD induction, as blocking of the IL-6R led to reduced GVHD pathology and Th1/Th17 cells in GVHD target organs, while absolute numbers of Tregs increased (145). However, another study showed that short-term administration of IL-6 could not confirm these beneficial effects (146). Differences between the design of these two studies indicate that the effect of IL-6 on GVHD development is dependent on conditioning, the used model, and the duration of therapy.

TNF-α, another Th1-associated cytokine, which also promotes Th17 differentiation, was shown to drive GVHD pathophysiology on several stages. For example, TNF-α is responsible for early intestinal GVHD-related toxicity (147) and TNF-receptor 1 (TNFR1) levels strongly correlate with GVHD severity (148). Additionally, the attenuating effect of TNF-blocking therapy in GVHD underlines the detrimental role of TNF-α in GVHD (149). Similarly, inhibiting the Th17 effector cytokines IL-21 and IL-23 decreased GVHD severity in various mouse models (150–152).

However, cytokines can derive from different cell types and do not necessarily represent the involvement of respective Th cell subsets. Hence, experiments examining subset defining TF knock-out CD4 T cells shed more light on the relevance of different Th cell subsets in GVHD and identified Th1 and Th17 cells as the relevant subsets promoting GVHD.

First TF-knock-out experiments to investigate the influence of Th1 differentiation on GVHD were performed with STAT6$^{-/-}$ and STAT4$^{-/-}$ T cells. STAT6$^{-/-}$ T cells are unable to differentiate to Th2 cells but instead show enhanced Th1 responses (23, 104, 153). In contrast, lack of STAT4 in T cells leads to impaired Th1

differentiation (154). Nikolic and colleagues investigated STAT6$^{-/-}$ and STAT4$^{-/-}$ T cells in a GVHD mouse model and found that STAT6$^{-/-}$ T cell recipients showed an earlier and more severe course of GVHD with severe inflammation in the GI tract in comparison to STAT4$^{-/-}$ T cell recipients, while only the latter group displayed severe skin disorders (107). These results indicate the detrimental role of Th1 cells in GVHD mainly affecting the GI tract but not liver and skin. Recipients of T cells with STAT1 KO, another critical STAT TF for Th1 development, also resulted in the attenuation of GVHD and increased Treg expansion (155). Comparable results were obtained in GVHD experiments with c-Rel KO T cells, which showed a dramatically reduced ability to induce GVHD in various mouse models, defects in Th1 and Th17 differentiation, enhanced Treg differentiation and a preserved Graft-versus-leukemia (GvL) effect (156).

Experiments with T cells deficient for T-bet and RORγt, the master TFs of Th1 and Th17 cells respectively also confirmed that these subsets are the most involved Th cells in GVHD induction and development. Transplanted T-bet deficient T cells screwed to Th2, Th17 and Treg subsets and led to attenuated GVHD, especially in the gut (157). The absence of RORγt in T cells only had little impact and RORγt was dispensable to induce GVHD development in two independent studies (157, 158), while one study reported an attenuated effect on GVHD if both isoforms, RORγ and RORγt were absent in CD4 transplanted T cells due to KO of the entire *Rorc* locus (159). However, T-bet and RORγt double KO T cells, which showed a defective differentiation of Th1 and Th17, and increased Th2 and Treg cells, induced less GVHD than T-bet KO T cells alone. This finding suggests a synergistic effect of RORγt-induced Th17 cells on Th1-mediated GVHD induction (157).

In addition, TFs linked to the reciprocal differentiation to Th17 versus Treg cells were also found to play a crucial role in attenuation of GVHD. For example, recipients of T cells with a STAT3 deficiency, a TF crucial for Th17 development, showed attenuated GVHD development and increased numbers of pTregs (160).

Summarized, Th1 and Th17 cells synergistically are the main Th subsets driving GVHD, especially with detrimental pathological effects on the GI tract. Blocking of Th1 and Th17-transcription factors was found to be a more effective strategy to prevent GVHD, than blocking Th1 and Th17-involved cytokines. Hence, the use of specific TF-blocking agents is a promising strategy to treat GVHD in the future. The following paragraph will give deeper insights in recent literature reporting the effect of a variety of Th-subset specific TF blocking agents in murine GVHD models.

# POTENTIAL STRATEGIES TO TARGET TRANSCRIPTION FACTORS OF T HELPER CELL DEVELOPMENT IN GVHD

As described earlier, experiment with various Th-differentiation associated TF knock-out T cells revealed efficient attenuation of GVHD in different transplantation models. Hence, inhibition of these TFs by target-specific inhibitory agents offers a potent strategy for GVHD prophylaxis and therapy.

Several commonly used GVHD therapeutics also rely on the modulation of TF expression or activity. Calcineurin inhibitors (CNIs) like Cyclosporine A (CyA) of tacrolimus (FK506) for example block TCR-proximal signaling by inhibition of NFAT. Even though CNIs remain standard of care for GVHD prevention, they also interfere with the valuable GVL-effect by impairing donor immunity and disruption of Treg function and survival (161–165). Combinatorial therapy with mTor inhibitors like Rapamycin (Sirolimus) and/or low-dose IL-2 administration have already shown to improve Treg reconstitution after allo-hematopoietic cell transplantation (164, 166–170).

The following section will provide more detailed information on various new therapeutic agents, divided by substance classes, which have been successfully evaluated in GVHD alone or in combination with standard of care therapeutics in the recent years (**Tables 1**, **2**). Importantly, if not indicated by the respective clinical trial number or mentioned explicitly, this paragraph mostly summarizes results from pre-clinical GVHD mouse models and not from studies in patients. Most of them rely on the strategy of targeting TFs that mediate the reciprocal effect between Th17/Th1 and Treg differentiation, hence inducing a homeostatic effect by skewing CD4 T cell differentiation towards Tregs while preserving the GVL effect.

## Epigenetic Modulators

Epigenetic modulation of transcription is a promising approach to indirectly inhibit TF expression. The acetylation of histones, regulated by histone acetyl transferases (HATs) and histone deacetylases (HDACs), is an epigenetic mark, which influences chromatin structure and ultimately gene expression. The use of HDACs and HDAC-inhibitors (HDACi) can modulate this balance and subsequently alter gene expression.

Valproic acid (VPA), a HDACi of the short-chain fatty acid category, was shown to indirectly decrease STAT5 phosphorylation and dampen T-bet expression in NK cells (202). In a mouse model, the administration of VPA attenuated aGVHD by downregulation of Th1 and Th17 cells (171). This effect was associated with a direct inhibition of Akt (171), a kinase which promotes Th1, Th17 and Tfh but inhibits Treg development by activation of mTOR which in turn induces T-bet, RORγt and HIF1α and inhibits FOXO1-dependent FoxP3 transcription (203–205). Importantly, the GVL-effect was preserved during VAP therapy.

Another HDACi, which showed promising effects on GVHD in preclinical models is Ex-527, a Sirtuin-1 (Sirt-1) inhibitor. Sirt-1 represses AP-1, Smad3 and FOXO-transcription factors which regulate pTreg differentiation *via* the CNS1 regulatory element (206–209) and was identified as a direct negative regulator of FoxP3 (210). Pre-clinical experiments in a murine GVHD mode showed that Sirt1$^{-/-}$ T cells were impaired in inducing aGVHD and showed an enhanced pTreg differentiation in which FoxP3 stability was increased. Ex-527 administration induced comparable effects while preserving GVL effects (172). Stabilization of FoxP3 expression by Ex-527 had already been reported earlier and associated to increased Treg suppressive function (210, 211). Another Sirt-1 inhibitor, Sirtinol, was found to decrease RORγt and IL-17A expression in CD4 T cells *in vitro* and to screw Th17/Treg differentiation towards Tregs, leading to a prolonging allograft

**TABLE 1** | Summary of pre-clinical studies on Th-differentiation targeting TF inhibitors.

| Class | Sub-class | Compound | Target | murine aGVHD model | Effect GVHD | Effect GVL | Effect Th differentiation | Reference |
|---|---|---|---|---|---|---|---|---|
| Epigenetic regulators | HDACi (short-chain fatty acid) | Valproic acid (VPA) | AKT | MHC mismatch model: BL/6→BALB/c | ameliorated | preserved | Th1 ↓ Th17 ↓ | (171) |
| | HDACi (sirtuin inhibitor) | Ex-527 | Sirt-1 | MHC mismatch model:BL/6→BALB/c | ameliorated | preserved | Th1 ↓ Th17↓ Tregs ↑ | (172) |
| | HDACi (hydroxamic acid) | Vorinostat (SAHA) | STAT3/STAT1 | MHC mismatch model:BL/6→BALB/c | ameliorated | - | - | (173) |
| | HDACi(cyclic peptides) | Romidepsin | STAT3/STAT1 | MLR | ameliorated | - | - | (174, 175) |
| Kinase inhibitors | JAK/STAT Inhibitors | Ruxolitinib (INCB018424) | JAK1/2 | MHC mismatch model:B6→BALB/c | ameliorated | preserved | Th1 ↓ Th17↓ Tregs↑ | (176, 177) |
| | JAK/STAT Inhibitors | Itacitinib (INCB039110) | JAK1 | MHC mismatch model:B6→BALB/c; xenogeneic model | ameliorated | preserved | Tregs ↑ | (178–180) |
| | JAK/STAT Inhibitors | Pacritinib | JAK2 | minor histocompatibility antigen-mismatched model BALB/b→BALB/c;MLR (human);human skin graft rejection model | ameliorated | preserved | Th1↓ Th17↓ Th2↑ | (181) |
| | JAK/STAT Inhibitors | Pacritinib +S3I-201 +Rapamycin (Sirolismus) | JAK2+STAT3 +mTOR | xenograft model | ameliorated | preserved | Th1 ↓only PAC/ SIR or S3I/SIR: Th17↓ Tregs↑ | (166) |
| | JAK/STAT Inhibitors | Fedratinib (TG101348) | JAK2/STAT3 axis | MLR | ameliorated | - | Th1↓ Th17↓ Tregs↑ | (182) |
| | JAK/STAT Inhibitors | Tofacitinib (CP-690550) | JAK3 | semiallogeneic MHCII-disparate model B6→(B6xbm12)F1;MLR | ameliorated | - | Th1↓ | (183) |
| | ROCK1/2 Inhibitors | Fausidil | Rho kinase (ROCK1 and ROCK2) | semiallogeneic MHC-disparate modelC3H→ (B6C3)F1 | ameliorated | – | – | (184) |
| | ROCK1/2 Inhibitors | Belomosudil (KD025) | ROCK2 | major MHC mismatch model of multiorgan cGVHD; minor MHC mismatch model of sclerodermous GVHD | ameliorated | – | Tfh ↓ Tfregs↑ | (185) |
| | other Inhibitors | ONO-7790500 | ITK | semiallogeneic MHC-disparate modelB6→ (B6D2)F1 | ameliorated/ delayed | preserved | Th1 ↓Th2 ↓ Th17↓ | (186) |
| | other Inhibitors | 6-bromoindirubin 3'-oxime (BIO) | glycogen synthase kinase 3 (GSK3) STAT3STAT1 | xenograft model | prevented | preserved | Th1 ↓Th2 ↓ | (187) |
| other TF Inhibitors | peptide antibiotic | Echinomycin (NSC-13502) | HIF-1α | MHC mismatch model:B6→BALB/c | ameliorated | preserved | Th1 ↓ Th17↓ Tregs↑ | (188) |
| | | IT-603 | c-Rel | MHC mismatch model:B6→BALB/c | ameliorated | preserved | - | (189) |
| | | IT-901 | c-Rel | MHC mismatch model:B6→BALB/c | ameliorated | preserved | - | (190) |
| | | syntheticretinoid (SR11302) | AP-1 | MHC mismatch model:B6→BALB/c | ameliorated | - | Th1 ↓ Th17↓ Tregs↑ | (191) |
| | | S3I-201 | STAT3 | MLR (human); human skin graft rejection; xenograft GVHD model; human GVHD | ameliorated | preserved | Th1↓ Th17↓ iTregs↑ | (192–194) |
| | nitrofuran antibiotic | nifuroxazide | STAT3 | MHC mismatch model:B6→BALB/c | ameliorated | - | Th1↓ Tregs↑ | (195) |
| | | bile acid 3-OxoLC(bile acid) | indirectly RORγt | MHC mismatch model:B6→BALB/c | ameliorated | preserved | Th17 ↓ Treg↑ | (196) (197) |

survival in a mouse transplantation model (212). However, the effects of Sirnotol in GVHD were not reported yet.

Givinostat (ITF2357), a HDACi of the hydroxamic acid category, was also reported to suppress Th17 polarization and enhance FoxP3 expression and hence Treg differentiation *via* decreased STAT3 phosphorylation and RORγt expression downstream of IL-6R signaling. Administration of Givinostat inhibited experimental colitis development by skewing the Th17/Treg balance in the lamina propria (213) and reduced release of inflammatory IFN-γ and TNF-α in systemic inflammation (214). Virinostat (SAHA), another hydroxamic acid HDACi, inhibits STAT3 and also STAT1 phosphorylation, and was shown to

**TABLE 2** | Summary of clinical trials on Th defining TF inhibitors.

| Class | Sub-class | Compound | Target | Clinical trial number | Indication | Co-medication | Effect GVHD | Reference |
|---|---|---|---|---|---|---|---|---|
| Kinase inhibitors | JAK/STAT Inhibitors | Ruxolitinib (INCB018424) | JAK1/2 | NCT02953678 NCT02913261 | Steroid- refractory aGVHD | Corticosteroids, BAT | Ameliorated | (198–200) |
| Kinase inhibitors | JAK/STAT Inhibitors | Itacitinib (INCB039110) | JAK1 | NCT02614612 | Steroid-naïve & steroid-refractory GVHD | Corticosteroids | ameliorated | (201) |
| Kinase inhibitors | JAK/STAT Inhibitors | Pacritinib | JAK2 | NCT02891603 | aGVHD | Rapamycin (Sirolismus), Tacrolismus | ameliorated | (166) |

*BAT, best available therapy.*

attenuate GVHD and inhibit proinflammatory cytokine production during the initiation phase of GVHD (173). Additionally, blocking of STAT3 by both Givinostat and Virinostat, was shown to enhance indoleamine 2,3-dioxygenase (IDO) expression in APCs which suppresses APC allo-stimulatory functions and reduced GVHD in a murine allogeneic BM-transplantation model (215). Hence, Givinostat and Virinostat attenuate GVHD *via* multiple mechanisms, targeting inflammatory cytokine release, antigen presentation and T cell differentiation (216).

Romidepsin (Istodax), a cyclic peptide class HDACi, was shown to effectively suppress allo-responses in a mixed lymphocyte reaction (MLR) (174). Recently, Romidepsin was also shown to inhibit the activation of STAT1 and STAT3 *via* induction of suppressor of cytokine signaling 1 (SOCS1) (175). However, its effect in GVHD has not been fully assessed yet as a first study in patients was terminated due to slow accrual (clinical trail.gov, NCT02203578).

Overall, epigenetic modulators like the describes HDACi, were shown to efficiently inhibit GVHD by altering Th polarization *via* TF modulation. The success of HDACi in preventing GVHD is also displayed by multiple clinical studies validating their beneficial effect often in combinatory therapy in patients [reviewed by Xu et al., (217)].

## Kinase Inhibitors

Another indirect way to target TFs is the inhibition of Kinases, which catalyze the transfer of activating phosphate from ATP to substrates withing signaling pathways. Hence, kinase inhibitors can indirectly block activation of the respective kinase substate like TFs and hence modulate transcription.

The most prominent examples in GVHD therapy are Janus-Kinase (JAK) inhibitors. These inhibitors block JAK/STAT signaling pathways, which have a crucial function of transmitting cytokine-receptor signals intracellularly. Early expression profiling studies and the detection of activated STAT1 and STAT3 in GHVD target organs and alloreactive donor T cells already indicated a link between GVHD and cytokine signaling through the JAK/STAT pathway (218–220).

Subsequent experiments, disrupting JAK/STAT1 signaling by the use of T cells lacking STAT1, a Th1 specific TF responding to IFN-γ Receptor (IFNγR) signaling, reported ameliorated GVHD outcomes in a minor antigen-mismatched and fully-MHC mismatched GVHD model (155). Shortly after, Ruxolitinib (INCB018424), a bioavailable JAK1/2 inhibitor, was reported to have similar mitigating effects on GVHD as IFNγR$^{-/-}$ T cells while the GvL effect was preserved (176, 177, 221, 222). Further mechanistical analyses revealed, that Ruxolitinib ameliorates GVHD by disrupting Th1 and Th17 differentiation but promoting Treg differentiation *via* indirect STAT1 and STAT3 inhibition (223). Overall, these pre-clinical data suggested Ruxolitinib as a promising candidate for GVHD treatment, which indeed has shown remarkable results in the application for steroid refractory GVHD in various clinical studies (224).

Besides Ruxolitinib inhibiting JAK1 and JAK2 simultaneously, selective JAK1, JAK2 and JAK3 inhibitors have also been investigated as potent treatment options in GVHD. The JAK3 inhibitor Tofacitinib (CP-690550) was reported to ameliorate GVHD *in vivo* and *in vitro* by selectively inhibiting Th1 differentiation but not Th17 polarization or CD4 T cell proliferation (183). Itacitinib (INCB039110), a selective JAK1 inhibitor, disrupts the JAK1/STAT3 signaling pathway and was shown to improve GVHD outcomes and survival in various mouse models, partially by reduction of CD4 and CD8 T cell numbers in the inflamed colon tissue, indicating a loss of Th17 phenotype (178–180). Itacitinib also showed promising efficiencies in the treatment of steroid-naïve and steroid-refractory GVHD in a first clinical study (201). Selective inhibition of the JAK2/STAT3 axis, an IFN-γ, IL-6 and IL-23 receptor signaling response element, by Pacritinib (SB1518) was also shown to significantly reduce GVHD in murine models (181, 225). Similar to the effects of the JAK/STAT3 inhibitor Fedratinib in early MLR experiments; Pacritinib, led to impaired expansion of Th1 and Th17 cells while Treg and Th2 responses were sustained (181, 182). A recent study also reported a successful combinatory therapy of acute GVHD with Pacritinib the STAT3 inhibitor S31-201 and the mTOR inhibitor Rapamycin in a xenogeneic mouse model and with Rapamycin and the calcineurin inhibitor Tacrolismus in patients (166).

Despite the advanced clinical validation of JAK/STAT inhibitors in GVHD [reviewed by Assal and Mapara, (224)], few other agents of the Kinase-inhibitor group have also shown beneficial effect on GVHD in pre-clinical studies. Inhibition of the glycogen synthase kinase 3 (GSK3) by the small molecule 6-bromoindirubin 3'-oxime (BIO), prevented mice from lethal GVHD in a xenogeneic model by STAT1/3 suppression and subsequent decrease of Th1 effector cytokines (187). Recent studies suggested the IL-2 inducible kinase (ITK) inhibitor ONO-7790500 as another potent therapeutic in GVHD, as administration inhibited Th1, Th2 and Th17 differentiation, inflammatory cytokine production and alloreactive T cell proliferation and significantly delayed GVHD onset and mortality (186). An earlier study with ITK$^{-/-}$ donor T cells in

an allo-HSCT mouse model has already reported comparable beneficial effects on GVHD and observed reduced expression of IRF4, JAK1, JAK2, and STAT3 as well as phosphorylated forms of JAK1, JAK2 and STAT3 if ITK was absent in T cells, which might explain impaired differentiation capacities observed in the ITK inhibitor study (226). Rho-Kinase (ROCK) inhibitors represent a further group evaluated in pre-clinical GVHD settings. While Fausidil, a small molecule inhibiting ROCK1 and ROCK2 only had moderate ameliorating effects on GVHD-associated colitis (184), the ROCK2 inhibitor Belomosudil (KD025), which shifts the Th17/Treg balance towards homeostasis *via* an STAT3/STAT5-dependent mechanism, efficiently ameliorated chronic GVHD in multiple models and first clinical studies (185, 227). However, the effect of Belomosudil on aGVHD remains to be determined.

## Other Direct and Indirect Transcription Factor Inhibitors

Besides epigenetic regulators and kinase inhibitors other small molecules targeting TFs in a direct or indirect manner have been assessed in pre-clinical GVHD models in the last decade.

As already implicated by the successful use of JAK/STAT inhibitors, the repression or STAT3, an important activator of RORγt during Th17 differentiation, was investigated as potent strategy to prevent severe GVHD. Betts at al. reported, that the small molecule S3I-201 efficiently inhibits STAT3 expression, leading to suppressed proliferation of allo-sensitized T cells and impaired Th17 differentiation while iTreg polarization was enhanced. Mechanistically, the group uncovered that S3I-201 polarized the phosphorylation of STAT5 over STAT3 and led to activation of FoxP3 in iTregs (192). Hence, S3I-201 shifts the Th17/Treg balance towards regulatory T cells, as already reported for other STAT3 inhibitors in this review earlier. A later study of the group connected increased pSTAT3 and RORγt levels with severe aGVHD. They found that RORγt suppression was enhanced by combinatory treatment with Rapamycin and S3I-201, which abrogated the proliferation of Rapamycin-resistant T cells upon allo-sensitization in a MLR model (193). Additionally, they reported successful prevention of acute GVHD in a xenogeneic mouse model, using a combinatory treatment with S31-201, the JAK2 inhibitor Pacritinib and Rapamycin in a recently published study, as referred to earlier (166). Moreover, S3I-201 treated iTregs were found to efficiently reduce skin graft rejection and GVHD in a xenograft mouse model by reducing Th1- and Th17-mediated allorectivity, while preserving the GVL effect (194). Similarly, the STAT3 inhibitor nifuroxazide also attenuated GVHD symptoms in skin, liver and GI-tract and efficiently delayed aGVHD-associated lethality (195). Blocking of the TF AP-1 by the synthetic retinoid SR11302 also inhibited Th1/Th17 proliferation and enhanced Treg expansion by indirectly pSTAT3 blockage and STAT5 dependent FoxP3 expression, leading to diminished GVHD-associated pathology and lethality (191). Another study, which investigated the effect of GRIM19 overexpressing donor BM and T cells in GVHD, also found decreased disease-severity, Th17 polarization, and alloreactive activation due to diminished STAT3 expression. Comparable to the effect of other STAT3 inhibitors, GRIM19 overexpression also led to enhanced STAT5 expression and Treg differentiation suggesting GRIM19 induction as another potent strategy for STAT3 inhibition in the future (228).

Alongside STAT3, the inhibition of other Th1 and Th17-differentiation inducing TFs was shown to efficiently ameliorate GVHD. Inhibition of HIF1α, a key TF in Th17/Treg reciprocal differentiation, by Echinomycin (NSC-13502) was shown to efficiently attenuate GVHD and preserve anti-leukemic activity by inducing Treg expansion while diminishing Th17 responses (229). The TF c-Rel plays a role in differentiation of Th1, Th17 and Treg cells. Studies on the c-Rel inhibitor IT-603 showed ameliorating effects on GVHD, mediated through reduced alloreactivity, defective gut homing and impaired negative feedback on IL-2 production by effector T cells leading to an expansion of regulatory T cells. The attenuating effects on GVHD were additionally accompanied by a preserved graft-versus-tumor (GVT) effect and promising effects against lymphomas (189, 190). Bile acid synthesized form cholesterol, called 3-oxoLC was discovered as an inhibitory ligand of the RORγt. It efficiently altered Th17/Treg polarization towards regulatory T cells in the lamina propria suggesting a beneficial effect of bile acid metabolites in controlling intestinal-microbiome tolerance but also immune responses in GI-associated GVHD (197). Indeed, a shortly later published study reported, that the bile acid pool was reduced in patients with GVHD, and that application of bile acids reduced GVHD in several transplantation mouse models but was rather associated to alterations in antigen presentation that in Th17 differentiation (196). However, these studies suggest bile acids as potent immune modulators in the GI-tract during GVHD, partially acting through Th-subset determining TF inhibition.

# CONCLUSION

Summarized, these data show that specific targeting of Th cell-differentiation involved transcription factors might represent a potent therapeutic strategy to prevent or ameliorate GVHD in addition to standard of care medication. However, most of the presented therapeutics have only been assessed in pre-clinical models yet and beneficial effects for patients remain to be proven. In addition, the immune modulatory effect of the presented therapeutic strategies may lead to a higher susceptibility for infections. This includes the re-activation of latent viral infections [e.g. cytomegalovirus (CMV)] but also the predisposition for newly acquired infections due to major immune suppression of especially Th1 T cells but also other immune cell populations required for viral clearance. First clinical trials with the HDACi Vorinostat and Panobinostat in GVHD patients did not show an augmentation for risk of infections while Romidepsin treated patients with T cell lymphoma more often experiences infections (230–232). Studies on the JAK1/JAK2 inhibitor Ruxolitinib also reported an increased susceptibility for viral re-activation of Hepatitis-B and varicella zoster virus in treated patients with myeloproliferative neoplasm and polycythemia vera, but also a modestly higher incidence of infection and reactivated CMV infection in patients with steroid-refractory GVHD (198, 233, 234). However, first line and second

line therapies in GVHD also harbor the risk of viral re-activation and overall significant improvement in efficacy outcomes by more target specific TF inhibitors probably weights more than a moderate elevated risk for infection (198, 235). Additionally, the above-mentioned examples from clinical trials show that the risk of an enhanced susceptibility towards infections under TF inhibitor treatment is highly dependent on the drug target and specificity so that these more specific TF inhibitors might exhibit superior protection from infections that other commonly used therapeutics.

Together, given the promising results of some TF-modulators in clinical studies, we expect a fundamental contribution of TF-inhibitors to improve GVHD therapy in the future.

## AUTHOR CONTRIBUTIONS

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# Post-Transplantation Cyclophosphamide Uniquely Restrains Alloreactive CD4$^+$ T-Cell Proliferation and Differentiation After Murine MHC-Haploidentical Hematopoietic Cell Transplantation

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Post-transplantation cyclophosphamide (PTCy) reduces the incidence and severity of graft-versus-host disease (GVHD), thereby improving the safety and accessibility of allogeneic hematopoietic cell transplantation (HCT). We have shown that PTCy works by inducing functional impairment and suppression of alloreactive T cells. We also have identified that reduced proliferation of alloreactive CD4$^+$ T cells at day +7 and preferential recovery of CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells ($T_{regs}$) at day +21 are potential biomarkers associated with optimal PTCy dosing and timing in our B6C3F1→B6D2F1 MHC-haploidentical murine HCT model. To understand whether the effects of PTCy are unique and also to understand better the biology of GVHD prevention by PTCy, here we tested the relative impact of cyclophosphamide compared with five other optimally dosed chemotherapeutics (methotrexate, bendamustine, paclitaxel, vincristine, and cytarabine) that vary in mechanisms of action and drug resistance. Only cyclophosphamide, methotrexate, and cytarabine were effective in preventing fatal GVHD, but cyclophosphamide was superior in ameliorating both clinical and histopathological GVHD. Flow cytometric analyses of blood and spleens revealed that these three chemotherapeutics were distinct in constraining conventional T-cell numerical recovery and facilitating preferential $T_{reg}$ recovery at day +21. However, cyclophosphamide was unique in consistently reducing proliferation and expression of the activation marker CD25 by alloreactive CD4$^+$Foxp3$^-$ conventional T cells at day +7. Furthermore, cyclophosphamide restrained the differentiation of alloreactive CD4$^+$Foxp3$^-$ conventional T cells at both days +7 and +21, whereas methotrexate and cytarabine only restrained differentiation at day +7. No chemotherapeutic selectively eliminated alloreactive T cells. These data suggest that constrained alloreactive CD4$^+$Foxp3$^-$

conventional T-cell numerical recovery and associated preferential $CD4^+CD25^+Foxp3^+$ $T_{reg}$ reconstitution at day +21 may be potential biomarkers of effective GVHD prevention. Additionally, these results reveal that PTCy uniquely restrains alloreactive $CD4^+Foxp3^-$ conventional T-cell proliferation and differentiation, which may explain the superior effects of PTCy in preventing GVHD. Further study is needed to determine whether these findings also hold true in clinical HCT.

**Keywords: graft-versus-host disease (GVHD), haploidentical, alloreactive, bendamustine, methotrexate, regulatory (Treg) cell, post-transplantation cyclophosphamide (PTCy), allogeneic hematopoietic cell transplantation (HCT)**

## INTRODUCTION

Allogeneic hematopoietic cell transplantation (HCT) is the only potentially curative therapy for many life-threatening hematologic diseases, but historically was not accessible to many patients for lack of a suitable human leukocyte antigen (HLA)-matched donor. HLA-haploidentical donors are available for nearly all patients, but early results of HLA-haploidentical HCT showed unacceptably high rates of graft failure, graft-versus-host disease (GVHD), and transplant-related mortality due to strong bi-directional alloreactivity (1). The administration of the chemotherapeutic cyclophosphamide on days +3 and +4 post-transplant (post-transplantation cyclophosphamide, PTCy) can greatly reduce the incidence and severity of acute and chronic graft-versus-host disease (GVHD) after HLA-haploidentical or HLA-partially mismatched unrelated donor HCT and consequently has been widely adopted (2). Despite very encouraging clinical outcomes, how PTCy works to prevent GVHD has not been well understood.

A better understanding of the immunological mechanisms by which PTCy works to prevent GVHD may allow for rational modifications of this platform in attempts to improve outcomes for patients. In murine HCT models, we have shown that PTCy works by inducing alloreactive T-cell functional impairment and subsequent suppression by $CD4^+Foxp3^+$ regulatory T cells ($T_{regs}$) (3–5). We also have shown in our B6C3F1→B6D2F1 MHC-haploidentical murine HCT model that optimal dosing and timing of PTCy are associated with reduced proliferation of alloreactive $CD4^+Foxp3^-$ conventional T cells at day +7 and preferential recovery of $CD4^+CD25^+Foxp3^+$ $T_{regs}$ at day +21 (5, 6). We have proposed that together these T-cell endpoints may be potential biomarkers of effective GVHD control by PTCy (6).

The relative survival and recovery of T-cell subsets after optimally timed and dosed PTCy may be due to differential and dynamic expression of important drug resistance pathways, including aldehyde dehydrogenase (ALDH) and ATP-binding cassette (ABC) transporters (3, 4, 7). Human and mouse $T_{regs}$ upregulate expression of ALDH, the main *in vivo* detoxifying pathway for cyclophosphamide (8), after alloantigen stimulation, contributing to resistance to cyclophosphamide-induced cell death in this specific context (3, 4, 9). These pathways also contribute to human $CD8^+$ T-cell survival and recovery after cyclophosphamide (7). ALDH and ABC transporters are not only widely expressed throughout the hematopoietic system (10, 11), but also confer differential degrees of resistance to virtually all classes of chemotherapeutics (12–14). Each chemotherapeutic also may have additional specific mechanisms of resistance.

It is unclear whether the effects of cyclophosphamide (CY) given in the early post-transplant period are unique. In 1971, survival after murine MHC-haploidentical HCT was compared after treatment with cyclophosphamide, methotrexate, mercaptopurine, chlormethine, or cortisol, each administered on days +5, +8, +11, and +14 (15). Cyclophosphamide was the only drug found to be effective, while all other drugs had minimal impact (15). However, since 1971, not only are there more chemotherapeutics available, but we also have identified that PTCy is maximally effective in HCT when given between days +3 to +5 (6). Additionally, the relative effects of PTCy compared with other chemotherapeutics on T-cell subsets have not been examined. The only exception has been recent interest in post-transplantation bendamustine (BEN), which has been shown in pre-clinical studies to produce engraftment and GVHD prevention results similar to PTCy, while maintaining the graft-versus-leukemia effect (16–18); yet, phase I/II trials thus far have shown mixed results (17, 18).

To assess whether the biological effects of PTCy may be unique and provide further insights into our mechanistic understanding of the immunological mechanisms by which PTCy prevents GVHD, we investigated in our murine MHC-haploidentical HCT model (5) the relative efficacy of five other chemotherapeutics (methotrexate, bendamustine, paclitaxel, vincristine, and cytarabine). We specifically chose these drugs as they represent an array of mechanisms of action, metabolism, and drug resistance (**Table 1**) and include methotrexate, which has a long history of clinical use for GVHD prophylaxis, and bendamustine, which has recently been explored as an alternative to PTCy; topoisomerase inhibitors and other alkylators beyond bendamustine were intentionally excluded over theoretical concern for therapy-related myeloid neoplasms in any clinical application of these studies.

## MATERIALS AND METHODS

### Mice

B6C3F1/Crl (donor) and B6D2F1/Crl (recipient) female mice, 10-12 weeks old at the time of transplant, were obtained from the Charles River Laboratories. Mice were housed in specific-pathogen-free conditions at the NCI and were provided food and water *ad libitum*.

**TABLE 1** | Putative pathways of resistance to chemotherapeutics tested in this study as assessed by available literature (8, 12–14, 19–44).

| Drug | Class | ALDH | ABC | Other Mechanisms of Resistance |
|---|---|---|---|---|
| **Cyclophosphamide (CY)** | Alkylating agent | +++ | + | Inactivation *via* glutathione S-transferase |
| **Bendamustine (BEN)** | Alkylating agent + antimetabolite | NR | + | Only partial cross-resistance to other alkylating agents.<br>Mechanisms of resistance understudied. |
| **Methotrexate (MTX)** | Antimetabolite (antifolate) | + | ++ | Reduced uptake *via* the human reduced folate carrier.<br>Increased dihydrofolate reductase activity.<br>Decreased polyglutamylation. |
| **Cytarabine (ARA-C)** | Antimetabolite (antinucleoside) | + | + | Reduced uptake *via* human equilibrative nucleoside transporter.<br>Decreased activation *via* deoxycytidine kinase deficiency or increased expression of 5' nucleotidases.<br>Deactivation *via* increased expression of cytidine deaminase.<br>Increased expression of DNA polymerase α. |
| **Paclitaxel (PTX)** | Antimicrotubular/taxane | + | ++ | Alterations to the tubulin/microtubule system. |
| **Vincristine (VCR)** | Antimicrotubular/vinca alkaloid | + | ++ | Alterations to the tubulin/microtubule system. |

*+, some evidence in literature suggesting involvement; ++, more numerous reports of involvement and/or likely a major mediator of resistance, but other pathways also may play important roles; +++ established involvement as a key mediator of resistance. NR, not reported.*

## HCT

Spleens, tibias, and femurs were aseptically collected from donor B6C3F1 mice and processed as previously described, including red blood cell lysis of splenocytes and T-cell depletion of bone marrow (5). Recipient B6D2F1 mice were irradiated to 10.5 Gy in a single fraction and 6-8 hours later received $10x10^6$ B6C3F1 T-cell-depleted bone marrow +/- $40x10^6$ red blood cell-depleted B6C3F1 splenocytes *via* tail vein injection. Recipient mice received levofloxacin-treated water from days 0 to +14. Survival was followed daily, and blinded assessments of weights and clinical scores using a standardized rubric (5) were performed every three days. Tissue specimen preparation and blinded histopathologic assessments were performed as previously described (5).

## Drug Preparation

On the day of administration, methotrexate (MTX, Intas Pharmaceuticals), paclitaxel (PTX, Athenex), vincristine (VCR, Hospira), and cytarabine (ARA-C, Hospira) were diluted with sterile PBS to appropriate concentrations, while bendamustine (BEN, TEVA Pharmaceutical Industries) was reconstituted using sterile water to 5 mg/ml per the manufacturer's instructions and then further diluted to the appropriate concentrations with sterile PBS. Cyclophosphamide (CY, Baxter Oncology) was prepared as previously described (5) and diluted to a 1 mg/ml concentration with sterile PBS on the day of administration for injection. All drugs were diluted to concentrations allowing for administration of 300-500 µl per mouse and administered *via* intraperitoneal injection. Doses were based on the weight on the day of injection. Vehicle-treated mice received similar volumes of sterile PBS intraperitoneally.

## Flow Cytometry

Blood and spleens were collected and processed as previously described (5). Viable cell counts were performed with dual-fluorescent imaging with a Cellometer Auto 2000 Cell Viability Counter (Nexcelom). $2x10^6$ viable cells/sample were stained sequentially with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Thermo Fisher), extracellular antibodies, fixation/permeabilization (eBioscience Foxp3/Transcription Factor Staining Kit), and intracellular antibodies. Single stains were used to generate compensations, and fluorescence-minus-one controls were prepared for CD25, Ki-67, and phospho-STAT5. Data were acquired on an LSRFortessa (BD Biosciences) and analyzed using FCS Express (De Novo Software). Cell subsets had to be at least 50 cells to allow for reliable further subsetting; subsets with denominators less than this threshold were excluded from further subsetting analyses.

Fluorochrome-conjugated monoclonal antibodies used for flow cytometry included BUV395 anti-CD3 (clone 145-2C11), BV786 anti-CD8a (clone 53-6.7), PE-CF594 anti-CD25 (clone PC61), AF700 anti-CD44 (clone IM7), BUV737 anti-CD62L (clone MEL-14), PE anti-$H2k^k$ (clone 36-7-5), and BV711 anti-$H2k^k$ (clone AF3-12.1) from BD Biosciences; PE-Cy5 anti-CD8 (clone 53-6.7), PE-Cy7 anti-$H2k^d$ (clone SF1-1.1), and BV605 anti-Ki67 (clone 16A8) from BioLegend; and APC-eFluor780 anti-CD4 (clone GK1.5), PerCP-eFluor710 anti-Vβ6 (clone RR4-7), eFluor450 anti-Foxp3 (clone FJK-16s), and PE anti-phospho-STAT5 (Tyr694) (clone SRBCZX) from Invitrogen.

## Statistics

Survival distributions were compared using the exact log-rank test. Weight and clinical score area-under-the-curve (AUC) comparisons were performed using Wilcoxon's rank sum test and were restricted to intervals in which ≥70% of vehicle-treated mice survived. Weight and clinical score data are shown as the mean +/- SEM. Due to strong serial correlations, weight and clinical score SEMs were not corrected for multiple measurements. Cell counts and median fluorescence intensities were natural logarithmically transformed and cell subset percentages were transformed using an arcsine transformation prior to one-way ANOVA. ANOVA results were followed with the Holm-Sidak *post hoc* correction for the multiple comparisons to the control group. Non-transformed data are displayed and are shown as box-and-whisker plots for ease of understanding, but transformed data were used for statistical testing. SAS/STAT software (SAS Institute Inc.), version 14.3, was used for analyses of survival, weight, and clinical score data. GraphPad Prism

(GraphPad Software), version 8.4.3 was used for all other statistical analysis. *P* values <0.05 were considered statistically significant in an exploratory mode of analysis of repeated measurements of correlated immunologic outcomes.

## Study Approval

Mice were treated under a protocol approved by the NCI Animal Care and Use Committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

# RESULTS

## MTX 5 mg/kg/day, BEN 10 mg/kg/day, PTX 1 mg/kg/day, VCR 0.05 mg/kg/day, and ARA-C 25 mg/kg/day Are Optimal Doses of Each Drug When Given on Days +3 and +4 in the B6C3F1→B6D2F1 MHC-Haploidentical HCT Model

We have previously shown that PTCy is maximally effective in our B6C3F1→B6D2F1 MHC-haploidentical HCT model when given between days +3 to +5 with dosing on days +3 and +4 being among the best dosing schedules and also is what is used clinically (2, 6). Therefore, we sought to identify the best dose of each alternative chemotherapeutic (MTX, BEN, PTX, VCR, ARA-C) when given on days +3 and +4 in this HCT model; we explored a wide range of doses, spanning what were thought to be below effective doses to near-lethal ranges based on prior studies (15, 16, 19, 45–56). MTX 5 mg/kg/day, BEN 10 mg/kg/day, PTX 1 mg/kg/day, VCR 0.05 mg/kg/day, and ARA-C 25 mg/kg/day were determined as best for each drug, although some had minimal to no efficacy in preventing fatal GVHD (**Figure 1** and **Supplementary Figure 1**).

## CY 25 mg/kg/day Is Superior to All Other Chemotherapeutics in Preventing Severe GVHD

We next compared the optimal doses of these other chemotherapeutics with the previously established optimal dose of CY (25 mg/kg/day) in this MHC-haploidentical HCT model (5, 6), with all drugs being administered as daily doses on days +3 and +4. CY, MTX, and ARA-C all were partially effective in preventing fatal GVHD (**Figure 2A**). BEN slightly delayed survival in a subset of mice, while VCR and PTX were completely ineffective in preventing fatal GVHD (**Figure 2A**). In fact, PTX led to more rapid mortality (**Figure 2A**).

CY was superior to all other chemotherapeutics in ameliorating clinical GVHD (**Figure 2A**). CY-treated mice had significantly improved body weights and clinical scores compared with vehicle-treated mice as well as with MTX- and ARA-C-treated mice (**Figure 2A**). MTX and ARA-C had similar weights but significantly better clinical scores than vehicle-treated mice. BEN, VCR, and PTX did not significantly improve clinical scores compared with vehicle-treated mice (**Figure 2A**), while VCR had significantly lower body weights and PTX trended towards lower body weights compared with vehicle-treated mice (**Figure 2A**). Higher mean body weight in BEN-treated mice was a result of substantial weight gain from ascites, rather than clinical benefit from the chemotherapeutic (**Figure 2A**). Autopsy suggested that these mice were developing ascites secondary to GVHD-induced liver failure and protein-losing enteropathy, also evident in high average liver GVHD scores at day +21 (**Supplementary Figure 2**).

Histopathological scoring at day +7 revealed that mice receiving CY, MTX, BEN, and PTX all had significantly reduced total histopathological GVHD severity scores compared with vehicle-treated mice (**Figure 2B** and **Supplementary Table 1**). However, by day +21, only CY significantly reduced histopathological GVHD compared with vehicle-treated mice (**Figure 2B** and **Supplementary Table 2**). MTX and ARA-C both had lower median histopathological GVHD severity scores compared with vehicle-treated mice at day +21, but the lack of statistical significance was due to wider variability of scores between mice in those groups (**Figure 2B**).

## The Partially Effective Chemotherapeutics (CY, MTX, and ARA-C) All Constrain T-Cell Recovery at Day +21

Some have contended that PTCy prevents GVHD *via in vivo* T-cell depletion, but our recent work showed that day +7 total numbers of T cells in blood, spleens, peripheral lymph nodes, and liver after CY 25 mg/kg/day, the optimal dose in this model, were similar to or in the same log range as vehicle-treated mice (5). Similar to these findings, total T-cell numbers in blood and spleen from CY and most other tested chemotherapeutics were not significantly reduced at day +7 when compared with vehicle-treated mice (**Figure 3A**). The only exception was that MTX reduced total T-cell concentrations in blood at day +7, which was attributable to a decrease in total $CD8^+$ T-cell concentrations at that timepoint (**Figure 3A**). CY did not significantly reduce total T-cell numbers in either blood or spleen, but did result in a significant reduction of $CD4^+$ T cells in the spleen at day +7 (**Figure 3A**). By contrast, CY, MTX, and ARA-C all significantly reduced total T-cell, including $CD4^+$ and $CD8^+$ T-cell subset, numbers at day +21, whereas the ineffective chemotherapeutics had total T-cell numbers similar to vehicle-treated mice (**Figure 3B**). This suggests that effective GVHD control may be associated with constrained T-cell recovery at day +21.

## The $CD4^+/CD8^+$ T-Cell Ratio Is Not Affected by Chemotherapeutics Other Than MTX, Which Increases It, and CY, Which Decreases It

MTX administration facilitated a markedly distinct recovery of $CD4^+$ versus $CD8^+$ T cells compared with all other treatment groups and opposite that of CY-treated mice (**Figures 3C, D** and **Supplementary Figure 3**). At day +7, MTX demonstrated higher percentages of $CD4^+$ T cells in both blood and spleen compared with vehicle-treated mice, while CY-treated mice had similar percentages of $CD4^+$ T cells in the blood and reduced

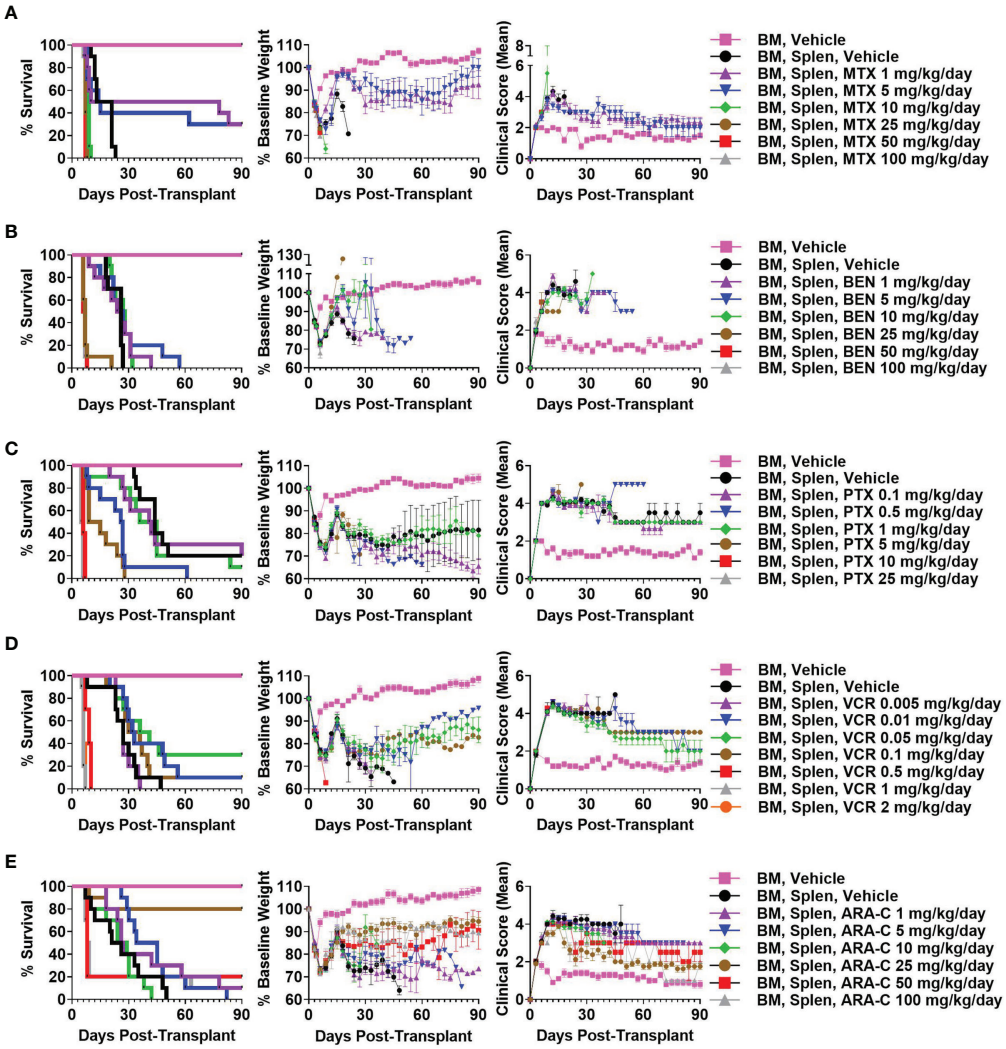


**FIGURE 1** | Methotrexate (MTX) 5 mg/kg/day, bendamustine (BEN) 10 mg/kg/day, paclitaxel (PTX) 1 mg/kg/day, vincristine (VCR) 0.05 mg/kg/day, and cytarabine (ARA-C) 25 mg/kg/day are optimal doses of each drug when given on days +3/+4 in the B6C3F1 → B6D2F1 MHC-haploidentical hematopoietic cell transplantation (HCT) model. On day 0, recipient 10-12-week-old female B6D2F1 mice were irradiated to 10.5 Gy in a single fraction and transplanted 6-8 hours later *via* intravenous injection with 10 x $10^6$ T-cell-depleted bone marrow (BM) cells +/- 40 x $10^6$ red-blood-cell-depleted splenocytes (Splen) from 10-12-week-old female B6C3F1 donors. Phosphate buffered saline (PBS) vehicle or the chemotherapeutic of interest was administered intraperitoneally on days +3 and +4. Although different chemotherapeutics had varying efficacy in mitigating fatal or severe graft-versus-host disease (GVHD), **(A)** MTX 5 mg/kg/day, **(B)** BEN 10 mg/kg/day, **(C)** PTX 1 mg/kg/day, **(D)** VCR 0.05 mg/kg/day, and **(E)** ARA-C 25 mg/kg/day were determined as optimal doses for each drug due to superior survival, weights, and/or clinical scores. Combined results from two independent experiments of n = 5 mice/group/experiment are shown.

percentages in the spleen (**Figure 3C**). At day +21, splenic $CD4^+$ T-cell percentages remained high in MTX-treated mice, while CY again reduced percentages in both blood and spleens (**Figure 3D**). The ineffective chemotherapeutics all had similar percentages of $CD4^+$ T cells compared with vehicle-treated mice across tissues at both days +7 and +21 (**Figures 3C, D**). This unique, contrasting T-cell recovery after MTX compared with that observed after PTCy is especially interesting considering that MTX was found to be effective in this model (**Figure 2**), albeit less so than PTCy, suggesting that MTX and PTCy may have distinct mechanisms of GVHD prevention.

## Unlike CY, MTX and ARA-C Do Not Control CD25 Expression by $CD4^+Foxp3^-$ Conventional T Cells at Day +7

Consistent with increased percentages of $CD4^+$ T cells in MTX-treated mice (**Figure 3C**), total numbers of $CD4^+Foxp3^-$ conventional T cells were similar to slightly higher in MTX-treated compared with vehicle-treated mice at day +7 (**Figure 4A**). Furthermore, at day +7, much higher percentages of $CD4^+Foxp3^-$ conventional T cells appeared to have an activated phenotype ($CD25^+Foxp3^-$) in MTX-treated mice and, to a lesser extent, ARA-C-treated mice, whereas this percentage

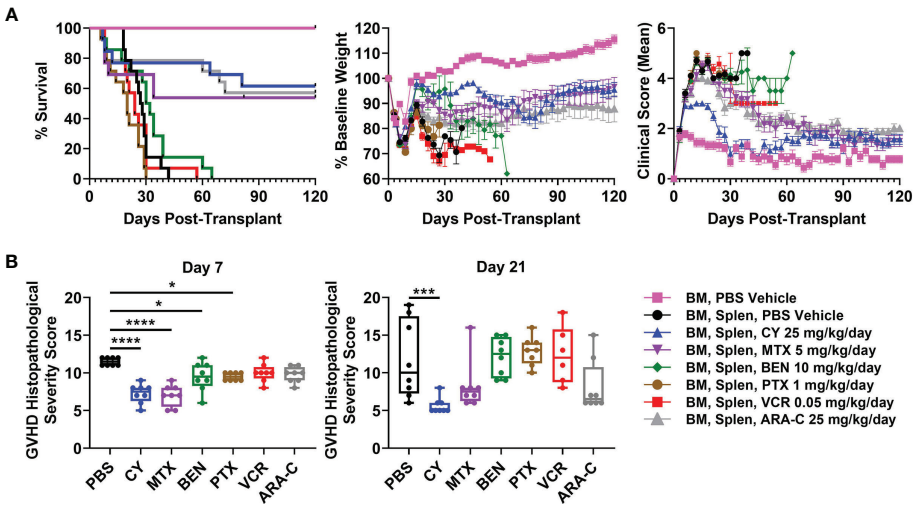


**FIGURE 2** | Post-transplantation cyclophosphamide (PTCy), MTX, and ARA-C all are partially effective in mitigating severe GVHD with PTCy having the most efficacy. Mice were transplanted as in **Figure 1** and were given either PBS or the previously determined optimal dose of one of the tested chemotherapeutics. **(A)** CY (HR 0.12, p = 0.0008), MTX (HR 0.28, p = 0.012), and ARA-C (HR 0.11, p = 0.0004) all significantly prolonged survival compared with vehicle-treated mice, whereas PTX led to more rapid mortality (HR 2.42, p = 0.038). MTX and ARA-C had similar survival compared with CY, but BEN (HR 6.86, p = 0.0008), PTX (HR 8.41, p = 0.0005), and VCR (HR 5.86, p = 0.0012) had worse survival. However, of the three partially effective chemotherapeutics, only CY-treated mice had significantly higher weights (p = 0.015) compared with vehicle-treated mice (p > 0.10 for MTX and ARA-C). Conversely, VCR-treated mice had significantly lower weights (p = 0.011) and PTX trended towards lower weights (p = 0.075) compared with vehicle-treated mice. CY-treated mice had higher weights compared with PTX-treated (p = 0.0068), VCR-treated (p = 0.0011), and ARA-C-treated (p = 0.02) mice and also had marginally higher weights compared with MTX-treated mice (p = 0.052). CY (p < 0.0001), MTX (p = 0.03), and ARA-C (p < 0.0001) led to better clinical scores than vehicle-treated mice, and CY was significantly better than all other treatment groups including both MTX and ARA-C (p < 0.0001 for each). Statistical comparisons for clinical scores and weights are for the area-under-the-curve (AUC) calculations over the period of time in which ≥70% of vehicle-treated mice were alive. **(B)** Mice were taken for histopathology of GVHD target organs on day +7 or +21. Several chemotherapeutics (CY, MTX, BEN, PTX) significantly reduced histopathological GVHD at day +7, but only CY continued to significantly reduce histopathological GVHD at day +21. *p < 0.05, ***p < 0.001, ****p < 0.0001 on one-way ANOVA followed by the Holm-Sidak *post hoc* test using the vehicle-treated group as the control. Only significant results are shown; all other comparisons between treatment groups and the vehicle group are non-significant. Combined results from two independent experiments are shown; n = 5/group/experiment for the weights and clinical score assessments in A except TCD BM + PBS (n = 9 total), and n = 4/group/experiment for all groups in B except VCR at day +21 (n = 6 total) due to excess early deaths. Extra mice set up in B for both experiments were also followed and included in the survival graphs [n = 4 independent experiments with total n=14/group except CY (n = 13), MTX (n = 13), and TCD BM + PBS vehicle (n = 9)].

was reduced in CY-treated mice (**Figures 4B, C**). Interestingly, this difference in CD25 expression of $CD4^+Foxp3^-$ conventional T cells did not equate to differences in STAT5 phosphorylation, which was not decreased in CY-treated mice (**Supplementary Figure 4**). Corresponding to the higher CD25 expression within $CD4^+Foxp3^-$ conventional T cells after MTX and ARA-C at day +7, MTX- and ARA-C-treated mice had worse weights and clinical scores than CY-treated mice (**Figure 2A**). This clinical effect was seen despite increased percentages of $CD4^+CD25^+Foxp3^+$ $T_{regs}$ at day +7 in MTX-treated mice, whereas these percentages were reduced in the spleens of CY-treated mice at this timepoint (**Figure 4B**).

## All Partially Effective Chemotherapeutics Facilitate Preferential $T_{reg}$ Recovery at Day +21

We have shown that $T_{regs}$ are necessary for GVHD prevention by PTCy and that this role is increasingly important as time progresses post-transplant in suppressing surviving alloreactive T cells (3–5). PTCy also allows preferential recovery of $T_{regs}$ in mice and patients (3–6); indeed, increased percentages of $T_{regs}$ at day +21 are associated in our MHC-haploidentical HCT model with more effective dosing schedules of PTCy (5, 6). Consistent with our previous work, CY facilitated increased percentages of $T_{regs}$ at day +21 in both blood and spleens (**Figures 4D, E**), an effect that also was seen with the two other partially effective chemotherapeutics, MTX and ARA-C (**Figures 4D, E**). Conversely, the ineffective drugs, BEN, PTX, and VCR, were not associated with increased percentages of $T_{regs}$ at either timepoint (**Figures 4B, E**). These data further support preferential recovery of $T_{regs}$ at day +21 as a potential biomarker of successful GVHD prevention, as this T-cell endpoint has been consistent between all effective chemotherapeutics here and also for maximally effective dosing schedules of PTCy (5, 6).

## Alloreactive T Cells Persist After All Chemotherapeutics, but Alloantigen-Specific $T_{regs}$ Are Increased at Day +7 After CY and MTX

Previously PTCy was thought to work *via* selective elimination of alloreactive T cells, since these cells would be proliferating rapidly

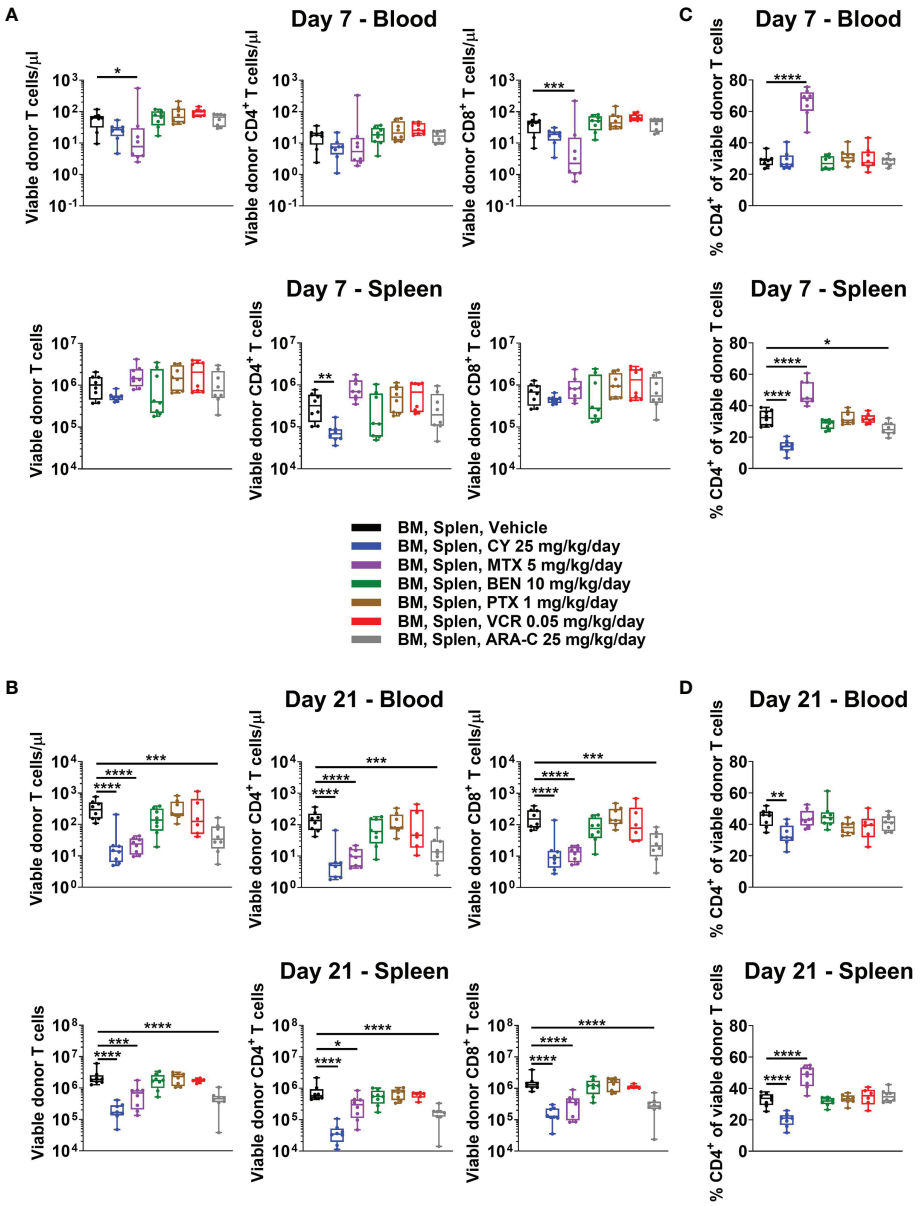


**FIGURE 3** | All partially effective chemotherapeutics constrain T-cell recovery at day +21, but MTX and CY differentially affect the balance of $CD4^+$ versus $CD8^+$ T cells, distinct from all other chemotherapeutics. Mice were transplanted as in **Figure 1** and received intraperitoneal injections on days +3 and +4 with either PBS vehicle or the optimal dose of one of the chemotherapeutics of interest. At day +7 or +21, mice were euthanized, and their blood and spleens were assessed by flow cytometry. **(A)** Total numbers of $CD3^+$ T cells and $CD4^+$ and $CD8^+$ T-cell subsets were not significantly reduced in BEN-, PTX-, VCR-, or ARA-C-treated mice at day +7. However, MTX significantly reduced total number of $CD3^+$ and $CD8^+$ T cells in the blood, while CY significantly reduced total numbers of $CD4^+$ T cells in spleens at day +7. **(B)** At day +21, CY, MTX, and ARA-C all constrained recovery of $CD4^+$ and $CD8^+$ T-cell subsets. **(C, D)** MTX and CY had opposite effects at both **(C)** day +7 and **(D)** day +21 on the balance of $CD4^+$ and $CD8^+$ T cells, divergent from effects seen in vehicle-treated mice and mice treated with other chemotherapeutics. Combined results from two independent experiments are shown with n = 4/group/experiment except for VCR at day +21 (n = 6 total) due to excess early deaths in one experiment prior to day +21. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 on one-way ANOVA followed by the Holm-Sidak *post hoc* test using the vehicle-treated group as the control. Only significant results are shown; all other comparisons between treatment groups and the vehicle group are non-significant.

in the early post-transplant setting. However, our recently published work showed that PTCy does not selectively eliminate alloreactive T cells (5, 6). Indeed, neither after PTCy, nor after any of the other chemotherapeutics tested, were alloreactive T cells eliminated (**Figures 5A, B** and **Supplementary Figure 5**). At day +7, the percentages of $CD4^+Foxp3^-$ T cells that were $V\beta6^+$ were slightly reduced after PTCy (**Figure 5A**). By contrast, these percentages, and those of $CD8^+$ T cells that were $V\beta6^+$ actually

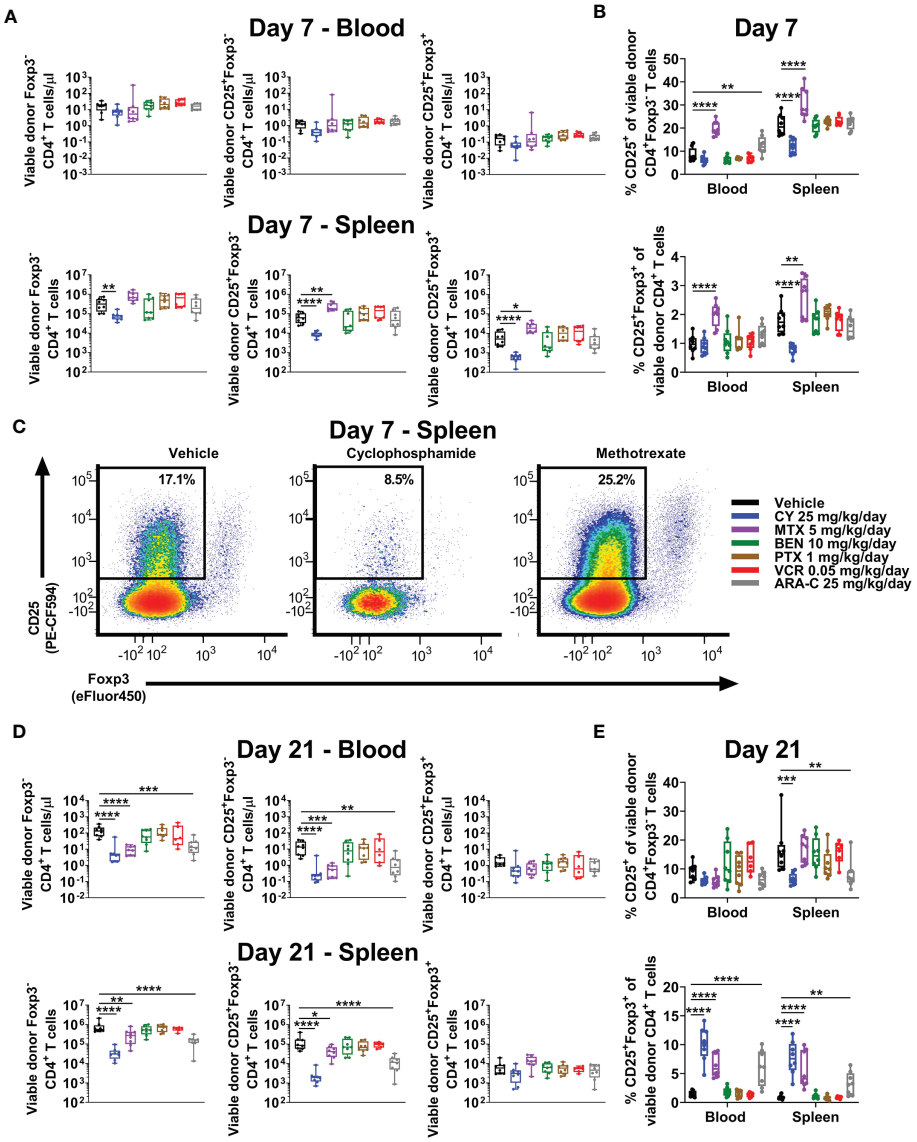


**FIGURE 4** | MTX and ARA-C do not control activated conventional CD4$^+$ T cells at day +7, but at day +21 CY, MTX, and ARA-C (all partially effective chemotherapeutics) constrain conventional CD4$^+$ T-cell recovery and facilitate preferential CD4$^+$CD25$^+$Foxp3$^+$ regulatory T-cell recovery. Mice were transplanted, treated with PBS or a chemotherapeutic on days +3 and +4, and euthanized for flow cytometric assessment at day +7 or +21 as in **Figure 3**. **(A)** At day +7, total numbers of CD4$^+$ T-cell subsets in the blood were not significantly different across treatment groups, but CY significantly reduced and MTX significantly increased total numbers of CD4$^+$ T-cell subsets in the spleen. **(B)** Although MTX increased percentages of CD25$^+$Foxp3$^+$ regulatory T cells ($T_{regs}$) at day +7 in both the blood and spleen, MTX also significantly increased percentages of conventional (Foxp3$^-$) CD4$^+$ T cells with an activated (CD25$^+$) phenotype. This was distinct from CY, which reduced percentages of both. All other chemotherapeutics did not significantly alter these percentages in comparison with vehicle-treated mice except for ARA-C, which increased percentages of activated (CD25$^+$) conventional CD4$^+$ T cells only in the blood. **(C)** Representative flow cytometric plots are shown of CD4$^+$ T cells gated on CD25 versus Foxp3 expression, showing percentages of CD25$^+$Foxp3$^-$ CD4$^+$ T cells at day +7 that were decreased after CY but increased after MTX. CD25-positivity was gated based on the use of a fluorescence-minus-one (FMO) control. **(D)** At day +21, total numbers of Foxp3$^-$ and CD25$^+$Foxp3$^-$ conventional CD4$^+$ T cells were decreased in mice treated with the partially effective chemotherapeutics (CY, MTX, and ARA-C), whereas total numbers of CD4$^+$CD25$^+$Foxp3$^+$ $T_{regs}$ were similar across treatment groups. **(E)** Due to this balance, CY, MTX, and ARA-C all were associated with increased percentages of CD4$^+$CD25$^+$Foxp3$^+$ $T_{regs}$ at day +21, while CY and ARA-C also reduced the percentages of activated (CD25$^+$) conventional CD4$^+$ T cells. Combined results from two independent experiments are shown with n = 4/group/experiment for **(A–E)** except for VCR (n = 6 total) in **(D, E)** due to excess early deaths. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 on one-way ANOVA followed by the Holm-Sidak *post hoc* test using the vehicle-treated group as the control. Only significant results are shown; all other comparisons between treatment groups and the vehicle group are non-significant.

were increased in the blood after MTX (**Figure 5A**). Interestingly, both PTCy and MTX were associated with increased percentages of alloantigen-specific ($V\beta6^+$) $T_{regs}$ at day +7 (**Figure 5A**). At day +21, across all T-cell subsets and treatment groups, there were no significant differences in percentages of alloreactive T cells compared with vehicle-treated mice (**Figure 5B**).

## CY Is the Only Effective Chemotherapeutic That Consistently Reduces Alloreactive $CD4^+$ T-Cell Proliferation at Day +7

Although PTCy does not selectively eliminate alloreactive T cells, effective GVHD control by PTCy is associated with a decrease in percentages of $CD4^+$, including alloreactive $CD4^+$ T cells, that are proliferating at day +7 (5, 6). Consistent with previous results, CY reduced percentages of proliferating $CD4^+$ T cells, including alloreactive ($V\beta6^+$) conventional T-cell subsets, in both blood and spleens (**Figures 6A, C**). Conversely, neither MTX nor ARA-C significantly reduced proliferation of alloreactive T cells at day +7, except within $CD4^+CD25^+Foxp3^-V\beta6^+$ T cells after ARA-C (**Figure 6C**). By contrast, proliferation of alloreactive T cells had normalized and was similar across treatment groups and T-cell subsets at day +21 (**Figures 6B, D**).

## All Partially Effective Chemotherapeutics Restrain Alloreactive $CD4^+$ Conventional T-Cell Differentiation at Day +7, but CY Has the Greatest Effect and Is the Only Drug That Maintains This Effect at Day +21

CY, MTX, and ARA-C, the three chemotherapeutics that were partially effective in ameliorating GVHD, all were distinct in

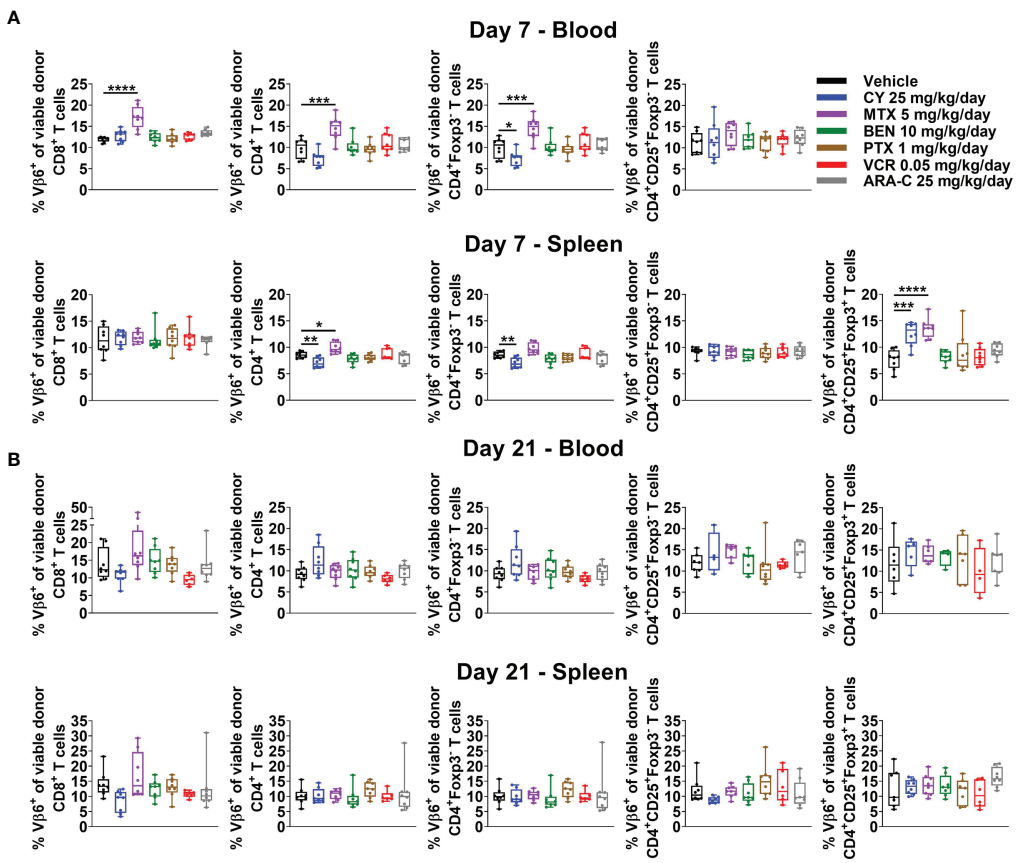


**FIGURE 5** | Alloreactive T cells persist after administration of all chemotherapeutics, but alloantigen-specific $T_{regs}$ are increased at day +7 after CY and MTX. Mice were transplanted, treated with PBS or a chemotherapeutic on days +3 and +4, and euthanized for flow cytometric assessment at day +7 or +21 as in **Figure 3**. Alloreactive $V\beta6^+$ T cells were not eliminated by any chemotherapeutic at either **(A)** day +7 or **(B)** day +21. **(A)** In fact, at day +7, percentages of $CD8^+$, $CD4^+$, and $CD4^+Foxp3^-$ T cells that were $V\beta6^+$ were increased in MTX-treated mice. The percentages of $CD4^+Foxp3^-$ T cells that were $V\beta6^+$ were slightly reduced after CY although other T-cell subsets, including percentages of $CD4^+CD25^+Foxp3^-$ that were $V\beta6^+$, were not affected. Interestingly, alloantigen-specific $CD4^+CD25^+Foxp3^+$ cells were increased in CY- and MTX-treated mice at day +7 in the spleen; percentages in the blood were not included due to low total numbers of $CD4^+CD25^+Foxp3^+$ cells at day +7 across treatment groups that did not permit reliable determination of further subsetting. **(B)** At day +21, there were no significant differences in percentages of $V\beta6^+$ T cells across T-cell subsets or treatment groups. Combined results from two independent experiments are shown with n = 4/group/experiment except VCR (n = 6 total) in B due to excess early deaths. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 on one-way ANOVA followed by the Holm-Sidak *post hoc* test using the vehicle-treated group as the control. Only significant results are shown; all other comparisons between treatment groups and the vehicle group are non-significant.

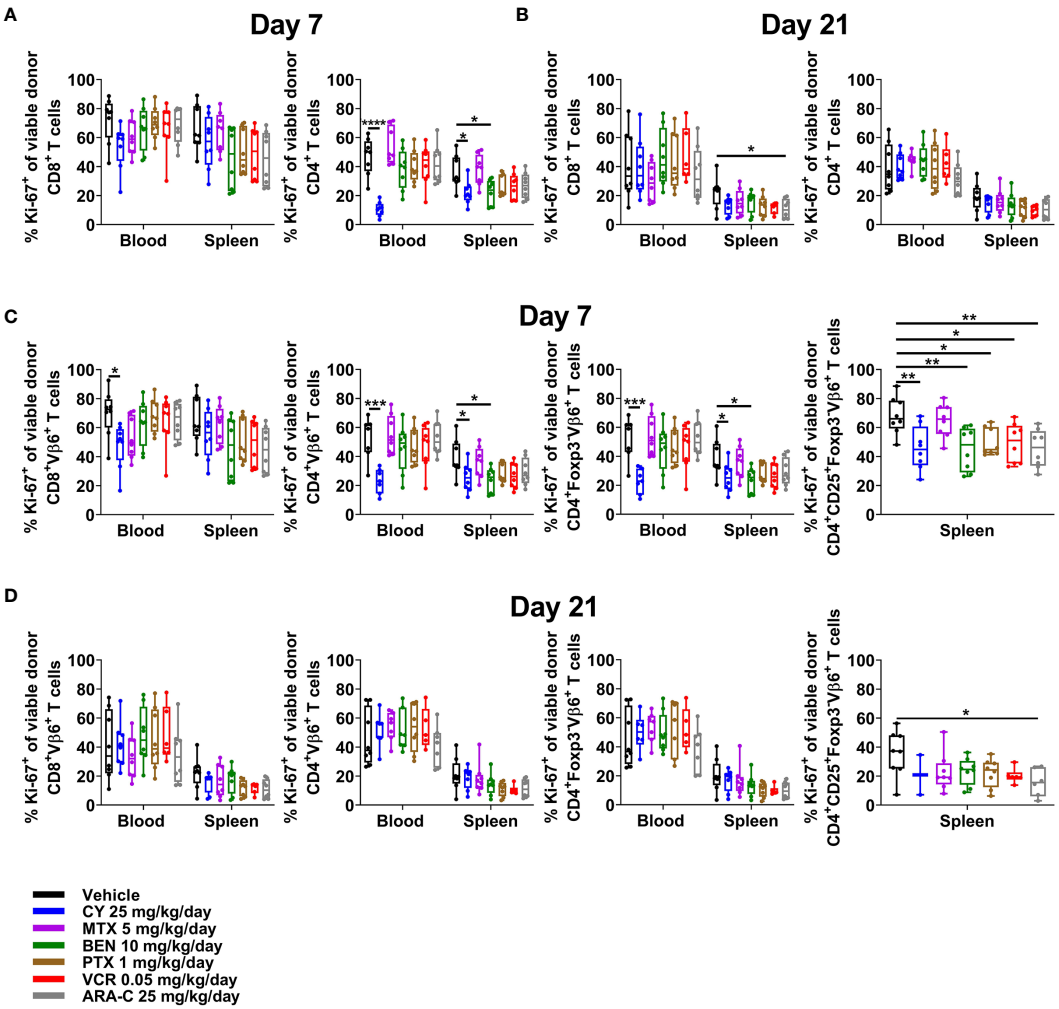


**FIGURE 6** | Only CY consistently reduces alloreactive CD4$^+$ T-cell proliferation at day +7. Mice were transplanted, treated with PBS or a chemotherapeutic on days +3 and +4, and euthanized for flow cytometric assessment at day +7 or +21 as in **Figure 3**. **(A)** In both the blood and spleens at day +7, CY reduced proliferation (Ki-67$^+$) of CD4$^+$ T cells. **(B)** At day +21, proliferation was generally similar across all groups. **(C)** CY also reduced proliferation of alloreactive (Vβ6$^+$) conventional CD4$^+$ T cells at day +7. Interestingly, BEN, PTX, VCR, and ARA-C significantly reduced percentages of proliferating alloreactive CD4$^+$CD25$^+$Foxp3$^-$ T cells at day +7, but did not affect proliferation of other alloreactive CD4$^+$ T cells. **(D)** At day +21, proliferation of alloreactive subsets again was generally similar across all groups. Percentages of proliferating alloreactive CD4$^+$CD25$^+$Foxp3$^-$ T cells in the blood are not shown in **(C, D)** due to low total numbers of CD4$^+$CD25$^+$Foxp3$^-$Vβ6$^+$ cells across treatment groups that did not permit reliable determination of further subsetting. Combined results from two independent experiments are shown with n = 4/group/experiment for **(A–D)** except for VCR (n = 6 total) in **(B, D)** due to excess early deaths. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 on one-way ANOVA followed by the Holm-Sidak *post hoc* test using the vehicle-treated group as the control. Only significant results are shown; all other comparisons between treatment groups and the vehicle group are non-significant.

restraining alloreactive CD4$^+$Foxp3$^-$ T-cell differentiation at day +7, with less effector/effector memory and more naïve/central memory phenotypes (**Figure 7A**). This effect was most pronounced for CY and even more evident when looking at all CD4$^+$Foxp3$^-$ T cells (**Supplementary Figure 6**), which also would include alloreactive T cells beyond those that are Vβ6$^+$. Furthermore, the restraint of alloreactive CD4$^+$ T-cell differentiation continued after CY at day +21, whereas MTX-treated mice actually had slightly more highly differentiated alloreactive CD4$^+$ T cells at that time point (**Figure 7B** and **Supplementary Figure 6**).

## DISCUSSION

Using our previously described MHC-haploidentical murine HCT model (5), we tested the relative efficacy of five other chemotherapeutics (MTX, BEN, PTX, VCR, and ARA-C) in comparison with cyclophosphamide when given as GVHD prophylaxis early post-transplant. We demonstrated that PTCy not only was superior to all other tested chemotherapeutics in ameliorating severe GVHD clinically and histopathologically, but also showed that some of the effects of PTCy on T-cell subsets appear unique. Similar to PTCy, the other partially effective

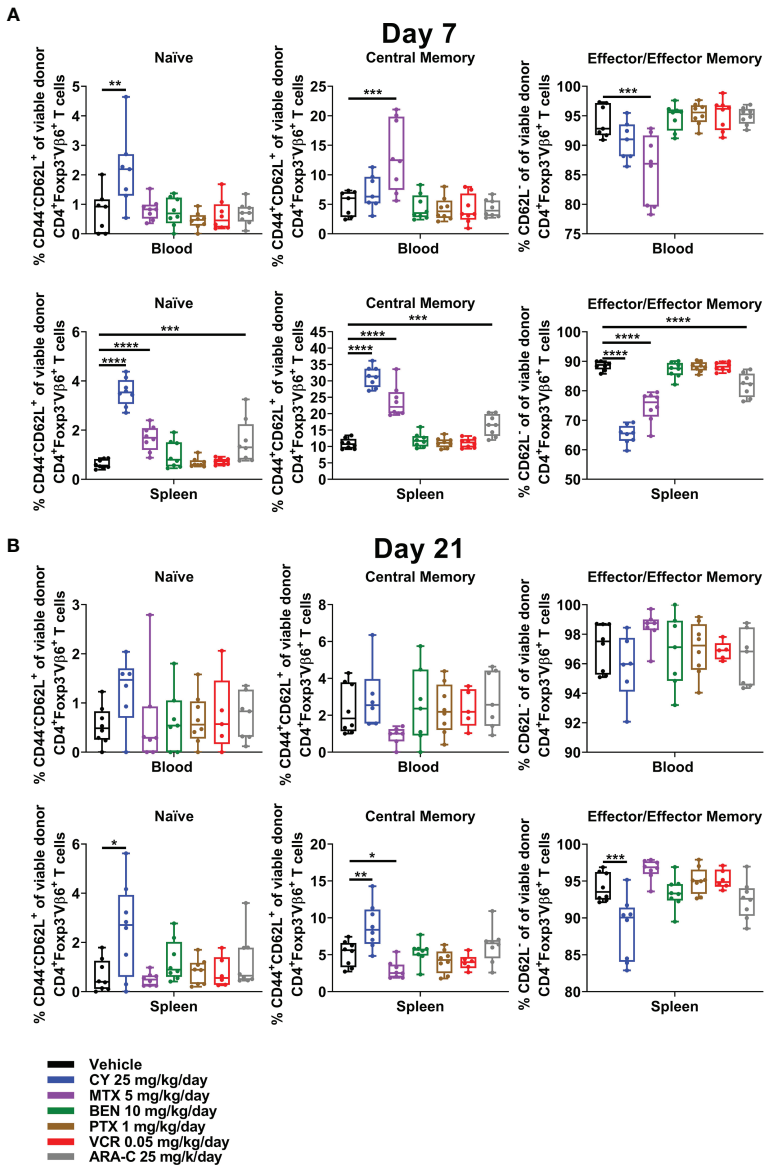


**FIGURE 7** | CY uniquely restrains T-cell differentiation at both early and later timepoints. Mice were transplanted, treated with PBS or a chemotherapeutic on days +3 and +4, and euthanized for flow cytometric assessment at day +7 or +21 as in **Figure 3**. **(A)** At day +7, CY decreased percentages of $CD4^+Foxp3^-V\beta6^+$ T cells that were phenotypically effector/effector memory ($CD62L^-$). Consequently, percentages of naïve ($CD44^-CD62L^+$) and central memory ($CD44^+CD62L^+$) $CD4^+Foxp3^-V\beta6^+$ T cells were increased by CY at day +7. This same effect was achieved to a lesser extent after both MTX and ARA-C. **(B)** This restrained differentiation was persistent after CY at day +21 but was completely lost after MTX, wherein differentiation seemed to be overall accelerated. Combined results from two independent experiments are shown with n = 4/group/experiment except for VCR (n = 6 total) in **(B)** due to excess early deaths. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 on one-way ANOVA followed by the Holm-Sidak *post hoc* test using the vehicle-treated group as the control. Only significant results are shown; all other comparisons between treatment groups and the vehicle group are non-significant.

chemotherapeutics, MTX and ARA-C, did constrain conventional T-cell numerical recovery at day +21 and facilitated preferential recovery of $T_{regs}$ at day +21. But, unlike PTCy, MTX and ARA-C did not reduce percentages of alloreactive $CD4^+$ conventional T cells that were activated (as measured by CD25 expression) or proliferating at day +7 and only restrained alloreactive $CD4^+$ conventional T-cell differentiation at day +7 (not day +21); indeed, MTX was associated with a more differentiated phenotype at day +21 compared with even vehicle-treated mice. These findings provide further support for reduced alloreactive $CD4^+$ conventional T-cell proliferation at day +7 and preferential $T_{reg}$ recovery at day +21 as potential biomarkers for effective GVHD control by PTCy as identified in our previous publications on optimal dosing and timing of PTCy (5, 6). Additionally, we

propose restrained alloreactive $CD4^+$ conventional T-cell activation and differentiation as possible additional elements that may further explain PTCy's superior efficacy in preventing GVHD. The discrepancy between activation markers of CD25 and phospho-STAT5 positivity within $CD4^+$ conventional T cells in PTCy-treated mice may suggest that activation is incomplete or impaired after PTCy, and we are working to better understand this phenomenon in the laboratory.

It is difficult to determine if PTCy causes preferential deletion of differentiated effector/effector memory alloreactive $CD4^+$ conventional T cells or has a direct effect on alloreactive $CD4^+$ conventional T cells that prevents their subsequent differentiation. Although this distinction is impossible to definitively tease out with this model system, the continued increased percentages of naïve/central memory cells and decreased percentages of effector/effector memory cells at day +21 seen in PTCy-treated mice would support the latter possibility. We are continuing to study in the laboratory the nature of the alloreactive T-cell dysfunction induced by PTCy that may contribute to GVHD prevention and restrained T-cell proliferation and differentiation.

PTCy previously was thought to work *via* the selective elimination of alloreactive T cells, as these cells would be rapidly proliferating in the early post-transplant time period. However, this hypothesis was extrapolated from data in MHC-matched murine skin-allografting models that were extremely contextual and had questionable relevance to HCT (5, 57–60). Our recently published work disproved this hypothesis in HCT, showing that alloreactive T cells persisted after PTCy administration even at high, intolerable PTCy doses (5). Both in our current and prior studies (5, 6), we did find a small transient reduction in the percentages of alloreactive $CD4^+$ conventional T cells after PTCy in some organs, and here we also found that CD25-expressing activated alloreactive $CD4^+$ conventional T cells were reduced. We cannot exclude that PTCy in some organs may preferentially diminish alloreactive T cells, particularly activated and proliferating alloreactive T cells, but any such effect is minor, incomplete, and short-lived. Alternatively, this effect could be explained by relative restraint of activation, proliferation, and expansion of surviving alloreactive T cells rather than selective killing, consistent with our findings of similar pSTAT5 signaling but reduced CD25 expression in PTCy-treated mice. Even so, all our prior and current data clearly show that alloreactive T cells are not selectively eliminated by PTCy (5, 61).

In retrospect, the lack of selective alloreactive T-cell elimination should not be surprising since cyclophosphamide, as an alkylator, is a non-cell-cycle-specific chemotherapeutic. Thus, it appears that PTCy may be killing a substantial percentage of T cells in a dose-dependent manner, but this effect is broad and not selective for alloreactive T cells (5, 61). Interestingly, none of the chemotherapeutics tested in these experiments selectively eliminated alloreactive T cells, despite the use of some cell-cycle-specific chemotherapeutic agents; by contrast, the percentages of alloreactive T cells were even higher after MTX at day +7. Even though none of these other chemotherapeutics greatly affected either the relative percentages of alloreactive T cells or global T-cell proliferation, all drugs except MTX did slightly reduce the proliferation of activated ($CD25^+$) alloreactive $CD4^+$ conventional T cells at day +7. Overall though, the decline was modest and not associated with substantial clinical or histopathologic reduction in GVHD severity for most drugs.

Moreover, no chemotherapeutic induced pan T-cell depletion at day +7 compared with vehicle-treated mice, which may in part be attributable to intermediate rather than maximally tolerated doses being tested as intermediate doses were the most optimal for survival for each chemotherapeutic. Even so, we have previously shown that very high doses of PTCy, which did greatly reduce T-cell counts at day +7, resulted in preferential survival of alloreactive T cells at day +7 and actually led at day +21 to a tremendous rebound in alloreactive T-cell counts, consequent blunting of regulatory T-cell recovery, and worse GVHD in our model compared with intermediate dosing (5). Indeed, early results from a PTCy dose de-escalation clinical study at our institution suggest that intermediate-dose PTCy maintains excellent protection against acute GVHD (62). Whether such results also are true for other chemotherapeutics is unknown, but is important to understand, particularly given the recent attempts to implement post-transplantation bendamustine clinically (17, 18).

A limitation of this study is that all the alternative chemotherapeutics tested were administered on days +3/+4 to best compare against PTCy each chemotherapeutic's effects on GVHD and immune subsets; PTCy is administered in this manner in clinical practice and administration on days +3/+4 is among the most effective PTCy dosing schedules in our MHC-haploidentical murine HCT model (6). We have hypothesized that PTCy administration on days +3/+4 is particularly effective because during this critical window specific T-cell subsets are metabolically primed, based on differential dynamic expression of ALDH and ABC transporter activity, for differential sensitivity to cyclophosphamide (3–7, 9). In previous experiments using our MHC-haploidentical murine HCT model, earlier administration of PTCy on days +1/+2 was associated with a less robust decrease in alloreactive $CD4^+$ conventional T-cell proliferation at day +7 and later dosing on days +5/+6 was associated with a blunted relative recovery of $T_{regs}$ by day +21, likely contributing in either case to the lower efficacy seen when compared with PTCy on days +3/+4 (6). Clinical success of PTCy was not achieved until the dose and timing were better optimized; in fact, early clinical studies using serial administration for 100 days of low dose PTCy suggested partial efficacy that was inferior to cyclosporine when each was combined with methylprednisolone (63). Moreover, it is possible that other chemotherapeutics may be more effective when given in different dosing schedules, and such optimal dosing schedules might vary depending on the relative metabolism of each drug. For example, MTX's current dosing schedule for acute GVHD prophylaxis, which was derived from animal studies (45, 64, 65) and then tested clinically (46, 47, 66), is distinct from PTCy with administration on days +1, +3, +6, +/- +11. Nevertheless, additional studies on optimal timing for administration of other chemotherapeutics would be necessary to determine if the

day +3/+4 timing is universally optimal or whether another chemotherapeutic administered in a different schedule or in combination with PTCy may produce similar or superior results to PTCy alone.

Our data showed no clear association between drug metabolism or resistance pathways and effective GVHD control. We had specifically chosen chemotherapeutics with varying involvement of ABC transporters and ALDH in resistance pathways (**Table 1**) to explore their relative efficacy. Interestingly, resistance patterns of the partially effective drugs (CY, MTX, and ARA-C) diverge (8, 12–14, 20–23), but may explain why each had differing effects on T-cell subset activation, proliferation, differentiation, and recovery kinetics. Yet, resistance patterns are not very dissimilar for the ineffective drugs compared with the effective drugs (**Table 1**) (8, 12–14, 19–36), suggesting either a small therapeutic index to effectively prevent GVHD or that the cellular impact of these drugs and/or their metabolism may be more complicated than we currently appreciate. Furthermore, MTX still facilitated the preferential recovery of $T_{regs}$ by day +21 and even expanded $T_{regs}$ at day +7. However, when considering ALDH versus ABC transporter activity, ABC transporters, which $T_{regs}$ lack (67), likely play a comparatively larger role in resistance to MTX (**Table 1**) (12–14, 21–23, 30, 31, 36). By contrast, MTX has not been reported to be metabolized *via* ALDH which appears important for mediating $T_{reg}$ resistance to cyclophosphamide (3, 4). Even so, the recovery kinetics of $T_{regs}$ in MTX-treated mice were distinct from all other chemotherapeutics as was the balance of $CD4^+$ versus $CD8^+$ T cells. MTX uniquely facilitated increased percentages of $T_{regs}$ at day +7, which may have mitigated effects of the increased percentages of activated ($CD25^+$) alloreactive $CD4^+$ conventional T cells at that timepoint. The divergence in some immunologic effects of MTX and PTCy suggests that GVHD control by MTX may occur *via* a different mechanism than PTCy and may explain why MTX is only effective for acute GVHD clinically, while PTCy can prevent both acute and chronic GVHD.

An additional interesting finding of this study is that BEN was ineffective in ameliorating severe and fatal GVHD in our MHC-haploidentical HCT model. Recently, post-transplantation BEN has shown promise in pre-clinical murine HCT studies (16) and mixed results in early phase clinical trials (17, 18). It is possible that our conflicting findings may be explained in part due to differences in models or relative doses of CY or BEN, which were higher in the CB6F1→CAF1/J model (16) than we have determined to be optimal at preventing GVHD in our B6C3F1→B6D2F1 model (5). Additionally, a recent clinical study examining post-transplantation BEN showed that patients developed severe cytokine release syndrome, often manifesting with liver dysfunction, at unacceptably high rates (18). This may parallel the ascites secondary to liver failure that developed in our BEN-treated mice. Ultimately, our results and the recent clinical study (18) suggest the need for caution in considering BEN as a suitable, safe, and effective alternative to PTCy until more mature clinical data are available.

In conclusion, our results provide further insight into the mechanisms of PTCy and the biology of GVHD prevention. The clinical and immunologic effects of cyclophosphamide given in the early post-transplant period appear unique and not fully reproducible by another alkylating agent or four other chemotherapeutics of multiple classes given over the same dosing schedule. Our data show that effective GVHD prophylaxis is associated with distinctive effects on constraining alloreactive conventional T-cell numerical reconstitution and facilitating preferential $T_{reg}$ recovery at day +21, but also uncover that PTCy uniquely restrains alloreactive $CD4^+$ conventional T-cell proliferation and differentiation. To what extent these findings hold true in patients, particularly those receiving adjunct immunosuppression beyond PTCy or to patients undergoing combined HCT/solid organ transplantation (68, 69), requires further exploration.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by National Cancer Institute Animal Care and Use Committee.

## AUTHOR CONTRIBUTIONS

CK designed the study. AH contributed to the study design. AH, NN, SK, RF, and AP performed the experiments. AH and CK analyzed the data. ME performed blinded assessments of histopathology. DV designed and performed the statistical analyses. All authors interpreted the data. AH and CK designed and created the tables and figures. AH and CK wrote the manuscript, and all authors revised the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.796349/full#supplementary-material

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# Targeting the Retinoid X Receptor Pathway Prevents and Ameliorates Murine Chronic Graft-Versus-Host Disease

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Most allogeneic hematopoietic stem cell transplant (allo-HSCT) recipients receive peripheral blood stem cell grafts resulting in a 30%–70% incidence of chronic graft-versus-host disease (cGVHD), a major cause of mortality and morbidity in long-term survivors. While systemic steroids remain the standard of care for first-line therapy, patients may require long-term administration, and those with steroid-resistant or refractory cGVHD have a worse prognosis. Although durable and deep responses with second-line therapies can be achieved in some patients, there remains an urgent need for new therapies. In this study, we evaluated the efficacy of IRX4204, a novel agonist that activates RXRs and is in clinical trials for cancer treatment to prevent and treat cGVHD in two complementary murine models. In a major histocompatibility complex mismatched, non-sclerodermatous multiorgan system model with bronchiolitis obliterans, IRX4204 prevented and reversed cGVHD including associated pulmonary dysfunction with restoration of germinal center T-follicular helper: T-follicular regulatory cell balance. In a minor histocompatibility antigen disparate sclerodermatous model, IRX4204 treatment significantly prevented and ameliorated skin cGVHD by reducing Th1 and Th17 differentiation due to anti-inflammatory properties. Together, these results indicate that IRX4204 is a promising therapeutic option to treat cGVHD with bronchiolitis obliterans or sclerodermatous manifestations.

**Keywords: RXR, TFH, TfR, IL-17, germinal center B cells**

## INTRODUCTION

Chronic graft-versus-host disease (cGVHD) is a life-threatening complication following allogeneic hematopoietic stem cell transplantation (allo-HSCT), which causes late non-relapse morbidity and mortality (1, 2). Although recent studies have advanced the understanding of GVHD pathophysiology, the first-line therapy remains corticosteroids, which can achieve a complete response in only 20%–50% of patients (3, 4). For patients that do not respond to steroid therapy, the

mortality rate is high (5). Furthermore, allo-HSCT recipients undergoing broad immunosuppressant therapy are more prone to tumor relapse, infections, and drug-related toxicities. Therefore, novel targeted immunomodulatory strategies are highly warranted to improve clinical outcomes in allo-HSCT.

Chronic GVHD patients with either bronchiolitis obliterans (BO), an obstructive pulmonary disease, or scleroderma complications are poor responders to standard available therapies (6–9). Moreover, complete and durable responses in steroid refractory or dependent cGVHD are infrequent. Hence, multiple preclinical cGVHD models have been developed to represent aspects of the spectrum of cGVHD manifestations and further elucidate cGVHD pathogenesis in an attempt to develop therapeutic strategies (10–15). Since cGVHD with BO has a poor prognosis, a cGVHD BO model was developed as a platform to evaluate new therapies. Toward that end, B10.BR ($H2^k$) recipients were conditioned with high-dose cyclophosphamide and total body irradiation prior to reconstitution with major histocompatibility complex (MHC)-disparate C57BL/6 ($H2^b$) bone marrow and a low dose of T cells, recapitulating many of the clinical, functional, and pathological manifestations of cGVHD with BO (13, 16). Chronic GVHD pathogenesis with BO was dependent upon alloreactive donor CD4+ T cells that differentiated into T-follicular helper (Tfh) cells to activate germinal center (GC) B cells, resulting in pathogenic antibody production and disposition onto cGVHD target organs. Monocyte and macrophage recruitment into areas of lung injury results in stimulation of fibroblast release of profibrogenic molecules and fibrosis in target organs except the skin (17). In a minor histocompatibility antigen disparate scleroderma model, BALB/c ($H2^d$) recipients underwent total body irradiation prior to reconstitution with B10.D2 ($H2^d$) bone marrow and T cells resulting in fibrotic skin disease mediated by donor T helper 1 (Th1) and Th17 cells (18).

Retinoid X receptors (RXRs) are key members of the nuclear receptor (NR) superfamily due to their diverse roles in modulating various physiological processes (19). RXRs form homodimers (RXR-RXR) and heterodimers with other NRs, namely, retinoic acid receptors (RARs), thyroid hormone receptor, liver X receptors (LXRs), vitamin D receptor, farnesoid x receptor, nuclear receptor related 1 protein (Nurr1, Nr4a2), nerve growth factor IB (Nur77, Nr4a1), and peroxisome proliferator-associated receptors (PPARs) (20). Studies in mice have reported the direct role of RXR in controlling Th1 differentiation with loss of RXRα in CD4+ T cells leading to increased Th1 polarization and interferon gamma (IFN-γ) production (21). Since RXRα is also required to maintain the suppressive function of T regulatory cells (Tregs), RXR agonists could have dual benefits as a therapeutic strategy for controlling inflammatory disorders.

Rexinoids are RXR ligands that selectively bind and activate RXRs. Although rexinoids can have high therapeutic value in treating various metabolic disorders and cancers (22, 23), off-target responses due to RXR promiscuity with NRs other than RARα limited their clinical applications (24–26). For instance, in addition to RARα, Food and Drug Administration (FDA)-approved synthetic rexinoid bexarotene binds with liver-X-receptor (LXR) and PPAR (27–29). IRX4204 is a highly selective rexinoid with a potency of 100-fold more than bexarotene in activating RXRs. Specifically, IRX4204 does not transactivate RXR heterodimers of RAR, LXR, or PPAR (30), consistent with the selective biological function of IRX4204 and pointing to its use as a therapeutic agent. Recent studies have demonstrated the therapeutic efficacy of IRX4204 in controlling murine autoimmune disease and acute GVHD (aGVHD) (20, 31). Administration of IRX4204 in a murine model of multiple sclerosis attenuated the severity of the disease (20). We previously showed that IRX4204 treatment ameliorated aGVHD while retaining graft-versus-tumor (GVT) responses against leukemia and lymphoma cells (31). Since the pathophysiology of cGVHD is distinct from aGVHD, we sought to test the prophylactic and therapeutic efficacy of IRX4204 in two pathologically distinct cGVHD models. Herein, we demonstrate that IRX4204 administration prevents and treats both BO and sclerodermatous manifestations of cGVHD. Mechanistically, IRX4204 impairs pathogenic donor Tfh, Th1, and Th17 differentiation leading to protection from cGVHD.

## MATERIALS AND METHODS

### Mice

Female C57BL/6 (B6;$H2^b$) and BALB/c ($H2^d$) mice were purchased from the National Cancer Institute. Female B10.BR ($H2^k$) and B10.D2 ($H2^d$) mice were purchased from The Jackson Laboratory. All mice ranged in age from 10 to 18 weeks. Mice were housed in a specific pathogen-free facility, and all studies were approved by the University of Minnesota's Institutional Animal Care Committee.

### BM Transplantation

For the BO model, B10.BR recipients were conditioned with cyclophosphamide on days −3 and −2 (120 mg/kg/day intraperitoneally). On day −1, recipients received total body irradiation (TBI) by X-ray [8.3 Gray (Gy) by X-ray)]. Recipients then received $10 \times 10^6$ B6 T-cell-depleted (TCD) bone marrow (BM) only or with $7–7.5 \times 10^4$ purified splenic T cells on day 0 (17, 32). In the scleroderma model, BALB/c mice were given lethal TBI (7 Gy by X-ray, day −1) and $10^7$ B10.D2 TCD-BM only or with $1.8 \times 10^6$ $CD4^+$ and $0.9 \times 10^6$ CD8 T cells (day 0) (32, 33). Mice were monitored daily for survival. Skin scores were assessed twice weekly (32).

### Pulmonary Function Tests

Pulmonary function tests (PFTs) were performed as previously described (16). Briefly, mice were anesthetized with Nembutal, intubated and ventilated using the Flexivent system (Scireq Montreal, QC). Pulmonary resistance, elastance, and compliance were reported using Flexivent software version 7. Chronic GVHD controls have increased pulmonary resistance and elastance along with decreased compliance as compared to BM-only controls in our BO cGVHD model (13, 16).

## Flow Cytometry

For Tfh and GC B cells, single-cell suspensions of spleens were obtained and stained with fluorochrome-labeled anti-CD4 (RM4-5, BD), anti-CXCR5 (SPRCL5, Thermo Fisher Scientific, MA, USA), anti-PD-1 (J43, Thermo Fisher Scientific), anti-CD19 (eBio1D3, Thermo Fisher Scientific), anti-GL7 (GL-7, Thermo Fisher Scientific), and anti-Fas (J02, BD). Live/dead fixable viability dye (Thermo Fisher Scientific) was used for live/dead discrimination. GC B cells were defined as Fas and GL7 double-positive CD19 B cells. Tfh cells were defined as PD1 and CXCR5 high CD4+ Foxp3− T cells. Tfr cells were defined as PD1, and CXCR5 high CD4+ Foxp3+ T cells. Tregs were detected by staining cells for surface antigens, followed by fixation, permeabilization using a Foxp3/transcription factor staining buffer set (Thermo Fischer Scientific), and labeling with anti-Foxp3 (FJK-16s, Thermo Fisher Scientific). For intracellular cytokine staining experiment, lymph node and spleen cells were stimulated with cell stimulation cocktail (plus protein transport inhibitors) (Thermo Fisher Scientific) for 5 h at 37°C. After surface staining, cells were fixed, permeabilized, and stained with anti-interleukin-17 (anti-IL-17) (eBio17B7, Thermo Fisher Scientific) and anti-IFN-γ (XMG1.2, Thermo Fisher Scientific). BD LSRFortessa (BD Biosciences, CA, USA) was used to acquire cells, and analyses were performed using FlowJo software.

## Immunofluorescence

For the GC detection, acetone-fixed 6-μm cryosections of spleens were stained with rhodamine-peanut agglutinin (Vector Laboratories, CA, USA). For CD4 and B-cell staining, sections were stained with CD4 FITC (RM4-5, Thermo Fisher Scientific) and B220 BV421 (RA3-6B2, BD). GCs are identified as PNA+ regions with B220+ and/or CD4+ areas surrounding them. Confocal images were acquired on an Olympus FluoView500 Confocal Laser Scanning Microscope at 200×, analyzed using FluoView3.2 software (Olympus), and quantified using a Voronoi tessellation methodology making use of EBImage (34).

## NP-OVA Immunizations

B6 mice were immunized with 4-hydroxy-3-nitrophenylacetyl hapten (NP)-OVA (100 μg, Bioresearch Technologies, Novato, CA, USA), diluted in complete H37 Ra (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) in each flank (35). For IRX4204 prophylaxis, mice were given IRX4204 on days 0–7 daily (i.p.), then sacrificed 7 days post-immunization, and the inguinal lymph nodes were harvested and analyzed by flow cytometry. For therapeutic purposes, mice were treated with IRX4204 on days 8–14 daily (i.p.) 7 days post-immunization. On day 14, inguinal lymph nodes were harvested and analyzed by flow cytometry.

## IRX4204

IRX4204 (Io Therapeutics, USA) was prepared in phosphate-buffered saline (PBS) [containing ~4% dimethyl sulfoxide (DMSO) and 1% Tween 80], once in a week and stored at 4°C (36). Chronic GVHD recipients were given vehicle or IRX4204 daily at a dose rate of 200 μg/mouse i.p as indicated.

## Histopathology and Trichrome Staining

Tissue sections were embedded in optimal cutting temperature (OCT) compound, snap-frozen in liquid nitrogen, and stored at −80°C. Lungs were inflated by 75% OCT before harvest and freezing. Acetone-fixed 6-μm cryosections were hematoxylin and eosin stained and evaluated as described (37). For trichrome staining, 6-μm cryosections were fixed for overnight in Bouin's solution and stained with the Masson's trichrome staining kit (Sigma HT15) for detection of collagen deposition.

## Statistical Analysis

GraphPad Prism 7 was used to conduct statistical analysis. One-way ANOVA with Bonferroni correction and Student's t-test were used for statistical analysis as indicated. Error bars indicate mean ± standard error mean (significance: $*p < .05$; $**p < .01$; $***p < .001$; $****p < .0001$).

# RESULTS

## IRX4204 Prevents and Reverses BO cGVHD

To evaluate the prophylactic efficacy of IRX4204 in cGVHD, we utilized a major MHC mismatched, murine multiorgan system model of cGVHD with BO. In this model, B10.BR recipients were preconditioned with cyclophosphamide and TBI followed by transplantation with donor B6 TCD BM alone or TCD-BM plus a low dose of T cells (13, 16). We previously reported that the loss of pulmonary function due to fibrotic change in the lung was detected as early as day 28 (13). Using a FlexiVent (SCIREQ) system, we performed PFTs on day 28 in cGVHD recipients treated with either vehicle or IRX4204. Recipients receiving IRX4204 daily from day 0 to 28 had significantly improved pulmonary function compared to those given vehicle (**Figure 1A**). These studies showed that cGVHD with BO was established by day 28 as demonstrated by PFTs and that IRX4204 given days 0–28 was sufficient to prevent cGVHD onset. Furthermore, mice given prophylactic IRX4204 showed improved PFTs by day 56 as compared to control recipients (**Figure 1B**).

We next sought to determine whether IRX4204 could reverse the established cGVHD by treating cGVHD mice beginning on day 28. On day 56, cGVHD mice that were continuously treated from day 28 showed improved PFTs as compared to vehicle-treated cGVHD recipients and comparable to BM-only recipients (**Figure 1B**) Consistent with the reduced cGVHD clinical signs, pathology scores of lungs and liver were significantly lower in recipients of either prophylactic or therapeutic IRX4204 as compared to vehicle controls (**Figure 1C**). No significant changes were observed in the spleen and colon of mice that received IRX4204.

Increased Tfh and GC B cells can promote while Tfrs can inhibit BO cGVHD pathogenesis by controlling GC formation and allo- or auto-antibody production and deposition in cGVHD

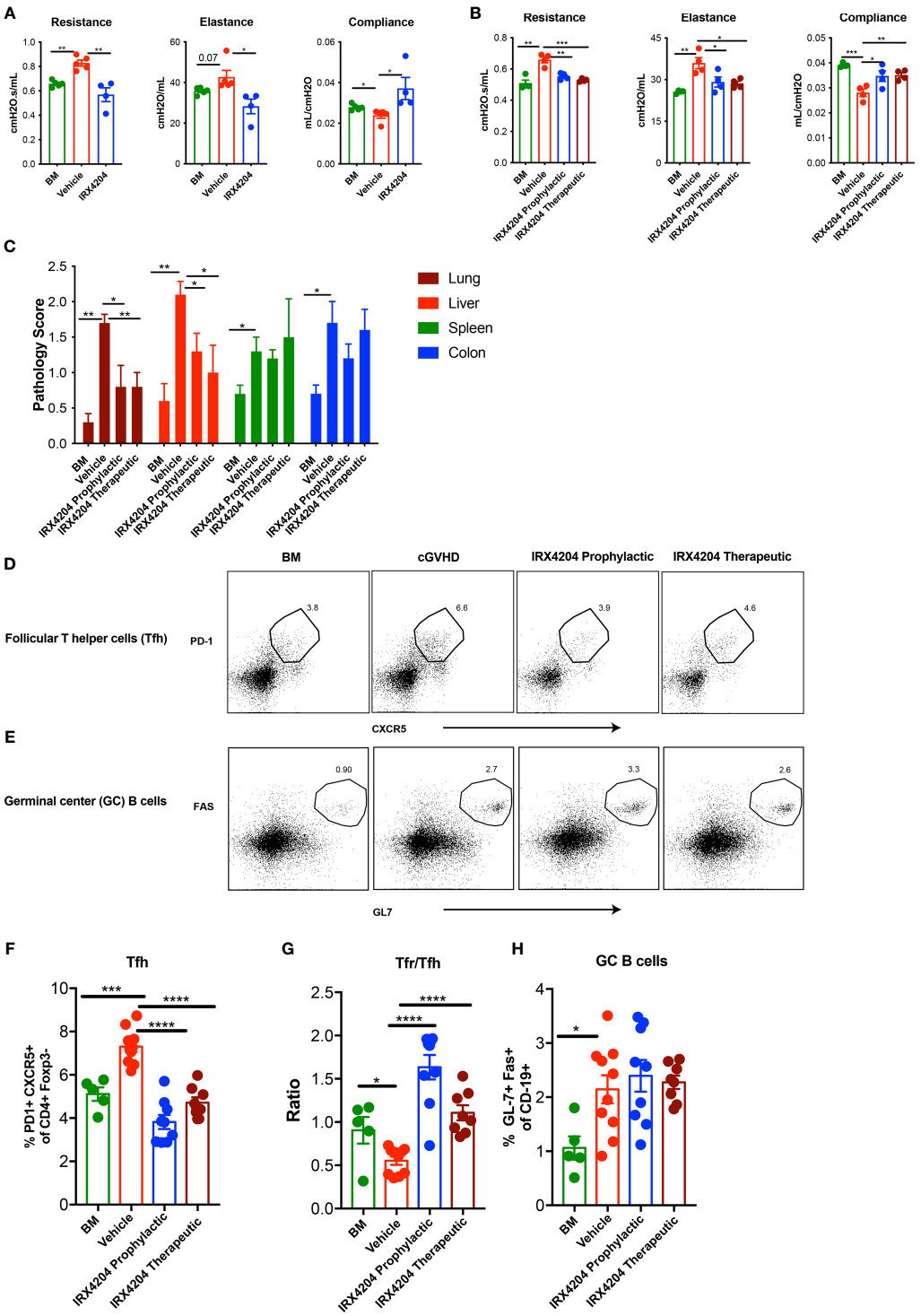


**FIGURE 1** | IRX4204 prevents and reverses established cGVHD-mediated BO. Conditioned B10.BR mice were transplanted with B6 donor BM ± T cells. A cohort of BM + T recipients were treated with IRX4204 either from days 0 to 28, or 28 to 56. Pulmonary tests including lung resistance, elastance, and compliance were performed on **(A)** day 28 and **(B)** day 56 post-transplantation n = 4 – 5/group. **(C)** Histopathology scores of hematoxylin and eosin–stained tissue sections from lung, liver, spleen, and colon on recipient on day 58. n = 5/group. Representative flow plots of **(D)** Tfh and **(E)** GC B cells. Frequency of Tfh **(F)**, Ratio of Tfr/Tfh **(G)** and frequency of GC B cells **(H)** in recipient spleen on day 58. *$p$ <.05; **$p$ <.01; ***$p$ <.001; ****$p$ <.0001. Error bars represent standard error of the mean (SEM); n = 5 - 10 per group.

target tissues (17, 38). To determine whether IRX4204 treatment induces immune cell changes that could preclude or disrupt GC formation in cGVHD mice, we analyzed the frequency of Tfh, Tfrs, and GC B cells in day 58 post-BMT spleens of recipients treated daily with IRX4204 from day 0 to 28 or day 28 to 56. IRX4204-treated mice had a reduced frequency of pathogenic Tfh with an increased frequency of immunoregulatory Tfrs, resulting in elevated Tfr/Tfh ratios (**Figures 1D, F, G**). Cumulatively, these data demonstrate that IRX4204 treatment is effective at preventing and reversing cGVHD with BO as measured by PFTs, lung and liver pathology scores, and Tfr/Tfh imbalance associated with diminution of the GC immune responses.

## IRX4204 Impairs GC Reactions

Although the GC B-cell frequency was not significantly different between the groups (**Figures 1E, H**) despite reduced Tfh and increased Tfr and Tfr/Tfh ratio, there was a statistical trend towards smaller GC size in recipients of IRX4204 prophylaxis (**Figures 2A, B**). Diminished GC size is consistent with the known cause–effect relationship between high GC numbers and size and cGVHD with BO (13, 17). Peri-bronchiolar collagen deposition is a characteristic feature of BO-cGVHD (13). We performed Masson's trichrome staining to identify collagen in tissue section. Compared to vehicle controls, mice treated with IRX4204 either as prophylaxis or therapy had a decrease in the accumulation of collagen in the lungs around the bronchioles (**Figure 2C**).

To determine IRX4204 effects on GC reactions due to nominal antigen delivered in adjuvant in a non-cGVHD setting, wild-type mice were immunized with NP-OVA, emulsified in complete H37 Ra. One cohort of mice was treated daily with IRX4204 as prophylaxis for GC formation beginning on the day of immunization. A second cohort was treated with IRX4204 beginning after GC formation beginning on day 7 after immunization. After 7 days of IRX4204 administration, draining lymph nodes were harvested and the frequency of Tfh and GC B cells assayed. IRX4204 treatment as either prophylaxis (**Figures 2D, E**) or therapy (**Figures 2F, G**) significantly inhibited the induction of GC B cells compared to the vehicle treatment. However, we found that IRX4204 had the ability to reduce Tfh induction only when administered prophylactically (**Figures 2D, E**). Despite reduced Tfh in mice given IRX4204 prophylaxis, Tfr/Tfh ratios were unchanged. Not surprisingly, as Tfh were not reduced by IRX4204 given as a therapeutic agent, Tfr/Tfh ratio did differ between IRX4204 and vehicle (**Figures 2F, G**).

## IRX4204 Is Effective as Prophylaxis and Therapy for Sclerodermatous cGVHD

Having demonstrated the potency of IRX4204 in attenuating cGVHD in the BO model that lacks skin manifestations, we next evaluated its efficacy in a classical model of sclerodermatous cGVHD. BALB/c recipients were lethally irradiated and transplanted with multiple minor mismatched B10.D2 donor BM ± T cells. In this model, cutaneous manifestations become apparent at approximately day 21 (39, 40). We tested the prophylactic and therapeutic efficacy of IRX4204 starting treatment from either day 0 or day 21 for a period of 50 days. We found that the administration of IRX4204 either as prophylaxis or therapy significantly improved cGVHD clinical and skin scores compared to vehicle controls (**Figures 3A–C**).

Sclerodermatous cGVHD is mediated by donor CD4+ T cells producing IFN-γ and IL-17 (18). To explore the cellular mechanisms associated with cGVHD protection in IRX4204-treated recipients, we examined the frequency of IFN-γ+ and IL-17+ producing CD4+ T cells in recipient peripheral lymph nodes and spleens. On day 50, compared to control, we found that IRX4204 treatment, both prophylactic and therapeutic, significantly reduced the frequency of CD4+ T cells producing IFN-γ and IL-17 in peripheral lymph nodes (**Figures 3D–G**). In addition, the frequency of splenic CD4+ IFN-γ+ but not IL-17 producing T cells was significantly reduced in the spleens of mice given IRX4204 as prophylaxis or therapy (**Figures 3H, I**). To evaluate whether IRX4204 reduces collagen deposition on skin, serially sectioned skin tissues were stained with Masson's trichrome stain. Compared to vehicle controls, mice treated with IRX4204 either as prophylaxis or therapy had a decrease in the accumulation of collagen in the skin (**Figure 3J**). Taken together, IRX4204 alleviated sclerodermatous cGVHD associated with reduced Th1 and Th17 differentiation in secondary lymphoid organs.

# DISCUSSION

Identification of novel immunomodulatory therapies to prevent and treat cGVHD remains the unmet clinical need in allo-HSCT. In the current study, we demonstrated that a highly potent rexinoid, IRX4204, prevented and treated cGVHD by impairing pathogenic donor T-cell responses in two preclinical models with distinct pathophysiology. Therefore, targeting the RXR pathway with IRX4204 is a promising therapeutic strategy to reduce cGVHD. Furthermore, IRX4204 impairs GC B-cell generation in mice immunized with NP-OVA.

In the MHC mismatched BO model, IRX4204 treatment prevented and reversed established cGVHD as reflected by improved PFTs. Although we found that IRX4204 was effective in reducing the cGVHD-mediated lung and liver pathology, there was no effect on spleen and colon pathology. The lack of effect may be due to contrasting mechanisms of cGVHD in different organs. We previously demonstrated that Tfh, a subset of CD4+ T cells, plays a key role in promoting BO cGVHD pathogenesis by supporting GC B-cell affinity maturation and differentiation to produce pathogenic antibodies (17). An earlier study reported that RXR activation has been shown to negatively affect Tfh differentiation in a chronic model of inflammation (41). A more recent study reported that RARα signaling enhances Tfh differentiation in an airway inflammation mouse model (42), perhaps through inhibiting IL2Rα upregulation on T cells, as IL2R signaling inhibits Tfh differentiation by repressing BCL6. In the current study, we reasoned that IRX4204 would modulate Tfh differentiation in cGVHD mice by directly impairing donor T-cell responses. Indeed, we found that IRX4204 treatment reduced the frequency of pathogenic Tfh cells, consistent with the known direct

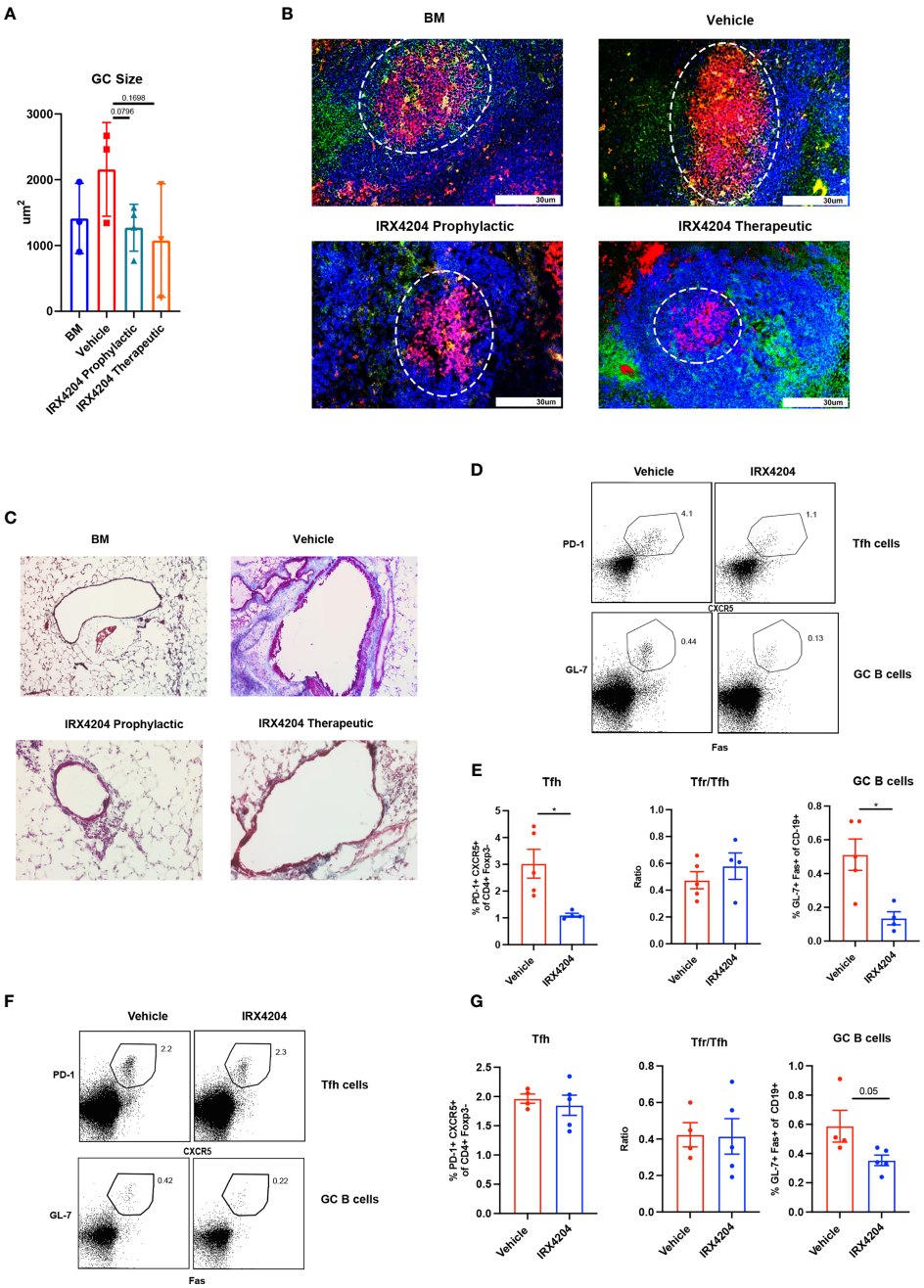


**FIGURE 2** | IRX4204 impairs GC B cell response in an immunization model but not in cGVHD. **(A, B)** Conditioned B10.BR mice were transplanted with B6 donor BM ± T cells. A cohort of BM + T recipients were treated with IRX4204 either from days 0 to 28, or days 28 to 56. n = 3 mice/group. **(A)** GC size and **(B)** representative splenic GC immunofluorescence images from BM only and BM plus T-cell mice on day 58 showing peanut agglutinin (PNA; Red). CD4 FITC (green) and B220 BV421 (blue). Germinal centers are highlighted in white circle. An Olympus FluoView500 confocal laser scanning microscope was used to acquire images at magnification 200×. **(C)** Representative images of Masson's trichrome staining. Collagen was identified as the area stained in blue. EVOS XL Imaging system was used to acquire images at magnification 200×. **(D–G)** WT mice were immunized with NP-OVA and treated with either vehicle or IRX4204 daily. **(D, E)** Flow plots of Tfh, GC B cells, and quantification of GC B cells, Tfh, and Tfr/Tfh ratio from inguinal draining lymph nodes 7 days post-immunization. IRX4204 or vehicle was given from day 0 to 7 (prophylactic). **(F, G)** Flow plots of Tfh, GC B cells, and quantification of GC B cells, Tfh, and Tfr/Tfh ratio from inguinal draining lymph nodes 14 days post-immunization. IRX4204 or vehicle was given from day 7 to 14 (therapeutic). *$p < .05$. Error bars represent standard error of the mean (SEM); n = 4/group.

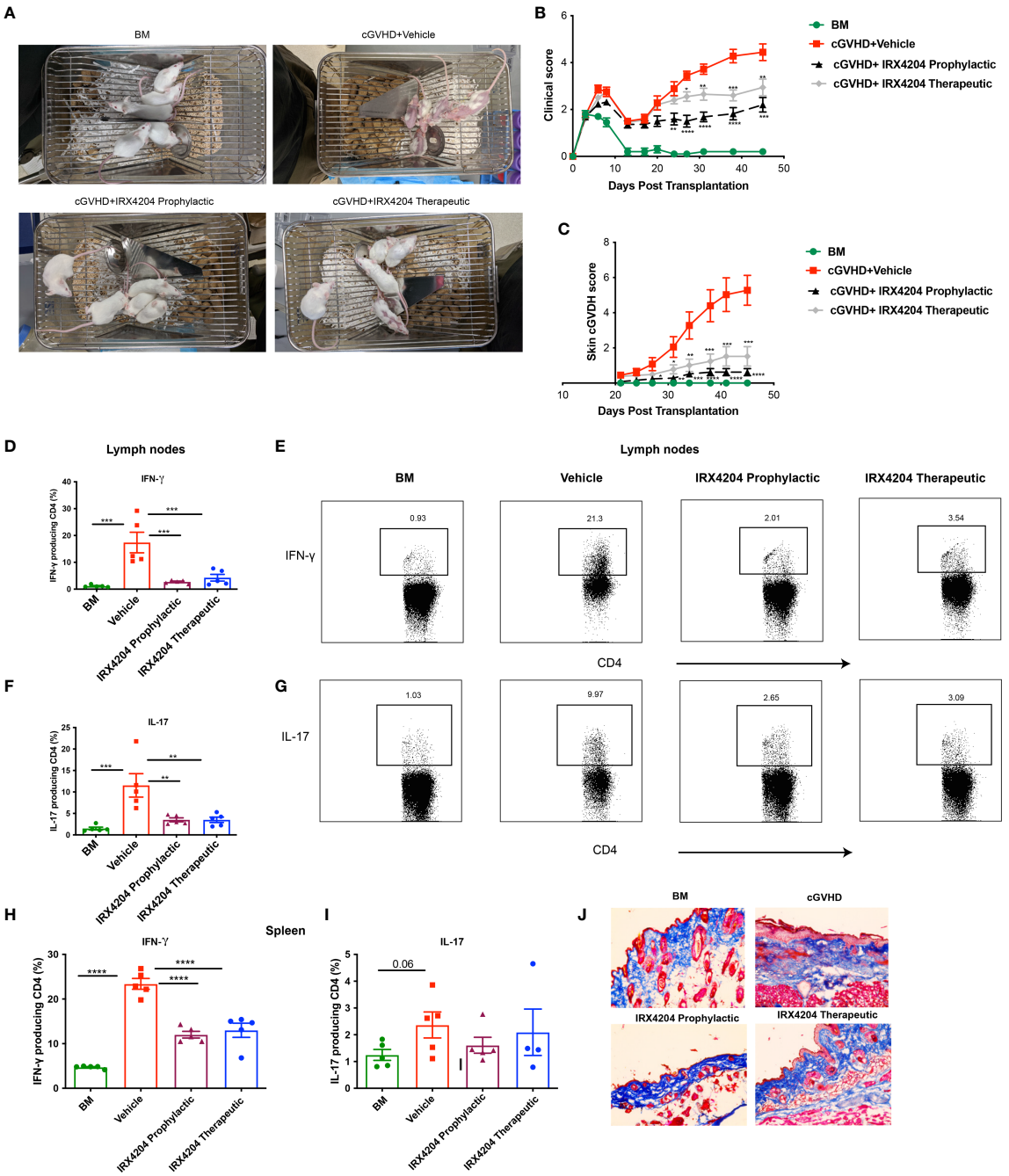


**FIGURE 3** | IRX4204 inhibits and treats sclerodermatous cGVHD. Lethally irradiated BALB/c mice were transplanted with B10.D2 BM only or with purified T cells (CD4, CD8: 1.8 x $10^6$ and 0.9 x $10^6$, respectively). Recipients were treated with IRX4204 either from day 0-21 (IRX4204 prophylactic) or day 21 (IRX4204 therapeutic). **(A)** Photographs of mice in BM, cGVHD + vehicle or IRX4204 treated cGVHD groups. **(B)** Clinical manifestations of cGVHD were assessed by giving scores to weight loss, activity, posture and fur condition. Healthy mice receive score 0. **(C)** Skin scores were assessed by measuring the area of skin with fur loss or sclerodermatous lesion. Intact skin was given a score of 0. **(D–I)** Peripheral lymph nodes (LNs) and spleens (SPL) were harvested on day 50 post-transplantation and stimulated with PMA and ionomycin *in vitro*. **(D–G)** The percentages and representative flow plots of **(D–E)** IFN-γ+ and **(F, G)** IL-17+ producing CD4+ T cells in LNs are shown. **(H, I)** The percentages of **(H)** IFN-γ+ and **(I)** IL-17+ producing CD4+ T cells in SPL are shown. **(J)** Trichrome staining of skin. Collagen was stained in blue. *$p < .05$; **$p < .01$; ***$p < .001$; ****$p < .0001$. Error bars represent standard error of the mean (SEM); n = 8 – 10 per group.

suppressive effect on the proliferation of murine and human T cells *in vitro* (31) and *in vivo* in IRX4204-treated aGVHD mice (31).

Chronic GVHD patients have a reduced frequency of Tregs (43), which leads to the lack of functional tolerance and subsequent immune dysregulation. Restoring immune tolerance through either expansion of Tregs *in vivo* or adoptive Treg transfer has been shown to be an effective strategy to reduce cGVHD (44–46). Here, we show the increased frequency of Tfrs when recipient mice were treated with IRX4204 compared with vehicle-treated recipients. IRX4204 is a potent activator of Nr4a2, which has been shown to promote Treg generation and stability (47). Consistent with the Nr4a2 activity, IRX4204 fostered *in vitro* induced Treg (20, 31) and pTreg generation in aGVHD mice (31).

Studies have shown that Tfrs impair the production of pathogenic antibodies by negatively regulating Tfh and GC B-cells responses (38, 48). Therefore, it is likely that both increased Tfr frequency and Tfr/Tfh ratios in IRX4204-treated mice contribute to the alleviated cGVHD. Furthermore, this cGVHD/BO model depends upon Tfh production of IL-21. IL-21 is counterregulatory to pTreg production, as we reported in an aGVHD model (49), and Treg function (50, 51). Taken together, increased Tfr in IRX4204-treated mice may be due to Nr4a2 pathway activation in donor T cells resulting in suppression of Tfh IL-21 production, fostering Treg suppressor function and the generation of Tregs and Tfrs over Tfhs. By employing the NP-OVA immunization model, we observed that prophylactic IRX4204 reduced the frequency of Tfh and GC B cells, although unexpectedly, we did not observe any significant differences in GC B cells between IRX4204 and vehicle-treated mice in cGVHD/BO mice. However, compared to vehicle-treated mice, IRX4204-prophylactically treated mice had a statistical trend toward smaller GC size.

Scleroderma is a serious and severe fibrosing disorder that occurs in the majority of cGVHD patients affecting the skin, subcutaneous tissue, and fascia (52). In an MHC-matched, multiple minor antigen mismatched scleroderma mouse model (32, 53), IRX4204 treatment as either a prophylactic or therapeutic significantly reduced sclerodermatous cGVHD with improved clinical outcomes. A previous study showed that in a scleroderma cGVHD model, transplantation of either donor IL-$17^{-/-}$ or IFN-$\gamma^{-/-}$ T cells significantly ameliorated the disease (18). In the current study, we observed a reduced frequency of Th1 and Th17 cells in IRX4204-treated recipients, consistent with previous studies showing that IRX4204 impaired Th1 and Th17 differentiation in aGVHD and experimental autoimmune encephalomyelitis, respectively (20, 31). RA signaling is required to maintain the stability of Th1 cells (54). We and others demonstrated that heightened RA synthesis during allo-HSCT exacerbated aGVHD lethality by enhancing Th1 differentiation of donor T cells (55–57). IRX4204 may directly suppress Th1 differentiation *via* Nr4a2 activation, as Nr4a2 has been shown to repress Th1 lineage commitment (47) or as a result of inadequate RA signaling in donor T cells.

Although RA signaling has been shown to exacerbate aGVHD (55–57), Nishimura and colleagues (18) demonstrated that the *in vivo* administration of synthetic retinoid attenuated scleroderma cGVHD by reducing the differentiation of Th1 and Th17. Since IRX4204 preferentially activates RXR homodimers, the endogenous RA signaling pathway that is heightened in GVHD may be impaired in cGVHD mice due to the competitive binding of RXRs to the agonist or other receptors, reducing binding with RARs. Whereas the RXR agonist tributyltin inhibited Th17 differentiation that mechanistically may be the consequence of LXR-RXR pathway activation (58), IRX4204 inhibition of Th17 differentiation *in vitro* and *in vivo* (20) does not require LXR activation, suggesting that RXR homodimers or other RXR binding partners are involved in controlling Th17 differentiation. Notably, IL-17 is known to support GC formation, ectopic lymphoid follicles, and antibody class switching in mouse B cells (59–61). In cGVHD/BO model, the *in vivo* administration of neutralizing anti-IL-17 antibody or small molecule RORγt inhibitors given as a therapeutic markedly alleviated cGVHD (62).

Taken together, our results suggest that targeting the RXR pathway with IRX4204 represents a novel immunomodulatory strategy to prevent or treat cGVHD. IRX4204 is currently in phase I and II clinical trials (NCT0154007 and NCT02991651) to treat cancer. Having shown the beneficial effects of IRX4204 in two distinct cGVHD preclinical models, our studies support consideration for clinical testing of IRX4204 in patients who do not respond to FDA-approved drugs for steroid refractory cGVHD.

## 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

The animal study was reviewed and approved by University of Minnesota.

## AUTHOR CONTRIBUTIONS

GT designed and performed research, provided, and analyzed the data, and wrote the paper. MZ, FM, RF, JD, SR, MR and EA performed experiments, analyzed the data and edited the manuscript. APM performed histopathological analysis. MS provided IRX4204 and edited the manuscript. BB designed, organized, and supervised research and edited the paper. All authors contributed to the article and approved the submitted version.

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# CpG-Activated Regulatory B-Cell Progenitors Alleviate Murine Graft-Versus-Host-Disease

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Development of Graft Versus Host Disease (GVHD) represents a major impediment in allogeneic hematopoietic stem cell transplantation (HSCT). The observation that the presence of bone marrow and circulating hematogones correlated with reduced GVHD risks prompted us to evaluate whether B-cell progenitors, which provide protection in various autoimmune disease models following activation with the TLR-9 agonist CpG (CpG-proBs), could likewise reduce this allogeneic disorder. In a murine model of GVHD that recapitulates an initial phase of acute GVHD followed by a phase of chronic sclerodermatous GVHD, we found that CpG-proBs, adoptively transferred during the initial phase of disease, reduced the diarrhea score and mostly prevented cutaneous fibrosis. Progenitors migrated to the draining lymph nodes and to the skin where they mainly differentiated into follicular B cells. CpG activation and IFN-γ expression were required for the protective effect, which resulted in reduced $CD4^+$ T-cell-derived production of critical cytokines such as TGF-β, IL-13 and IL-21. Adoptive transfer of CpG-proBs increased the T follicular regulatory to T follicular helper (Tfr/Tfh) ratio. Moreover, CpG-proBs privileged the accumulation of IL-10-positive $CD8^+$ T cells, B cells and dendritic cells in the skin. However, CpG-proBs did not improve survival. Altogether, our findings support the notion that adoptively transferred CpG-proBs exert immunomodulating effect that alleviates symptoms of GVHD but require additional anti-inflammatory strategy to improve survival.

**Keywords: allogeneic stem cell transplantation (allo-SCT), regulatory B-cell progenitors, CpG-proBs, cell therapy, fibrosis, Bregs: regulatory B cells, graft-versus host disease**

## INTRODUCTION

Graft-versus-host disease (GVHD), a donor cell-mediated immune disorder presenting in sequential acute and chronic forms, represents a major drawback for long-term effectiveness of allogeneic hematopoietic stem cell transplantation (HSCT) in hematologic malignancies. Efforts to improve immune regulation to prevent this disease have remained challenging. In addition to regulatory T cell deficiencies in both acute (1) and chronic (2, 3) GVHD, aberrant B cell homeostasis (4), with reduced generation of bone marrow (BM) B lymphoid progenitors (5), low frequencies of naive and memory cells, and a regulatory B cell (Breg) cell defect have recently been described (6, 7) in chronic GVHD. This led to the hypothesis that tolerogenic B-cell progenitors might play a role in the outcome of HSC transplantation. In accordance with this hypothesis, high numbers of donor BM B-cell progenitors were inversely correlated with the occurrence of GVHD in its acute (aGVHD) (8, 9) or chronic (cGVHD) form (10, 11) in HSC-transplanted patients. More recent studies have shown that their expansion at the time of engraftment heralded less frequent development of acute severe GVHD with increased mature B-cell counts and IgG levels post-HSCT (12, 13). Circulating B-cell progenitors have been detected in very low numbers in patients with low-grade acute GVHD scores (14). Whether they exhibit any suppressive properties either directly or by promoting the emergence of other regulatory cell types involved in GVHD inhibition remains unknown so far.

We have recently shown in mice that MyD88-dependent activation of BM cells by the Toll-like receptor-9 (TLR-9) agonist CpG-B as well as its injection *in vivo*, induced the emergence within the BM of a B-cell progenitor population, at the pro-B cell stage of differentiation, endowed with potent suppressive properties against autoreactive $CD4^+$ T cells. Importantly, these progenitors migrated into the autoimmune reaction sites and differentiated *in vivo* into several more mature B-cell subsets, which also shared suppressive properties (15–17). This *in vivo* maturation of the CpG-proBs into suppressive Bregs may account for the long-lasting effect of a single injection of CpG-proBs as well as for their remarkable suppressive potency. Indeed, as few as 60,000 CpG-proBs injected once at the onset of clinical signs were able to provide protection against nonobese type 1 diabetes (T1D) (15) and EAE (16), a murine model of multiple sclerosis.

The efficacy of CpG-proBs in murine autoimmunity models prompted us to examine whether this activated population could likewise provide protection in an allogeneic setting, namely a murine model of GVHD (18) that has been reported as developing along sequential acute and chronic phases and also for sharing features of autoimmune inflammation. To this end, we evaluated the effect of CpG-proBs on GVHD in terms of severity of diarrhea, skin fibrosis and survival. We examined how these cells migrated into diverse sites of the allogeneic response, including mesenteric lymph nodes (mLN), peripheral lymph nodes (pLN) and skin and analyzed their differentiation into more mature B-cell subsets. We further assessed their capacity to modulate the cytokine profile during GVHD and determined which cytokines were required for protection. Finally, we investigated how the administration of CpG-proBs affected the T follicular regulatory (Tfr) to T follicular helper (Tfh) cell ratio (Tfr/Tfh), which is key in controlling the $CD4^+$ T-B cell interaction taking place in GVHD.

**Abbreviations:** aGVHD, acute GVHD; BM, bone marrow; Bregs, regulatory B cells; cGVHD, chronic GVHD; CpG-proBs, CpG-activated proB cell progenitors; EAE, experimental autoimmune encephalomyelitis; Fo B, follicular B cells; GC, germinal center; HSC, hematopoietic stem cells; HSCT, hematopoietic stem cell transplantation; mLN, mesenteric lymph nodes; pLN, peripheral lymph nodes; T1D, Type 1 diabetes; TBCD-BM, T-and B-cell depleted bone marrow; TFh, follicular helper T cells; TFr, follicular regulatory T cells; TGF-β, Transforming growth factor-beta; Treg, regulatory T cells; SPF, specific pathogen free.

## MATERIALS AND METHODS

### Mice

Female Balb/c mice were obtained from Janvier Laboratories (Le Genest Saint Isle, France) and maintained under acidified water upon arrival. Donor cells were from specific pathogen free (SPF) C57BL/6J mice (from Janvier laboratories), congenic $CD45.1^+$ C57BL/6J, Actin-GFP knock-in (KI) C57BL/6J, IFN-γ deficient C57BL/6J mice, all raised in our accredited animal facility at Institut Necker Enfants Malades under pathogen-free conditions. All mice were backcrossed for at least ten generations.

### GVHD Induction and Clinical Scoring

Balb/c mice (female, 10 wk-old) were irradiated at 5.8 Gy in a Faxitron X-Ray irradiator at day 0 and reconstituted at day+1 by i.v. retro-orbital injection with 5 x $10^6$ T- and B-cell-depleted BM cells as well as 1 x $10^6$ splenocytes from C57BL/6J donors. Clinical evolution of GVHD was scored over 60-80 days, for survival, diarrhea, weight, posture, mobility and skin damage (18).

### T- and B-Cell Depletion of BM Cells

Donor T- and B-cell-depleted (TBCD) BM cells were isolated by flushing femurs and tibias from donor mice with RPMI 1640. After centrifugation, cells were stained for 15 min with anti-CD3-PE and anti-CD19-PE in PBS, 2% FCS and rat anti-mouse IgG and sheep anti-mouse IgM were added. Depletion was completed with anti-rat and anti-sheep beads, respectively (ThermoFisher Scientific) after 3 passages over a magnet in 5ml tubes. The TBCD-BM fraction contains mainly myeloid, precursor and stem cells.

### B-Cell Progenitor Sorting and Expansion

CpG-proB cells were isolated from C57BL/6J BM cell cultures activated with 1 μM CpG-1668 (CpG-B) (Eurogentec, Angers, France) for 17h in low endotoxin-RPMI medium (Fisher Scientific, Illkirch, France) supplemented with 10% (vol/vol) FCS and 1% antibiotics (penicillin and streptomycin). c-$kit^+$ cells were magnetically sorted using the Robosep automaton (StemCell Technologies, Grenoble, France) and thereafter stained with appropriately labeled mAbs and sorted by flow cytometry on a BD FACS Aria IIIu cell-sorter as c-$kit^+$Sca-$1^+$$B220^+$PDCA-$1^-$$IgM^-$

cells. Electronically sorted B-cell progenitors were cultured on plates at 20,000 cells/ml over OP-9 stromal cells in OPTIMEM medium (Gibco) supplemented with 10% FCS, 1% antibiotics, 0.1% β-mercaptoethanol and 20 ng/ml Flt3L, SCF (Immunotools, Frisoythe, Germany) and IL-7 (Peprotech France, Neuilly-sur-Seine, France), achieving on average a 10-fold expansion of sorted CpG-proBs over 6 days. Expanded CpG-proBs were further stained and electronically sorted as c-kit$^{low/-}$ Sca-1$^{+}$B220$^{+}$PDCA-1$^{-}$IgM$^{-}$ cells, routinely assessed as >95% pure, before i.v. injection through the retro-orbital sinus.

## Recovery of Cells From Lymph Nodes and Skin Samples

Inguinal (n=2), axillary and brachial (n=4), cervical (n=2) and mesenteric (n=3) lymph nodes were collected from GVHD controls and CpG-proB recipients, yielding equivalent cell counts in both groups. Skin samples were harvested and digested in RPMI medium (Fisher Scientific, Illkirch, France) supplemented with 1% (vol/vol) FCS, 1% antibiotics (penicillin and streptomycin), 1mg/mL collagenase D (Roche, Sigma COLLD-RO) and 1,000 IU DNase (Sigma-Aldrich, Fleury-Mérogis, France) for 45 min at 37°C [adapted from (19)].

## Flow Cytometry Analysis of Cell Subsets and Cytokine Expression

To block nonspecific Fc receptor binding, cells were pre-incubated for 10 min at room temperature with FcR blocker 2.4G2 mAb. Cells were then stained with appropriately labeled mAbs against CD4, B220, MHC II, PDCA-1, PDL-1, PDL-2, CD21, IgM, CD93, CD23, CD11b, F4/80 (eBioscience, ThermoFisher Scientific, Montigny-le-Bretonneux, France), c-Kit (CD117) (BioLegend, San Diego, CA), Sca-1 (anti-Ly6A/E), CD40, CD80, CD86, CD11c, CD8 (BD Bioscience/Pharmingen, Le Pont-de-Claix, France), CXCR5 (Sony, Weybridge, Surrey, UK) or GFP (ThermoFisher Scientific). Nuclear Foxp3 expression was measured by FACS analysis as per the manufacturer's instructions (eBioscience, ThermoFisher Scientific). Positive cells were defined using an isotype control antibody. Intra-cytoplasmic cytokine expression was assessed after a 4-h stimulation with PMA (10 ng/ml) plus ionomycin (500 ng/ml) in the presence of Brefeldin A (2 mg/ml), followed by fixation/permeabilization with PFA/saponin and subsequent staining with specific antibodies including PE-labeled anti-TGF-β, PE-labeled anti-IL-27p28, PE-labeled anti-GM-CSF, APC-labeled anti-IL-10, APC-labeled anti–IFN-γ, APC-labelled anti-IL-21 (eBioscience), APC-labeled anti-IL-17 (BD bioscience), FITC-labeled anti-IL-6, PE-labeled anti-IL-13, APC-labeled anti-IL-4 (Sony) and FITC-labeled anti-TNF-α (Biolegend). Positive cells were defined using isotype Ab-stained controls (BD Biosciences and eBioscience). Membrane and intracellular antigen expression was analyzed in a FACS Canto II cytometer (BD Biosciences) using FlowJo software (Treestar, Ashland, OR).

## qRT-PCR Microarray Analysis in Skin Samples

Skin samples (2cm$^2$) were collected from the back of GVHD controls or CpG-proB recipients at day+70, frozen in liquid nitrogen and stored at -80°C. Frozen tissues were then placed in Qiagen lysis buffer and dissociated using GentleMACS dissociator (Miltenyi Biotec, Paris, France). RNA was extracted with RNeasy Plus Universal mini-Kit (Qiagen, Courtaboeuf, France) following the manufacturer's instructions. The A260/A280 values of all RNA samples ranged from 2.06-2.1. Production of cDNA from 1ng of total extracted RNA was performed using random primers (Invitrogen, ThermoFisher Scientific, Montigny-le Bretonneux, France) and reverse transcriptase superscript II (Life Technologies, Villebon-sur-Yvette, France). qRT-PCR array for measuring the expression of 80 genes of interest (and 8 house-keeping genes), targeting cytokines and fibrosis-related genes, was performed on a custom-made plate (Anygenes, Paris, France) with SYBRGreen, using a qTower2 thermal cycler (Analytic Jena, Jena, Germany). See **Supplementary Table 1** for information on primers used in the qRT-PCR array.

Analysis was performed with Qlucore software (Lund, Sweden). Results are expressed as 2-(delta delta Ct) and gene expression was normalized using the geometrical mean of 6 housekeeping genes. The threshold for the selection of differentially expressed genes was an expression fold-change ≥1.4 and a $p \leq 0.05$.

## Histology

Skin sections (4 μm thick) recovered from the back of mice at day+70 were fixed in 4% paraformaldehyde, embedded in paraffin and stained with H&E. Epidermal thickness was measured on scanned images with NDP.view software (Hamamatsu City, Japan).

## Statistics

Statistical analysis was performed using GraphPad Prism (GraphPad Software, La Jolla, CA). Normality and variance equality were assessed for every data set with Shapiro-Wilk test (for samples with n>5) or D'Agostino-Pearson (for samples with n ≤ 5) and F Test respectively. Survival curves were analyzed with Kaplan-Meier estimates. Disease curves and multiple cytokine production were analyzed using a two-way ANOVA test, with Bonferroni multiple comparison post-test. Cell proportions were analyzed using two-way ANOVA with Bonferroni multiple comparison, Student's *t*-test or one-way ANOVA. Data are shown as mean ± SEM. $P \leq 0.05$ was considered statistically significant.

# RESULTS

## CpG-proBs Protect Against GVHD: Assessment of Cellular Dose and Therapeutic Window

After induction, GVHD went through an initial phase accompanied by diarrhea between day+2 and day+18 followed by a chronic stage from day+20 onwards, characterized by a second bout of diarrhea together with cutaneous manifestations. CpG-proBs were sorted as c-kit$^{+}$Sca-1$^{+}$B220$^{low}$PDCA-1$^{-}$IgM$^{-}$ cells, as reported before (16) (**Supplementary Figure 1A**). A dose of $10^5$ CpG-proBs, previously shown to be effective in autoimmune settings, did not significantly reduce the severity of

GVHD, when the adoptive transfer took place the day following reconstitution (**Supplementary Figure 1B**). To increase the amount of progenitors available for transfer, CpG-proBs were co-cultured with OP-9 stromal cells for 6 days. After a 10-fold expansion, on average, these progenitors were electronically sorted to routinely >95% purity. They shared a similar phenotype with CpG-proBs that had not been expanded, except for the loss of c-kit expression, presumably resulting from the presence of its ligand SCF in the expansion medium (**Supplementary Figure 1C**). When 7.5 x $10^5$ CpG-proBs per recipient were injected on day+2 post-irradiation (DPI), they provided significant protection, as assessed by reduced diarrhea and less skin damage but no significant increase in survival compared to controls with GVHD (**Figure 1**). By contrast, the same number of non-activated pro-B cell progenitors freshly sorted from the bone marrow as c-kit$^+$Sca-1$^-$ B220$^+$CD24$^{hi}$CD43$^{hi}$ cells (**Supplementary Figure 1C**) and expanded in the same conditions had no such effect (**Figure 1**). The same number of CpG-proBs adoptively transferred on day+9 conserved a reduced but still significant protection against disease symptoms, which was lost when injected on day+23 (**Figure 1**).

CpG induced a strong upregulation of MHC class II, together with the co-stimulatory molecule CD80, as well as high CD40 expression on proB cell progenitors, thereby improving their capacity to interact with T-cells. There was no significant difference between CpG-proBs and their unstimulated counterpart, in terms of FasL expression, while PDL-1 was upregulated, compared with unstimulated controls, which did not display this molecule at significant levels (**Supplementary Figure 2A**). However, the difference between CpG-proBs and proBs became less pronounced after expansion on the OP-9 cell layer. Finally, FACS analysis of PMA+ionomycin-activated proBs and expanded CpG-proBs revealed no significant difference between their cytokine expression profiles (GM-CSF, TNF-α, IL-10 and IFN-γ) (**Supplementary Figure 2B**).

## CpG-proBs Migrate Into Peripheral Organs Where They Differentiate

We took advantage of CpG-proBs derived from actin-GFP-knock-in (KI) mice to track their migration in recipients. On day+15, B220$^+$GFP$^+$ cells, gated as in **Figure 2A**, represented 20-30% of all B cells analyzed and were detected exclusively in CpG-proB recipients, in mesenteric (mLN) and peripheral lymph nodes (pLN) as well as in the skin (**Figure 2B**). Using a gating strategy based on relative expression of IgM, CD21, CD23 and CD93 (20–22) in all tissues examined, approximately 40% B220$^+$GFP$^+$ cells displayed a CD21$^{low}$CD23$^+$CD93$^-$IgM$^+$ phenotype (**Figures 2C, D**), similar to follicular B (FoB) cells, previously identified as the major CpG-proB progeny in NOD mice (15).

## Cytokines Are Expressed in the Peripheral CpG-proB Progeny

Twenty to 80% B220$^+$GFP$^+$ cells expressed various cytokines, including IL-10, TGF-β, IFN-γ, GM-CSF, TNF-α and IL-27, compared with only 10-25% positive cells among the non-CpG-proB-derived B220$^+$GFP$^-$ population. These observations suggest that the CpG-proB cell progeny is highly activated, especially in mLN, in which B220$^+$GFP$^+$ cells expressing these cytokines, notably IL-10 and TGF-β, were more frequent than in their pLN and skin counterpart (**Figures 2E, F**).

## Characterization of Two Distinct Phases of Cytokine T-Cell Response in Mice With GVHD

We investigated whether the two diarrhea phases occurring in this GVHD model, the first one between day+2 and day+18, the second one starting at day+20, concomitant with the onset of skin damage, corresponded to an initial acute inflammatory cytokine storm followed by a chronic phase characterized by a more systemic autoimmune disease associated with the alteration of regulatory mechanisms due to the alloreactive conflict. To this aim, we investigated the CD4$^+$ T-cell intracellular expression of cytokines by flow cytometry, in the mLN of control mice with GVHD at day+15 and day+25. Percentages of CD4$^+$ cells (**Figure 3A**) expressing TNF-α, IL-6, IL-17 and to a lesser extent IL-21 were already high at day+15 while IL-6 and IL-4 were statistically reduced at day+25. IL-17 expression also tended to be reduced between day+15 and day+25 but without reaching statistical significance, while TNF-α remained highly expressed at day+25. Conversely, low levels of GM-CSF, IFN-γ, IL-13 and IL-27 expression with nearly no detectable expression of TGF-β by CD4$^+$ T-cells were observed at day+15 while their expression was enhanced at day+25, with statistical significance for TGF-β and IL-27. IL-10 expression remained low and unmodified at day +15 and day+25. Such clear-cut shift in the cytokine expression pattern determining two distinct phases of the disease was even more conspicuous in a heatmap representation (**Figure 3B**). Therefore, the CD4$^+$ T-cell response is significantly distinct at day+15 and day+25 in this GVHD model, with an initial inflammatory phase at day+15 followed by a pro-fibrotic cytokine production at day+25, characteristic of the chronic phase of GVHD. Consequently, it was interesting to evaluate the effect of the adoptive transfer of CpG-proBs at these two phases of the GVHD model, i.e. day+15 and day+25.

## CpG-proBs Modulate Cellular Distribution and Cytokine Expression in GVHD Recipients

We analyzed the effect of adoptively transferred CpG-proBs on various recipient cell populations. On day+15, incidence and cell counts of CD4$^+$ T cells or CD4$^+$Foxp3$^+$ Treg cells were neither significantly different from controls nor did the cytokine expression by CD4$^+$ T-cells in mLN and pLN change (**Supplementary Figure 3A, B**). On day+25, once the chronic phase initiated, percentages of CD4$^+$, CD4$^+$Foxp3$^+$ Treg and CD8$^+$ T-cells as well as cell counts were not significantly modified (**Figures 4A, B**). However the proportion of CD4$^+$ T cells generating cytokines, such as TNF-α, TGF-β, IL-21 and IL-13, which are critically involved in chronic GVHD (23), was significantly reduced in mLN from CpG-proB recipients

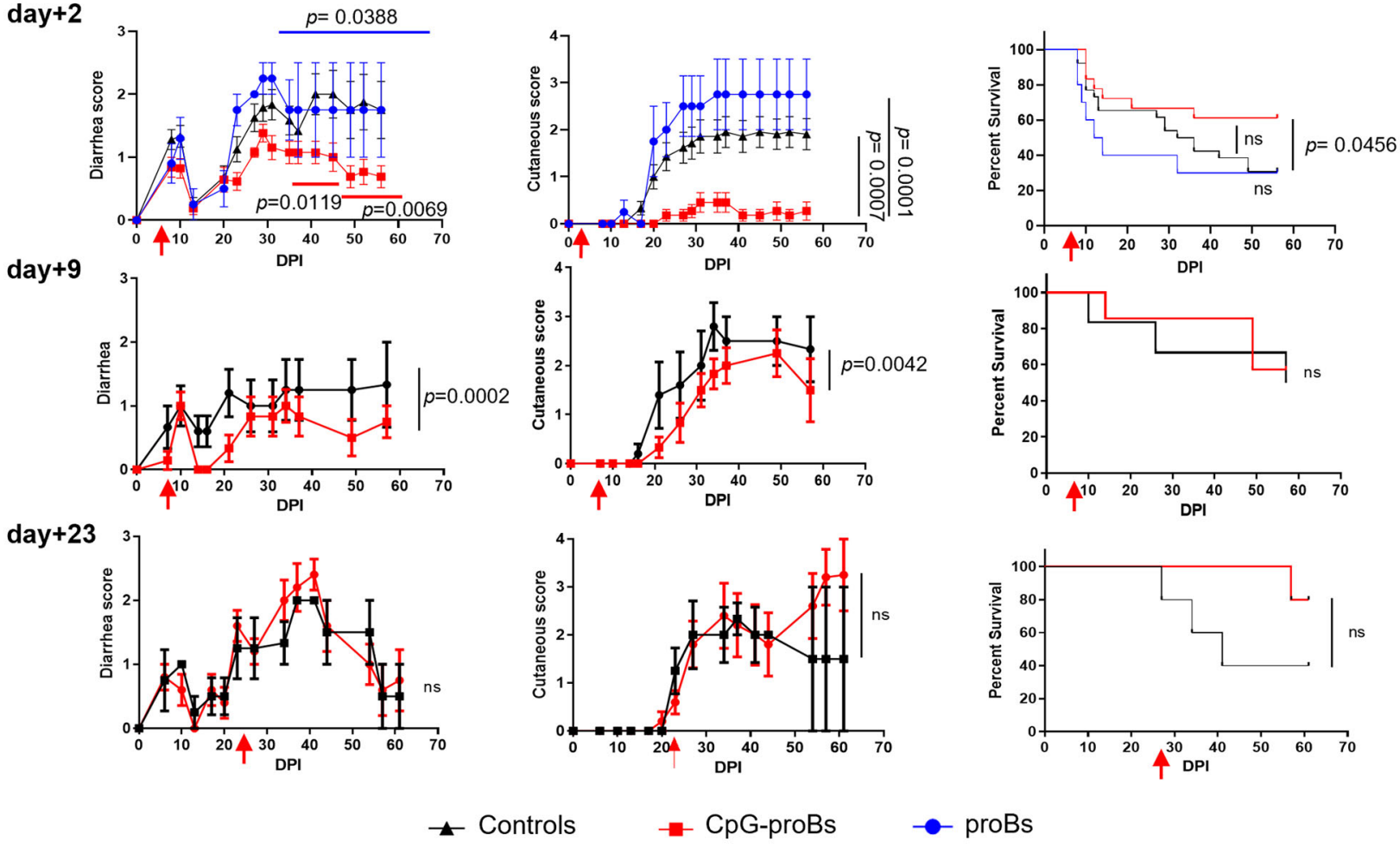


**FIGURE 1** | Effect of adoptively transferred CpG-proBs on GVHD symptoms. Balb/c recipients irradiated at 5.8 Gy on day-0, were reconstituted on day+1 with T- and B-cell depleted BM cells ($5x10^6$ cells) and splenocytes ($1 \times 10^6$ cells) from C57BL/6J donors. CpG-proBs ($7.5 \times 10^5$cells) or proBs prepared from C57BL/6J donors and expanded in co-culture with OP-9 stromal cells were adoptively transferred on day+2, day+9 or day+23 post-irradiation (DPI) as indicated. Diarrhea, cutaneous scores and survival are shown over a period of 60-80 days. Results are expressed as means ± SEM. Adoptive transfer (or PBS injection in control GVHD mice) was performed on day+2 in GVHD control mice (N=30, black line), CpG-proB recipients (N=19, red line), proB recipients (N=10, blue line); on day+9, in GVHD controls (N=6, black line) and CpG-proB recipients (N=7, red line); on day+23, in GVHD controls (N=7, black line) and CpG-proB recipients (N=6, red line). Statistical analysis was performed with two-way ANOVA with Bonferroni post-tests for diarrhea score and cutaneous score and Kaplan-Meier estimates for survival; *p* values as indicated; ns=non significant.

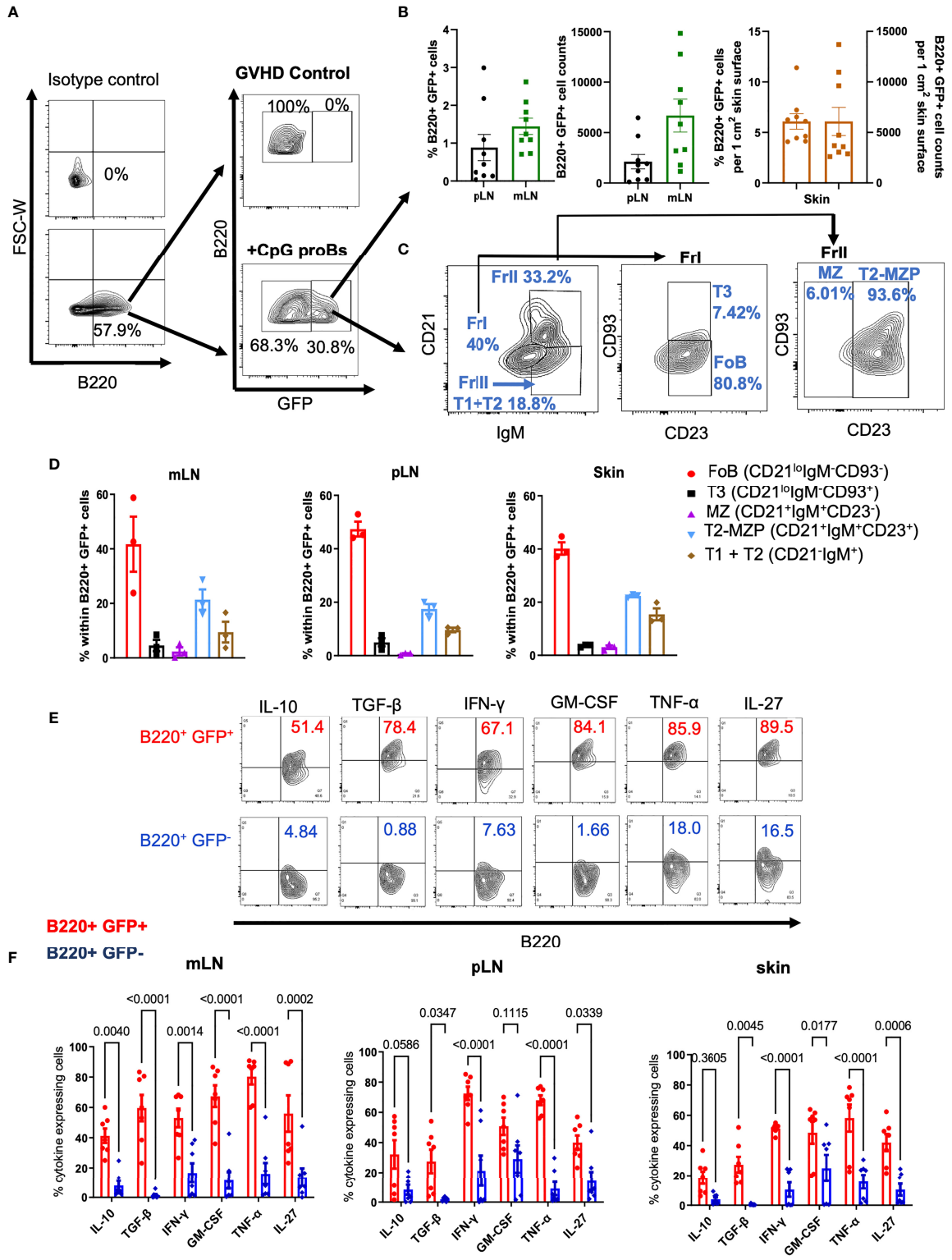


**FIGURE 2** | Continued

**FIGURE 2 |** Migration, differentiation and cytokine expression of CpG-proBs in GVHD mice. CpG-proBs, isolated from the BM of actin-GFP-KI C57BL/6J donors, were adoptively transferred on day+2 post-irradiation. **(A)** Gating FACS procedure of $B220^+$ $GFP^+$ cells, shown on day+15 in mesenteric lymph nodes (mLN), in controls with GVHD and in CpG-proB recipients, isotype antibody controls being used to define positivity. **(B)** The migration of $B220^+GFP^+$ cells was traced and analyzed by FACS on day+15 in peripheral and mesenteric lymph nodes (pLN), mLN) and skin. Indicated are percentages of $B220^+GFP^+$ cells among all recovered cells. In the skin, percentages and counts of $B220^+GFP^+$ cells are indicated per 1 $cm^2$ of skin surface. **(C, D)** Differentiation of CpG-proBs **(C)** and phenotype of the $B220^+GFP^+$ progeny assessed on day+15 in mLN. Isotype antibody controls were used to define positivity. The various B-cell subfractions were defined as FoB ($CD21^{lo}IgM^-CD93^-$), T3 ($CD21^{lo}IgM^-CD93^+$), MZ ($CD21^+IgM^+CD23^-$), T2-MZP ($CD21^+IgM^+CD23^+$) and T1+T2 ($CD21^-IgM^+$) cells. **(D)** CpG-proB differentiation on day+15 in mLN, pLN and skin. **(E, F)** Cytokine expression by the CpG-proB progeny on day+15. **(E)** FACS profiles of cytokine (IL-10, TGF-β, IFN-γ, GM-CSF, TNF-α and IL-27) expression by CpG-proB-derived $B220^+GFP^+$ and non-CpG-proB-derived $B220^+GFP^-$ cells in the mLN. **(F)** Percent cytokine expressing $B220^+GFP^+$ (red) and $B220^+GFP^-$ (blue) cells in mLN, pLN and skin. Statistical analysis was performed with two-way ANOVA with Bonferroni multiple comparisons. **(B, D, F)** Results are expressed as mean ± SEM of 3-9 mice per group.

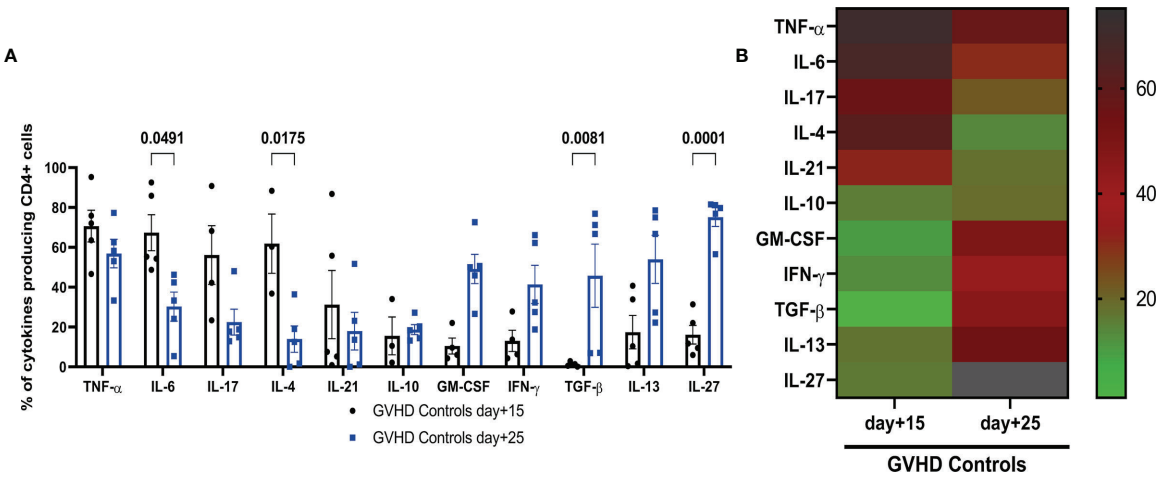


**FIGURE 3** | Characterization of two phases of cytokine expression by mLN CD4+ T-cell in controls with GVHD. **(A)** $CD4^+$ T cells were stimulated by PMA + ionomycin in the presence of brefeldin and their intracellular cytokine expression was analyzed by FACS in mLN of GVHD controls at day+15 (black) and day+25 (blue). Data are expressed as means ± SEM of 5 mice per group. Statistical analysis was performed with two-way ANOVA with Bonferroni multiple comparisons. *p* values as indicated, n.s., non significant. **(B)** Heatmap representation of the mean of percentages of $CD4^+$ T-cell expression of indicated cytokines in mLN of control mice with GVHD, at day+15 and day+25. Right: Colour scale of intensity of percentages.

(**Figure 4C**), while only IL-13-expressing $CD4^+$ T cells were diminished in pLN (**Figure 4D**). No significant difference was noted for IL-10 expression in $CD4^+$ cells (**Figures 4C, D** and **Supplementary Figure 3**), while it was slightly but non-significantly enhanced in mLN but not in pLN $CD8^+$ T-cells (**Figures 4E, F**).

## Adoptive Transfer of CpG-proBs Increases the Tfr/Tfh Ratio

T follicular helper (Tfh) cells, counterbalanced by T follicular regulatory (Tfr) cells, are known to play a key role in the $CD4^+$ T-B cell interaction (24). In addition, Bregs have been reported for interacting with both Tfh and Tfr subsets (25, 26). This led us to examine how CpG-proBs and their progeny affected the balance between these two populations. Tfh evaluation on day+15 disclosed no difference between GVHD controls and CpG-proB recipients (**Supplementary Figure 3C**). Conversely, on day+25, the ratio between $CD4^+CXCR5^+Foxp3^+$ follicular T regulatory cells (Tfr) and $CD4^+CXCR5^+Foxp3^-$ follicular helper T (Tfh) cells was markedly increased in both mLNs (**Figure 5A**) and pLNs (**Figure 5B**) of CpG-proB recipients relative to their counterpart in control mice undergoing GVHD. Moreover, the percentage of Tfh cells expressing IL-10 was increased in mLN, while Tfh cells expressing IL-21 were diminished in pLN of mice having received CpG-proBs relative to untreated GVHD controls (**Figures 5C, D**). Finally, percentages of $CD19^+GL7^+CD38^{low}$ GC B cells did not differ significantly in spleen and mLN (not shown). Altogether, these data show that CpG-proBs switch the follicular T-cell compartment towards regulation, by favoring the accumulation of Tfr over Tfh cells and by promoting their production of the immunoregulatory cytokine IL-10 over IL-21.

## The Protection Against GVHD by CpG-proBs Depends on IFN-γ Production

IFN-γ plays a key role in the protective effect of CpG-proBs in autoimmune T1D (15) and EAE (16). In GVHD mice, their migrated $B220^+GFP^+$ progeny expressed IFN-γ at similar levels, whatever the target tissue (**Figure 2F**), which prompted us to evaluate its role in the GVHD model. Using CpG-proBs isolated from IFN-γ-deficient mice, we found that graft recipients displayed exacerbated diarrhea and skin damage, compared with those having received WT CpG-proBs (**Figure 6A**). This finding proved the importance of IFN-γ in the protection against GVHD

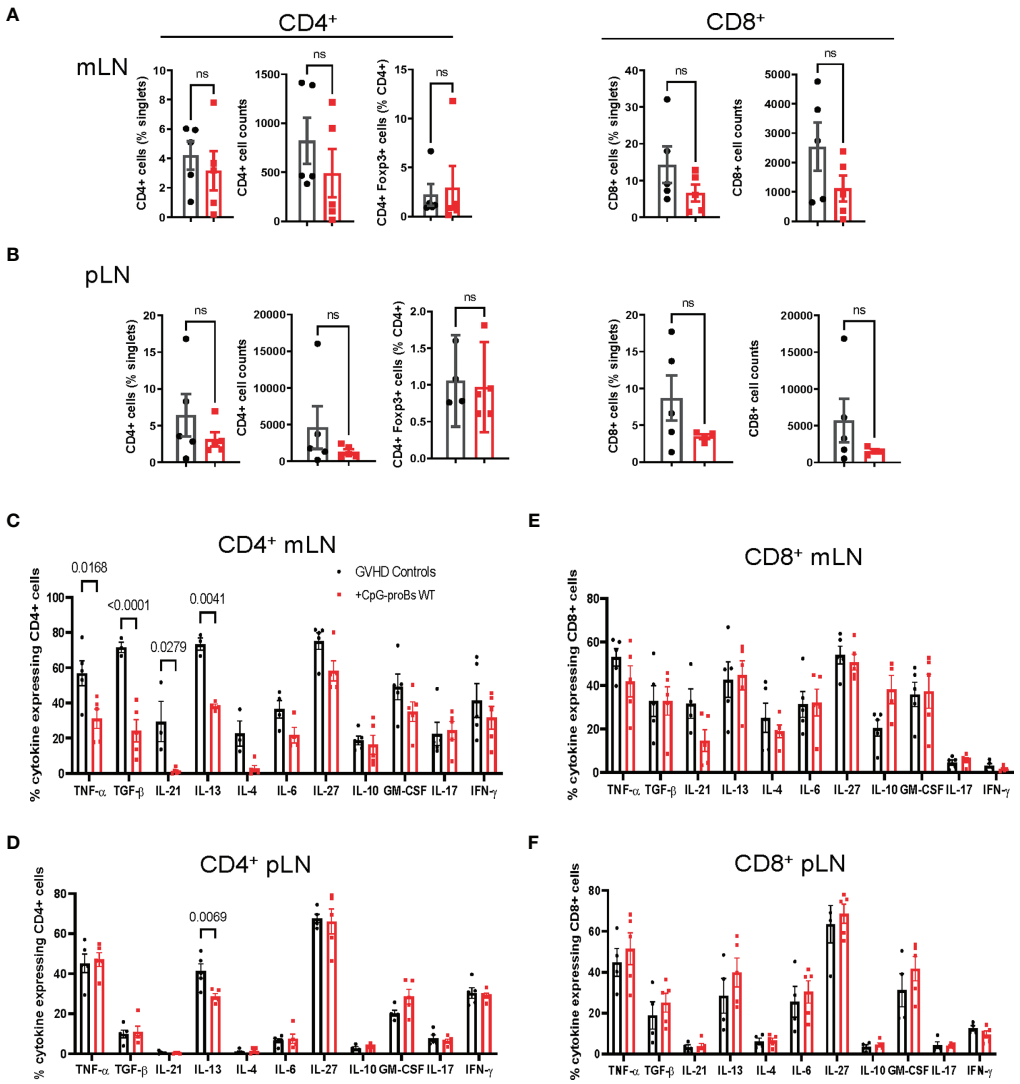


**FIGURE 4** | T-cell subset analysis in mLN and pLN of CpG-proB recipients and GVHD controls. **(A, B)** Quantification by FACS analysis on day+25 of $CD4^+$, $CD8^+$ (% and cell counts) and $CD4^+Foxp3^+$ (%) in mLN **(A)** and pLN **(B)** of GVHD controls (black) and CpG-proB recipients (red). **(C, D)** Cytokine expression by $CD4^+$ T cells in mLN **(C)** and pLN **(D)** of GVHD controls (black) and CpG-proB recipients (red). Data are expressed as means ± SEM of 5 mice per group. Statistical analysis was performed with unpaired Students'*t*- test **(A, B)** and two-way ANOVA with Bonferroni multiple comparisons **(C–F)**. *p* values as indicated, n.s., non significant.

by CpG-proBs. The progeny of IFN-γ deficient CpG-proBs having migrated to the mLN did not express IFN-γ as expected, but also generated less IL-10, compared to its WT counterpart (**Figure 6B**). Moreover, co-culturing peripheral and mesenteric lymph node cells isolated from naive mice with CpG-proBs significantly enhanced IL-10 expression in gated $CD4^+CXCR5^+PD1^+$ Tfh cells, only when the progenitors were competent IFN-γ producers (**Figure 6C** and **Supplementary Figure 5**).

## CpG-proBs Reduce Fibrosis and Regulate Gene Expression and Infiltrates in the Skin

GVHD recipients of CpG-proBs developed less alopecia and skin damage (**Figure 7A** right) compared with GVHD controls at day+70 (**Figure 7A** left). Histological analysis of H&E-stained skin sections recovered on day+70 revealed 50% reduced epidermal thickness (**Figure 7B**), consistent with diminished skin fibrosis. In addition, hair follicles that are a target of GVHD (27) are preserved in the skin of CpG-proB recipients, correlating with the observed reduced alopecia (**Figure 7A**). qRT-PCR microarray expression profiles, established at day+70, of genes involved in fibrosis and cytokine production (**Figure 7C**) disclosed that *Col3a1* (**Figure 7D**) as well as of *Pdgfa*, a Col3a1 inducer implicated in fibrosis were downregulated in samples from CpG-proB recipients. The expression of Pdgfa, a known inducer of CXCR4 (28), which attracts fibrocytes to fibrotic tissues (29, 30) was likewise reduced in the skin of CpG-proB recipients. By contrast, thrombospondin-2 (*thsb2*, TSP-2), an anti-angiogenic matricellular protein that improves wound healing (31) was upregulated in CpG-proB

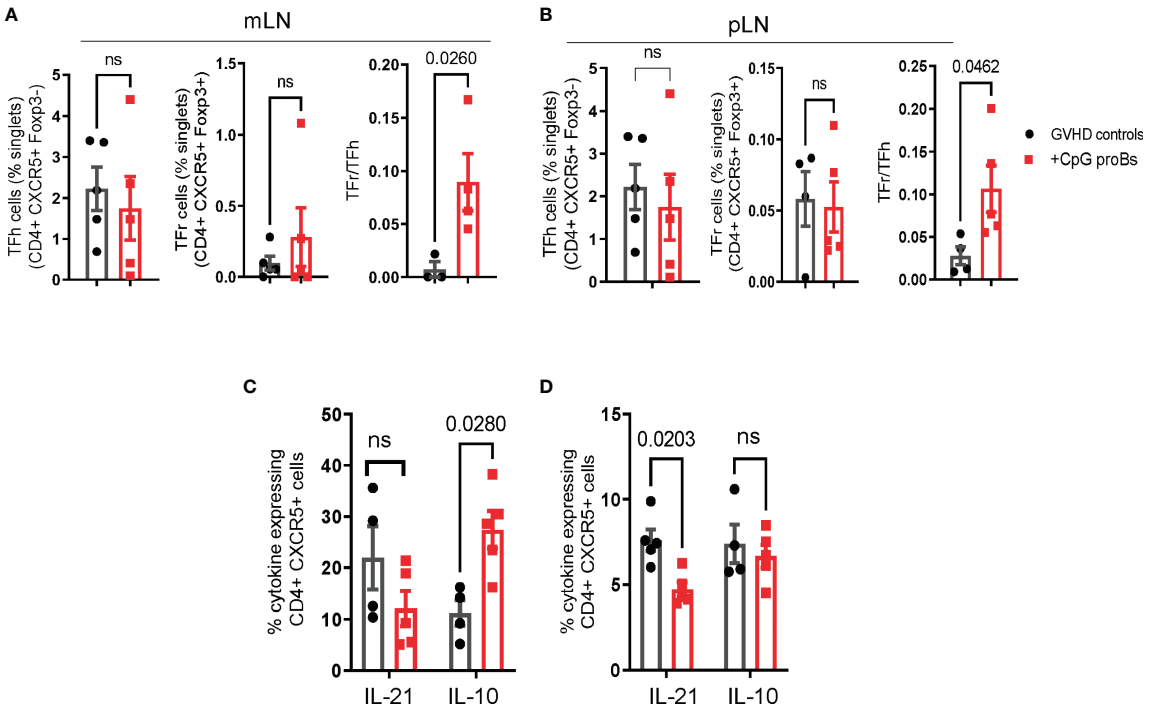


**FIGURE 5** | Follicular T-cell (Tf) analysis. **(A, B)** Percentages and counts of Tfh ($CD4^+CXCR5^+Foxp3^-$) and Tfr ($CD4^+CXCR5^+Foxp3^+$) cells as well as Tfr/Tfh ratios on day+25 in mLN **(A)** and pLN **(B)** of mice, either CpG-proB recipients (red) or GVHD controls (black), were established by FACS analysis. **(C, D)** Cytokine expression by $CD4^+CXCR5^+$ cells assessed by FACS analysis. Percent IL-21- and IL-10-expressing cells in mLN **(C)** and pLN **(D)** of GVHD controls (black) and CpG-proB recipients (red). Results are expressed as means ± SEM from 5 mice per group. Statistical analysis was performed with unpaired Students'*t*- test **(A, B)** and two-way ANOVA with Bonferroni multiple comparisons **(C, D)**. *p* values as indicated, ns= non significant.

recipients. The same applied to *MMP9*, which behaves like a collagenase (32) and can further regulate leukocyte infiltration into inflammatory tissues (33) by inactivating a number of chemoattractants. However, neither total immune cell nor T-cell infiltration was significantly different between GVHD controls and CpG-proB recipients on day+15 or day+42 (**Figure 7E**). The two-fold reduction in the total cell counts infiltrated at day+42 relative to day+15 (**Figure 7E**) observed in both control and CpG-proB-treated groups may reflect an initial transient wave of infiltration followed by a gradual inactivation of chemoattractants ligands or receptors occurring in the second phase of the model with profibrotic events taking over, thus contributing to enhance the cutaneous score observed in **Figure 1**. The enhanced *IL12rb* expression suggested a proTh1 effect of CpG-proBs on skin infiltrates, possibly controlling the deleterious Th2-driven fibrotic process. This conclusion was in keeping with the observed decrease in IL-13 expression by $CD4^+$ T-cells in the lymph nodes. Increased Stat6 expression in CpG-proB recipients (**Figure 7C**) was intriguing, knowing that this signal transducer can mediate skin fibrosis (34). However, this upregulation might result from increased expression of IL-33, which occurs upstream of IL-13 (35). Of note, IL-33 can substitute for IL-2 as an inducer of tissue $ST2^+$ Treg expansion (36). Although the proportions of $CD4^+Foxp3^+$ Tregs and $CD4^+IL\text{-}10^+$ Tr1 cells were not significantly increased in skin infiltrates, as measured by FACS analysis (**Figure 8**), IL-10-expressing $CD8^+$ T cells, reported for their ST-2 expression and responsiveness to IL-33 (37), markedly accumulated in the skin of CpG-proB recipients, both on day+15 and day+42, while total $CD8^+$ T-cell counts and percentages remained unchanged (**Figure 8**).

The proportion of IL-10 producers increased also among the $B220^+PDCA\text{-}1^-$ B subset as early as day+15, while on day+42, both $B220^+PDCA\text{-}1^-$ B cells and $CD11c^+CD11b^+$ dendritic cells expressing IL-10 accumulated (**Figure 8**). During GVHD, macrophages stimulated by Fc immunoglobulin fragments contribute to fibrosis by releasing TGF-β. *Csf1r* was enhanced in the microarray analysis of skin samples from CpG-proB recipients. However, FACS analysis of the skin cell infiltrate revealed that cell counts, percentages as well as IL-10 production by $F4/80^+CD11b^+$ macrophages remained unchanged on day+42 (**Supplementary Figure 4**). Moreover, microarray analysis detected no significant difference between *Arg* and *iNOS* expression. In mice, CSF1R is expressed by monocytes/macrophages, but also by conventional and plasmacytoid dendritic cells. However, the observed incremental increase in conventional (**Figure 8**) and plasmacytoid dendritic cell percentages and IL-10 expression (**Supplementary Figure 6**) did not reach statistical significance. A late accumulation of $csf1r^+$ cells in the skin analyzed on day+70, compared to the flow cytometry analysis performed on day+42, cannot be excluded.

Collectively, the analysis of skin samples and infiltrates revealed the histological effects of CpG-proBs resulting in

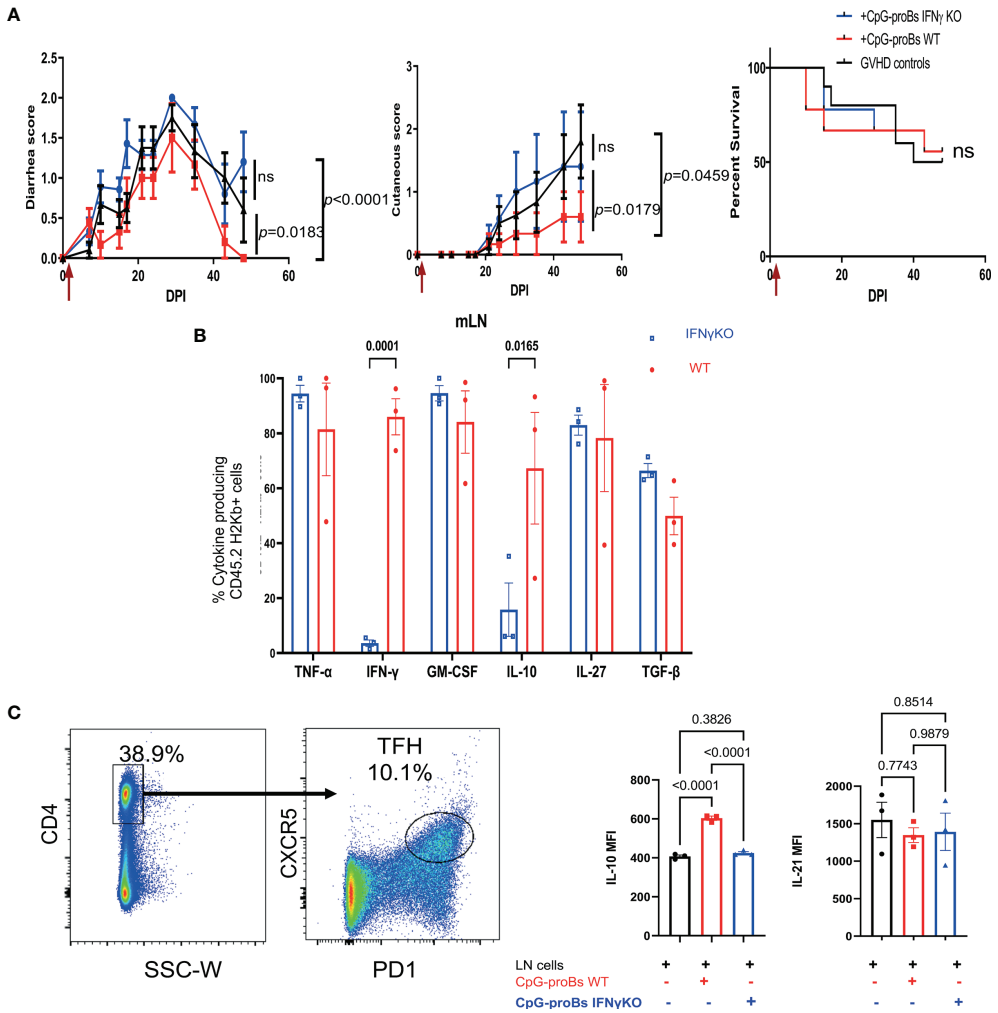


**FIGURE 6** | Role of IFN-γ in the protective properties of CpG-proBs against GVHD. CpG-proBs were prepared from either WT or IFN-γ deficient C57BL/6J donors and adoptively transferred (7.5 x $10^5$ cells/recipient) on day+2 post-irradiation (DPI). **(A)** Diarrhea, cutaneous scores and survival of GVHD controls (injected with PBS, black, n=10), WT CpG-proB recipients (red, n=9) and IFN-γ deficient CpG-proBs (blue, n=9). Statistical analysis was performed with two-way ANOVA with Bonferroni multiple comparisons for diarrhea and cutaneous scores. Results are expressed as means ± SEM. *P* values as indicated. ns= non significant. **(B)** CpG-proB progeny, derived from either WT (red) or IFN-γ deficient (blue) C57BL/6J CpG-proBs, was gated as CD45.2 $H2Kb^+$ cells in mLN of GVHD Balb/c (H2Kd) recipients of CD45.1 TBCD-BM and splenocytes from CD45.1 C57BL/6J donors and their cytokine expression analyzed by FACS as in **Figure 2E** on day+15 after adoptive transfer. N=3 mice per group. **(C)** Lymph node cells from naive C57BL/6J mice were co-cultured at a 1:1 ratio with WT or IFN-γ KO CpG-proBs at 5 x $10^5$ cells/ml for 3 days in RMPI 1640 medium, 10% FCS, 1% antibiotics, 0.1% β-mercaptoethanol in the presence of anti-CD3 (200 ng/ml) and analyzed by FACS for IL-10 and IL-21 expression in gated $CD4^+CXCR5^+$ Tfh cells. One experiment out of two. Statistical analysis was performed with one-way ANOVA for **(B, C)** Results are expressed as means ± SEM. *p* values as indicated.

reduced skin damage, including fibrosis, epidermal thickness and collagen accumulation. These findings correlated with immune tolerance evidenced by enhanced infiltration by IL-10-expressing DCs, $CD8^+$ T cells and B cells. The two latter populations were first to accumulate in the skin.

## DISCUSSION

Herein, we evaluated whether adoptive transfer of CpG-activated B-cell progenitors exerted immunomodulatory effects in a model of GVHD that displays sequentially an acute and a chronic sclerodermatous phase (18). This study was initiated by recent evidence for Breg deficiencies and impaired functions in patients suffering from this disease (3, 7), together with the observation that circulating hematogones and protection against GVHD (8–14) were correlated. These findings warranted further exploration of the regulatory functions of B-cell progenitors in the allogeneic model of GVHD, expanding our previous studies in experimental models of autoimmune diseases, such as T1D (15) and EAE (16).

A single injection of as few as 7.5 x $10^5$ CpG-proBs was sufficient to protect against GVHD, by reducing diarrhea and

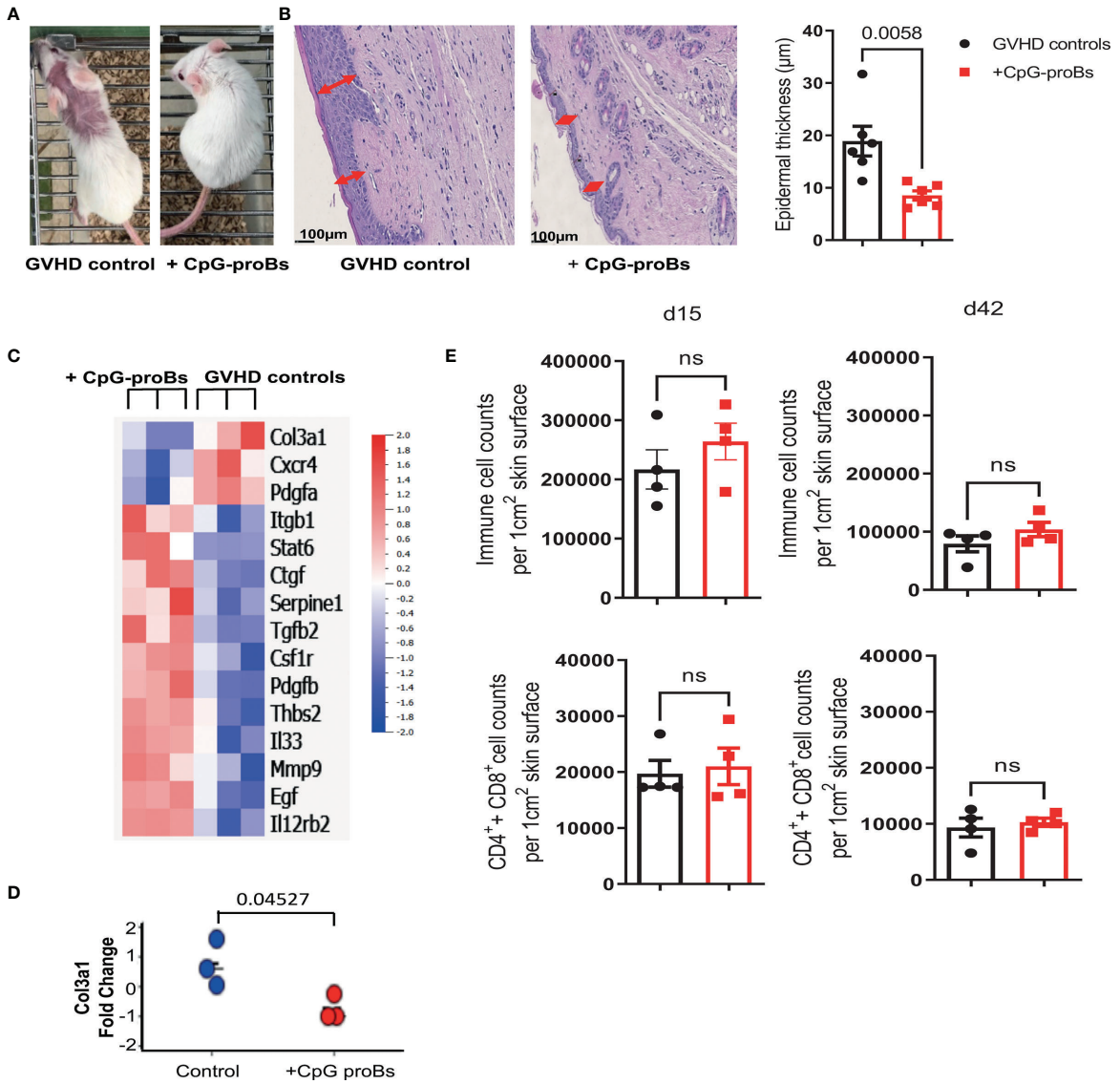


**FIGURE 7** | Analysis of skin histological modifications, gene expression and cellular infiltrate in GVHD controls versus CpG-proB recipients. **(A)** CpG-proB recipients were mostly protected from alopecia and skin damage induced by GVHD in Balb/c recipients. Picture at day+70 of one representative mouse per group. **(B)** H&E staining of representative skin sections at day+70 in GVHD controls versus CpG-proB recipients. Scale bar = 100 µm. Red arrows indicate the epidermal thickness. Forty measures were taken per skin section. Right: Histogram representation of epidermal thickness in GVHD controls (black) and CpG-proB recipients (red). Results are expressed as means ± SEM from 6 mice/group. *p* value as indicated. Analysis was performed with unpaired Student's *t*-test. **(C)** Heatmap showing significant fold-change expression of genes as measured by qRT-PCR microarray in skin fragments (2 $cm^2$) isolated at day+70 from GVHD controls (right) and CpG-proB recipients (left). N = 3 animals per group. Analysis was performed with Qlucore. Listed are genes showing ≥1.4 expression fold change with $p \leq 0.05$, considered significant. Right: Color scale of positive and negative fold-change gene expression. **(D)** Change fold of Col3A1 mRNA expression measured by qRT-PCR in skin samples recovered at day+70 from n=3 animals per group. **(E)** Flow cytometry analysis on day+15 and day+42 of total immune cell infiltrates as well as T-cell ($CD4^+$ and $CD8^+$) infiltrates in skin samples of GVHD controls (black) and CpG-proB recipients (red). Results are expressed as means ± SEM from 4 mice per group. Statistical analysis performed with unpaired Student's *t*-test, ns, non significant, *p* values as indicated.

skin fibrosis within a therapeutic window extending from day+2 up to day+9. The effect vanished when these cells were injected on day+23, indicating that they must intervene during the onset of disease to prevent its chronic phase. The easy access to the B-cell progenitors within the BM at the time of engraftment should facilitate their potential use as an addition to the HSC graft, as they provide long-lasting protection against diarrhea and skin fibrosis in GVHD. Protection required around 10-fold higher CpG-proB cell numbers than those needed in the case of organ-specific autoimmune disorders, presumably reflecting the necessity to migrate into the multiple tissues implicated in the allogeneic immune response.

Indeed, CpG-proB progeny was detected in the target sites of GVHD, including mLN, pLN and skin, as early as day+15, mainly

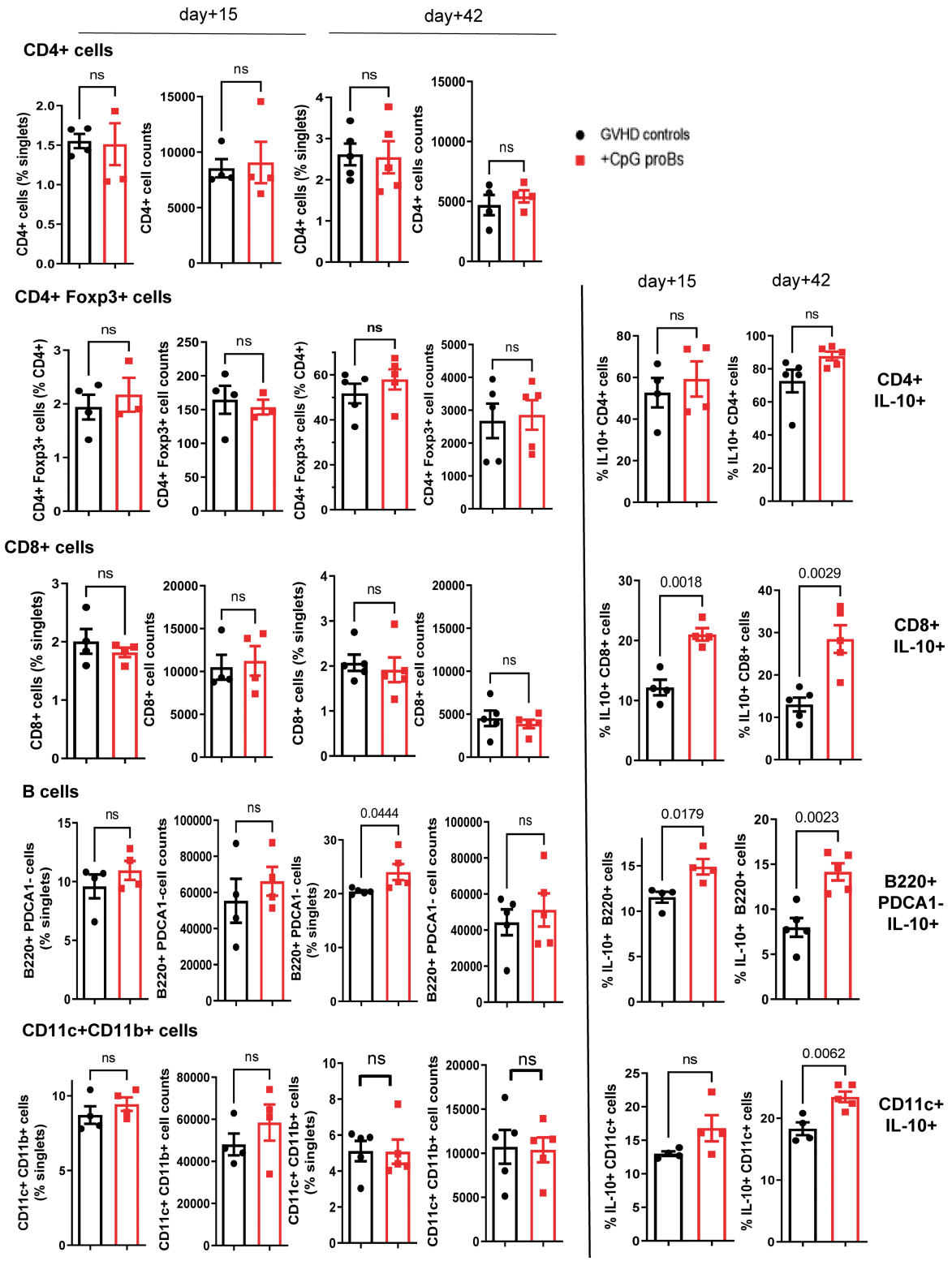


**FIGURE 8** | Flow cytometry analysis of skin infiltrates on day+15 and day+42 post-irradiation. $CD4^+$, $CD4^+Foxp3^+$, $CD8^+$, $B220^+PDCA\text{-}1^-$ B and $CD11c^+CD11b^+$ dendritic cell percentages and cell counts are shown in GVHD controls (black) and CpG-proB recipients (red). IL-10-expressing fraction of $CD4^+$, $CD8^+$, $B220^+$ and $CD11c^+$ cells on day+15 and day+42 in the skin of GVHD controls (black) and CpG-proB recipients (red). Results are expressed as means ± SEM for 5 mice per group. Statistical analysis was performed with unpaired Student's *t*-test, ns, non significant. *p* values as indicated.

differentiated into Fo B cells, as previously observed in the T1D model of NOD mice (15). Compared to non-CpG-proB-derived B cells in the same locations, the differentiated CpG-proBs were highly activated, as assessed by a 2-8 fold higher proportion of cells expressing cytokines, such as IFN-γ, GM-CSF, TNF-α, as well as IL-10, TGF-β and IL-27. Among these, IFN-γ production by CpG-proBs and their progeny proved to be critical for alleviating GVHD symptoms, particularly skin fibrosis, as previously shown in experimental models of autoimmune diseases, such as T1D (15) and EAE (16).

While CpG-proBs had to be adoptively transferred during the initial phase of GVHD for protection, their effect on the T-cell cytokine profile was observed mostly on day+25, when the expression of $CD4^+$ T-cell-derived cytokines involved in the inflammatory, humoral and fibrotic features of the chronic phase of GVHD, such as TNF-α, IL-21, TGF-β and IL-13, was significantly reduced in mLNs and pLNs of CpG-proB recipients compared to controls with GVHD. However, no effect was observed on GM-CSF, IL-17 and IFN-γ expression by $CD4^+$ T-cells in the mLN. The unmodified T-cell expression of these highly inflammatory cytokines in CpG-proB recipients may account for the lack of effect of the progenitors on mice survival. It remains to be evaluated whether performing a second progenitor cell transfer during the chronic phase might improve the mice survival. Alternatively, these observations suggest that CpG-proBs infusion should be associated with a supplementary strategy targeting anti-inflammatory cytokines beyond TNF-α, to be fully effective against GVHD. Conversely, as early as day+15, IL-10-expressing B cells and $CD8^+$ T-cells accumulated in the skin of CpG-proB recipients, suggesting an early major contribution of these cells to the protective effect induced by CpG-proBs particularly in skin. In both murine (38, 39) and human (40, 41) GVHDs, IL-10-expressing $CD8^+$ T cells have been reported for their regulatory effects, in particular for reducing collagen deposition in the skin of recipient mice (38). In the same line of evidence, we found IL-10-expressing dendritic cells accumulating on day+42 in the skin of CpG-proB recipients.

Fo B cells participate in germinal center (GR) responses generating long-lived plasma cells and memory B cells. The Tfh/Tfr balance plays a major role in GVHD, since Tfr cells can inhibit the interplay between Tfh and GC B cells (25, 26, 42–44). Bregs have been shown to take part in the crosstalk between these subsets (25, 26, 42). We found that the CpG-proB progeny belonged mostly to the Fo B phenotype and increased the Tfr/Tfh ratio. IFN-γ was essential for the capacity of the CpG-proB progeny to express IL-10 and enhance IL-10 expression by Tfh cells. We have previously reported that CpG-proB-derived IFN-γ induced eomesodermin in co-cultured $CD4^+$ T-cells (15). In turn, EOMES drives IL-10 expression, as shown in Tr1 cells that are protective against GVHD (45). Whether a similar mechanism takes place in Tfh cells remains to be assessed. Notably, an IL-10 expressing Tfh cell population with suppressive function was identified in chronic viral infection (46) as well as in inflammation associated with aging (47). Thus, CpG-proBs exert a profound influence on major participants of the $CD4^+$ T-B cell interaction that may limit the humoral response and IFN-γ production by CpG-proBs is required in both autoimmune and allogeneic settings.

IL-33 expression was enhanced in the microarray qRT-PCR study of skin tissue samples performed at day+70. Even though it has been reported that IL-33, released by epithelial and endothelial cells, induces cutaneous fibrosis, promoting the recruitment of BM-derived eosinophils as well as $CD3^+$ and $F4/80^+$ cell infiltration (48), we observed no accumulation of these cell types. Alternatively, IL-33 has also been described for its capacity to expand and stabilize ST2-expressing Tregs in tissues, thereby favoring tissue remodeling (36, 49). Treg frequency is inversely correlated with GVHD in patients (2, 50). Although we detected no accumulation of $Foxp3^+$ Tregs in CpG-proB recipients compared to GVHD controls, $IL\text{-}10^+CD8^+$ Tregs were more frequent early in the skin of CpG-proB recipients. These $IL\text{-}10^+CD8^+$ Tregs, which reportedly express ST2 (37), may play a key role in GVHD recovery. Interestingly, $IL\text{-}10^+CD8^+$ Tregs were shown to contribute to the GVL effect in allogeneic HSCT (51). In addition, Bregs were reported not to compromise GVL effects while protecting against acute GVHD (52). Altogether these observations suggest that it is likely that CpG-proBs, like mature Bregs, may not impair the GVL effect of HSCT.

cGVHD is characterized by the presence of hyperactivated B cells (53). Conversely, circulating Bregs are less frequent in cGVHD patients and less likely to produce IL-10 than those from healthy donors (3). In a murine sclerodermatous cGVHD model, reconstitution of donor-derived B10 cells participated in alleviating the disease (54). Interestingly, IFN-γ competence conditioned both IL-10 expression by the CpG-proB progeny and its protective effect against disease. Most Breg subsets reported so far for protective effects in cGVHD were mature B cells. Even cord blood B cells displaying regulatory functions against cGVHD belonged to naive and transitional B-cell subsets (6). Although an intriguing inverse correlation between BM and circulating B-cell progenitor frequencies and GVHD severity has been reported, evidence for a regulatory function of B-cell progenitors in GVHD has been lacking so far. Our findings acquired in a murine experimental model support the notion that innate activation with CpG confers tolerogenic properties to B-cell progenitors. Their immunomodulatory effect targets more specifically the chronic phase of the disease that exhibits autoimmune inflammatory features, whereas no effect was observed at the early phase. However, the lack of reduction of major pro-inflammatory cytokines such as GM-CSF, IL-17 and IFN-γ may preclude an improved survival in CpG-proB recipients. Evaluation of CpG-proBs in a more specific cGVHD model would also be interesting to perform.

The fact that the observed regulatory properties of CpG-proBs remain stable in highly inflammatory settings sheds a new light on Breg ontogeny (55). In depth examination of epigenetic and metabolic changes occurring in these B-cell populations may provide further insights into their tolerogenic imprinting.

## CONCLUSION

In this study we provided evidence that adoptive transfer of CpG-proBs at the early phase of GVHD alleviated disease symptoms, in particular skin fibrosis. Following their migration into lymph nodes

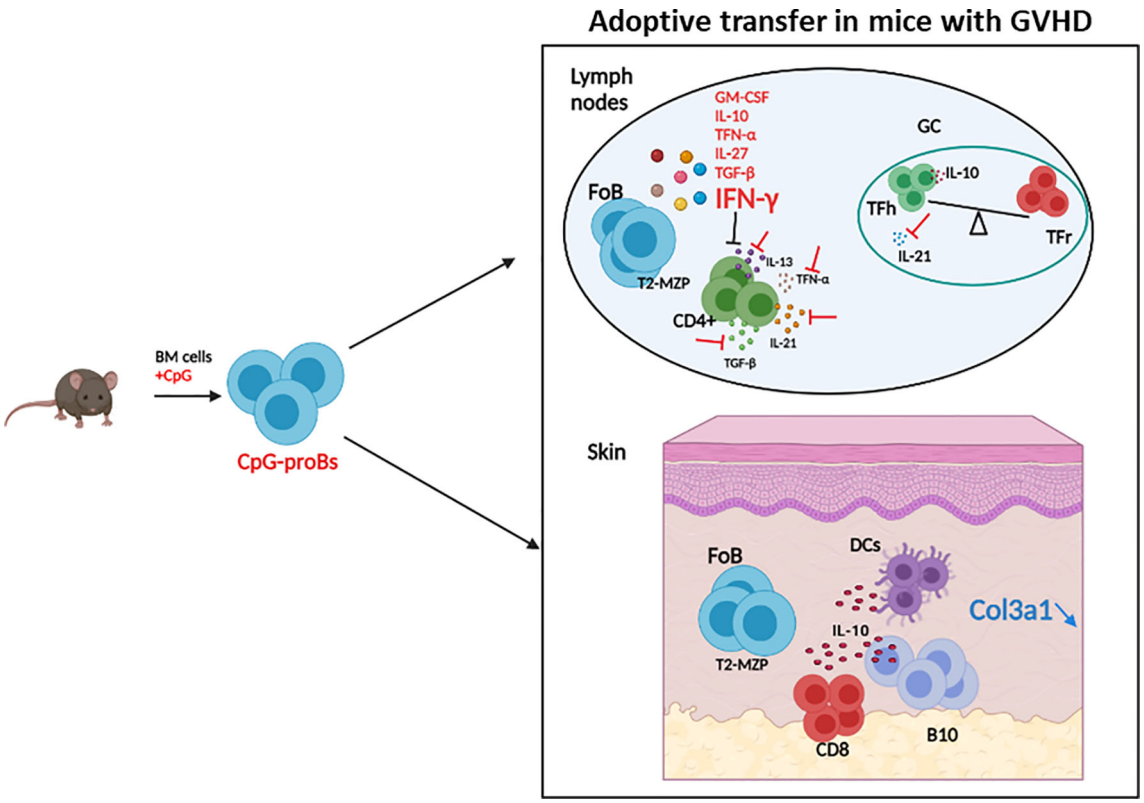


**FIGURE 9** | Graphical summary of the protective effects of CpG-proBs against GVHD. Adoptive transfer of CpG-proBs at the early phase of GVHD alleviated disease symptoms, in particular skin fibrosis. Following their migration into lymph nodes and skin, these progenitors produced many cytokines but depended on IFN-γ production for their protective effect. CpG-proB transfer reduced the $CD4^+$ T-cell production of profibrotic cytokines TGF-β, IL-21 and IL-13 and enhanced the Tfr/Tfh T-cell ratio in lymph nodes. They also promoted the accumulation of IL-10-producing $CD8^+$ T-cells, B-cells and dendritic cells in the skin. Figure created using BioRender.com.

and skin, these progenitors depended on IFN-γ production for their protective effect, as previously shown in experimental models of autoimmune diseases. CpG-proB transfer reduced the $CD4^+$ T-cell production of profibrotic cytokines, including TGF-β, IL-21 and IL-13 and enhanced the Tfr/Tfh T-cell ratio in lymph nodes. They also promoted the accumulation of IL-10-producing B-cells, dendritic cells and $CD8^+$ T-cells in the skin (**Figure 9**). However, they did not improve survival, presumably by failing to reduce a set of inflammatory cytokines. Taken together, our data support a potential benefit of CpG-proBs against GVHD that should be completed by an additional anti-inflammatory strategy. The data further suggest that circulating B-cell progenitors observed to correlate with reduced GVHD severity in patients may play an immunomodulatory role.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/, GSE182025.

## ETHICS STATEMENT

The animal study was reviewed and approved by Université Paris Cité Ethical Committee for Animal Experimentation and the French Ministry of Research and Higher Education, #21669-201807061804480v5.

## AUTHOR CONTRIBUTIONS

VAA, PG, ET, and FZ performed experiments, analyzed data and prepared figures. SK provided expertise in the model, analyzed and discussed data. VAA, SK, and FZ wrote the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.790564/full#supplementary-material

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