IMMUNE TOLERANCE POST ALLOGENEIC HEMATOPOIETIC CELL TRANSPLANTATION

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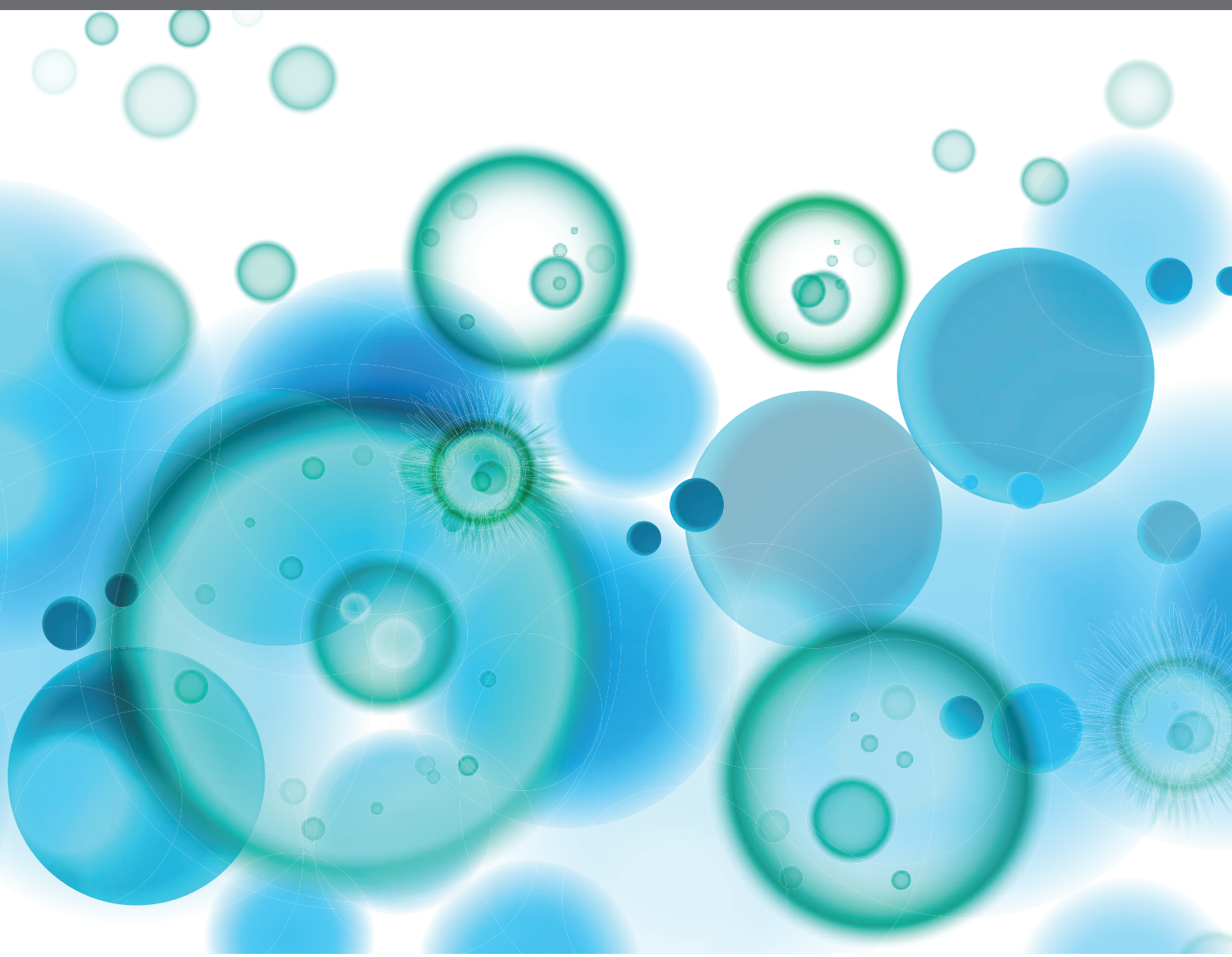

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IMMUNE TOLERANCE POST ALLOGENEIC HEMATOPOIETIC CELL TRANSPLANTATION

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Table of Contents

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EDITORIAL
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Editorial: Immune Tolerance Post Allogeneic Hematopoietic Cell Transplantation

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Keywords: allogeneic HCT, GvHD, immune tolerance, GVL effects, graft engineering

Editorial on the Research Topic

Immune Tolerance Post Allogeneic Hematopoietic Cell Transplantation

Within the last decade significant progress has been accomplished in the field of allogeneic hematopoietic cell transplantation (HCT). The articles in this special topics series present progress in improving clinical outcomes using some of the most vibrant current research and translational approaches, including novel reduced-intensity conditioning regimens, donor graft engineering, quantification of the microbiome, and tailored immunosuppressive therapies. Much of the progress described is due to improvements in immune reconstitution that results in durable allograft tolerance. Consequently, there is reduced graft-vs.-host disease (GVHD) and improvement in controlling infectious disease complications. In addition, improved immune reconstitution also appears to facilitate much-needed graft-vs.-leukemia effects, as relapse remains the major challenge of our field. Many of the articles in this special topics series are organized around our increasing understanding of GVHD and a suite of new tools and approaches to prevent and treat this dreaded immune complication.

Thangavelu and Blazar from the University of Minnesota provide an overview of our current understanding of GVHD pathophysiology and thoroughly review novel therapeutic strategies to induce immune tolerance focusing on biologicals, epigenetic modulation, and adoptive cell therapy. In particular, light is shed on the role of the intestinal microbiome for GVHD induction by Köhler and Zeiser: within the last years it became evident in various preclinical and clinical studies that changes of the bacterial composition affects the risk of intestinal GVHD which also constitutes a potential target to prevent deleterious damage of the gut. For patients with steroid-refractory acute and chronic GVHD, extracorporeal photopheresis (ECP) is an established procedure to induce immune tolerance and a significant impact of apoptotic bodies to modulate dendritic-cell function has been established. Ni et al. now suggest that also NK-cell subsets are influenced by ECP in such a way that $CD56^{high}CD16^{-}$ NK cells were decreased and cytotoxicity shifted toward a regulatory phenotype while maintaining antileukemic activity.

Efforts to define normal and healthy from abnormal and immune reconstitution that puts recipients at increased risk of GVHD continues to develop with the application of immune monitoring, as illustrated by Soares et al. who performed a prospective comparative analysis that suggests that thymic damage results in dysfunctional thymic output with increased $CD8^{+}$ terminally differentiated effector memory T cells and decreased T-cell receptor diversity. This study emphasizes the thymus as critical organ for central immune tolerance during immune reconstitution and sustained immune tolerance after allogeneic HCT. Simonetta et al. performed

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a comprehensive analysis of PD-1 expression on T cells following allogeneic HCT noticing an increase early after transplantation without impaired production of cytotoxic effector molecules. This study provides insight into dynamic T-cell regulation also suggesting that timing should be considered when check point inhibitors are applied.

Efforts to engineer donor grafts have shown evidence in pre-clinical and clinical studies of improved immune reconstitution. Bertaina and Roncarolo from Stanford University review such approaches focusing on T- and B-cell depletion strategies as well as regulatory T cells. In particular, three papers included in this Research Topic explore double-negative T cells, myeloid-derived suppressor cells (MDSCs) and invariant natural killer T (iNKT) cells for GVHD prevention. Haug et al. found that TCRαβ^{+}CD4^{-}CD8^{-} T cells inhibit mammalian target of rapamycin (mTOR) signaling and prevent metabolic adaption of conventional T helper cells resulting in decreased homing receptor expression and production of proinflammatory cytokines. The expansion of MDSCs from hematopoietic stem cells has been studied by Park et al. showing that these cells retain a suppressive phenotype and ameliorate GVHD in a xenogeneic GVHD model also resulting in improved survival. Jahnke et al. demonstrate that human iNKT cells that have been shown to promote immune tolerance after allogeneic HCT can also be expanded from cryopreserved donor lymphocytes efficiently lysing patient AML blasts.

Finally, two review articles provide detailed insights into innovative approaches of immune tolerance induction. Stahl et al. summarize preclinical and clinical data about the CD4 antibody MAX.16H5 that has been investigated in auto- and alloimmunity. Wajant and Beilhack from Würzburg highlight the impact of tumor necrosis factor signaling on the regulation of FoxP3 regulatory T cells being known as central players for sustained immune tolerance after allogeneic HCT.

This Research Topic bundles cutting-edge, innovative, and translational original research articles as well as excellent reviews from renowned scientists highlighting recent progress in the field of transplant immunology and immune tolerance.

AUTHOR CONTRIBUTIONS

DS and EM wrote the editorial.

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

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MINI REVIEW
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Intestinal Microbiota Influence Immune Tolerance Post Allogeneic Hematopoietic Cell Transplantation and Intestinal GVHD

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Under normal conditions our intestines are inhabited by trillions of diverse microorganisms composing the intestinal microbiota, which are mostly non-pathogenic anaerobic commensal bacteria vital for the maintenance of immune homeostasis. The composition and diversity of the intestinal microbiota can be disturbed by various factors including diet, antibiotics, and exposure to intestinal pathogens. Alterations of the intestinal microbiota contributes to many diseases including graft-vs.-host disease (GVHD), a life threatening complication that occurs after allogeneic hematopoietic cell transplantation (allo-HCT) caused by an allogeneic reaction of donor T cells against recipient target tissues. Intestinal GVHD is most difficult to treat and connected to a high mortality. Due to recent advances in high-throughput sequencing technology, composition of the microbiome during allo-HCT has been characterized, and some common patterns have been identified. Metabolites produced by intestinal bacteria were shown to promote intestinal tissue homeostasis and immune tolerance post-allo-HCT. In this review, we discuss the role of the intestinal microbiota and metabolites in the context of acute GVHD. Moreover, novel therapeutic approaches that aim at protecting or regenerating intestinal cell populations will be highlighted.

Keywords: GVHD, allo-HCT, microbiota, intestinal inflammation, microbial metabolites, Paneth cells

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INTRODUCTION

Allogeneic hematopoietic cell transplantation (allo-HCT) is the only curative therapy option for most acute leukemias. Prior allo-HCT patients undergo a conditioning regimen, consisting of chemotherapy with or without radiotherapy to decrease the number of malignant cells, and prevent rejection of the transplanted hematopoietic donor cells. Subsequently, hematopoietic stem cells (HSCs) from the bone marrow or peripheral blood of an allogeneic donor are infused. In addition to the HSCs, the graft contains allogeneic donor T cells, which can on the one hand attack residual malignant cells, known as graft-vs.-leukemia (GvL) effect, but on the other hand may attack healthy host tissues, resulting in graft-vs.-host disease (GVHD). Although the understanding of the disease pathophysiology has improved significantly in the last decades, acute GVHD is still a major cause of non-relapse morbidity, and mortality post-allo-HCT (1). In particular, patients developing acute GVHD that is refractory to standard steroid therapy are difficult to treat and have a dismal prognosis with only 5–30% overall survival (2–4).

The important influence of intestinal microbiota on immune responses, including post-allo-HCT, becomes more and more recognized and the gut is one of the main targets of acute GVHD. Severe gastrointestinal (GI) GVHD remains a major issue after allo-HCT, since it is difficult to treat and involvement of the GI tract is reported in almost all fatal acute GVHD cases (5). In addition to allogeneic donor T cells, recipient-derived myeloid cells have been shown to participate in GI GVHD development. A number of publications have demonstrated that innate immune cells, including neutrophils and inflammatory monocytes, are recruited to the gut shortly after allo-HCT and contribute to GVHD tissue damage (6–9). The recruitment of neutrophils was dependent on the translocation of bacteria into the intestinal wall and was abrogated in mice housed under germ-free conditions or subjected to antibiotics-based decontamination (6, 10). While in the early phase after allo-HCT antibiotics can reduce the number of transmigrated bacteria which is beneficial, the long-term effects of antibiotics are unfavorable because they limit the microbiota diversity.

INTESTINAL MICROBIOTA AND GVHD

Brief Historic Background

The first hint that the intestinal microbiota affect GVHD development dates back to the early 1970s, when first studies in murine models showed that GVHD was reduced and survival was prolonged in antibiotics-treated or germ-free mice undergoing allo-HCT (11–13).

Consequently, efforts were made to translate these insights into the human setting. Clinical trials investigated gut decontamination using antibiotics or laminar-airflow isolation rooms for patients undergoing allo-HCT, in order to minimize contact to microbiota (14–19). However, the study designs were heterogeneous and the outcomes showed inconsistent results. More recent trials even demonstrated that broad spectrum antibiotics increased GVHD-related mortality in allo-HCT patients and mice (20) and the lack of antibiotics that spare commensal bacteria remains a so far unresolved challenge (21). Therefore, to date no standardized protocol for prophylactic and peri-transplant antibiotic treatment has been established as standard of care across transplantation centers.

However, interest in the interplay between the microbiome and inflammatory conditions has been growing again in recent years. Major advances in modern high-throughput sequencing technology made it possible to investigate changes in the composition of the intestinal microflora after allo-HCT in depth and to analyze which bacteria might be detrimental and which may be beneficial with respect to immune tolerance post-allo-HCT.

Under physiological conditions, the intestinal microflora is highly diverse with a domination of anaerobic commensal bacteria, most importantly members of the *Firmicutes* and *Bacteroidetes* phyla (22, 23). During allo-HCT, this diversity is reduced and significant alterations in the microbial composition of the gut can be observed, indicating that the conditioning and/or transplantation induce an intestinal dysbiosis (24–26). Holler et al. demonstrated shifts in the intestinal microbiome after allo-HCT with a predominant increase in the proportion of *Enterococci* (24). This shift was associated with development of GI GVHD. The mean proportion of *Enterococci* was 21% in patients who did not develop GI GVHD as compared to 46% in those that subsequently developed GI GVHD and 74% at the time of active GVHD (24). Moreover, lower intestinal diversity has been shown to be associated with significantly worse mortality outcomes in allo-HCT patients, suggesting that the intestinal microbiota may be an important factor in the success or failure in allo-HCT (25). Looking more specifically at the composition of the microbiota of patients who died vs. patients who survived, greater abundance of *γ-Proteobacteria*, including *Enterobacteriaceae* correlated with increased mortality, whereas greater abundance of *Lachnospiraceae* and *Actinomycetaceae* was associated with favorable outcomes (25).

Since those first innovative studies, a lot of work has been done to investigate how the intestinal microbiota affects immune tolerance post-allo-HCT. A list of preclinical and clinical studies that have analyzed the role of specific bacteria during GVHD pathogenesis can be found in **Table 1** and has been reviewed in detail elsewhere (37, 38).

In the following, we will highlight the most recent of these findings as well as the latest clinical trials aiming to reduce GVHD by manipulating the intestinal microbiota.

Recent Developments

Following up on previous studies showing post-transplant monodomination of the gut microbiome with *Enterococcus* spp. in a smaller number of allo-HCT patients (24, 39), these findings were recently confirmed in a large cohort derived from three different centers (28). Monodomination with *Enterococcus* was significantly associated with severe acute GVHD. Moreover, oral administration of *Enterococcus faecalis* following transplantation significantly aggravated acute GVHD in a murine MHC-mismatched model, indicating a causative role for *Enterococcus* spp. in the pathogenesis of acute GVHD (28).

Another study found a significant depletion of anti-inflammatory *Clostridia* spp. (AIC) preceding the development of GVHD in pediatric allo-HCT patients (35). Treatment with anti-anaerobic antibiotics and subsequent depletion of AIC was associated with increased GVHD. These clinical observations were also validated in a murine GVHD model, where clindamycin depleted *Clostridia* and exacerbated disease, whereas oral AIC supplementation attenuated GVHD (35).

In a very recent monocentric study comprising 275 patients, the cumulative incidence of acute GVHD, in particular in the gut, was significantly increased in allo-HCT recipients treated with 4th generation cephalosporins peritransplant (40). A retrospective analysis of 857 allo-HCT recipients by Shono et al. demonstrated that treatment with the broadband antibiotics imipenem-cilastatin or piperacillin-tazobactam was associated with increased GVHD-related mortality at 5 years (20). However, in contrast with the aforementioned study (40), GVHD-related mortality was not correlated with cefepime or aztreonam therapy. Moreover, imipenem-cilastatin treatment caused destruction of the colonic mucus layer in mice and

TABLE 1 | Summary of studies investigating how microbiota changes affect GVHD (structured by phylum).

Bacterium	Role in GVHD	Species	References
PROTEOBACTERIA			
Escherichia coli	Murine GI GVHD was accompanied by flora shifts toward *E. coli* and this increase was significantly associated with GVHD severity and mortality.	Mouse	Heimesaat et al. (27)
FIRMICUTES			
Enterococcus spp.	Expansion post-transplantation and association with increased GI GVHD severity in allo-HCT patients.	Human	Holler et al. (24)
	Associated with increased GVHD severity in mice and in patients in three different centers. Aggravation of GVHD in a murine MHC-disparate model.	Human/Mouse	Stein-Thoeringer et al. (28)
Lactobacillus johnsonii	GVHD was accompanied by increase in Lactobacillales and decrease in Clostridiales in mice and patients. Ampicillin treatment before allo-HCT resulted in reduced survival in GVHD mouse models. L. johnsonii reintroduction prevented increased GVHD lethality and pathology and prevented *Enterococcus* expansion in mice.	Human/Mouse	Jenq et al. (29)
Lactobacillus rhamnosus GG	Oral administration reduced translocation of enteric bacteria and acute GVHD in a murine model.	Mouse	Gerbitz et al. (30)
	Randomized trial of probiotic treatment in 31 allo-HCT recipients. The trial was terminated when interim analysis did not detect an appreciable probiotic-related change in the gut microbiome or incidence of GVHD.	Human	Gorshein et al. (31)
Lactobacillus plantarum	Ongoing clinical trial aiming to prevent GVHD by orally-administered *L. plantarum* in children undergoing allo-HCT. Preliminary results demonstrated safety and feasibility.	Human	Ladas et al. (32)
Clostridiales spp.	Clinical trial (64 patients, stool analyzed 12 days after BMT) showing that *Blautia* is associated with reduced GVHD-related mortality. Data were confirmed in a 2nd cohort with 51 patients.	Human	Jenq et al. (33)
	Oral gavage with *Clostridia* spp. reduced GVHD severity and mortality in murine mouse models.	Mouse	Mathewson et al. (34)
	Depletion of *Clostridia* spp. was associated with increased GVHD in 15 pediatric allo-HCT patients. Treatment with clinda-mycin depleted *Clostridia* and exacerbated GVHD in mice, while *Clostridia* supplementation reduced murine GVHD severity.	Human/ Mouse	Simms-Waldrip et al. (35)
BACTEROIDETES			
Barnesiella spp.	*Barnesiella* spp. conferred protection against *Enterococcus* domination in allo-HCT patients and mice.	Human/ Mouse	Ubeda et al. (36)
Bacteroides/Prevotella spp.	*Bacteroides/Prevotella* spp. increased during GI GVHD in mice.	Mouse	Heimesaat et al. (27)
VERRUCOMICROBIA			
Akkermansia muciniphila	Allo-HCT recipients ($n = 857$) as well as GVHD mice treated with broad-spectrum antibiotics showed increased GVHD severity. Imipenem-cilastatin treatment caused destruction of the colonic mucus layer and expansion of *Akkermansia muciniphila* in mice.	Human/Mouse	Shono et al. (20)

expansion of *Akkermansia muciniphila*, which is a commensal bacterium with mucus-degrading capabilities (41, 42). These data suggest that mucus degradation might aggravate murine GVHD (20).

Based on these recent advances in the field, several clinical trials are currently aiming to reduce GVHD by manipulating the intestinal microbiota. Fecal microbiota transplantation (FMT), also known as stool transplantation, has been used to successfully treat recurrent *Clostridium difficile* infection in non-HCT patients (43). Very recently, first promising data from studies investigating FMT in allo-HCT patients were published (44, 45). A pilot study used FMT from healthy donors to treat eight patients with steroid refractory acute GI GVHD, who have a dismal prognosis (NCT03148743). Following FMT, bacterial diversity and proportion of beneficial bacteria, such as *Bacteroides*, increased and clinical symptoms relieved (44). Another recent pilot trial assessed the effect of third-party oral FMT early after allogeneic HCT (NCT02733744). First studies showed that FMT was feasible and associated with improvements in microbiome diversity in transplant recipients (45). Further studies assessing the safety and efficacy of FMT for GVHD prophylaxis are ongoing (NCT03214289, NCT03359980, NCT03549676, NCT03492502).

THE EFFECT OF MICROBIAL METABOLITES ON GVHD

Microbial metabolites comprise a multitude of different intermediate products and end products of intestinal microbiota

metabolism with various functions, yet their role in GVHD pathophysiology is merely starting to be discovered. Very recent publications revealed that one of the mechanisms, how intestinal bacteria can influence immune tolerance post-allo-HCT, is the production of metabolites (**Figure 1**). **Table 2** summarizes studies that have investigated the role of microbial metabolites in epithelial regeneration.

Indole and indole derivatives are produced by commensal bacteria using the enzyme tryptophanase and are known to enhance epithelial barrier integrity and to attenuate inflammation (57). Several reports have shown that in the GI tract, bacterial-, and diet-derived indoles engage the aryl hydrocarbon receptor (AhR) and thereby expand innate lymphoid cells (ILC3) and their production of IL-22 (47, 58–60). Specifically, the tryptophan metabolite indole-3-aldehyde produced by intestinal microbiota induces AhR-dependent *Il22* transcription and mucosal immune homeostasis (47). The important role of IL-22 in the protection of intestinal tissue is further discussed below. Very recently, indole and indole derivatives either produced by administered *E. coli* strains or administered exogenously have been shown to strongly reduce GVHD severity, damage of the intestinal epithelium, and transepithelial bacterial translocation, while the GvL effect was not compromised. Mechanistically it was demonstrated that the effects of indole administration were mediated through an increased type I interferon response in an IL-22 independent fashion (48).

Another study focusing on microbiota-derived metabolites could show that the short-chain fatty acid butyrate was significantly reduced in murine intestinal tissue post-allo-HCT resulting in diminished histone acetylation in intestinal epithelial cells (IECs) (34). Daily intragastric administration of butyrate improved IEC junctional integrity and reduced clinical scores and mortality in different murine GVHD models (34). Moreover, butyrate-producing bacteria in the gut were shown to be associated with increased resistance against respiratory viral infection in allo-HCT patients, indicating a favorable role of these bacteria for both immune regulation and prevention of infection after allo-HCT (61). Interestingly, a clinical trial analyzing the role of oral potato-based starch, which is able to increase microbial butyrate production (62), in GVHD prevention is currently recruiting (NCT02763033).

Focusing more on the receptors of bacterial metabolites, the group of Dr. Reddy recently revealed an important regulatory role for GPR43, a G-protein-coupled receptor on IECs recognizing the microbiota-derived short-chain fatty acids, butyrate, and propionate, during GVHD development. Sensing of the microbial metabolites by GPR43 reduced GVHD severity and mortality in murine models by activating the NLRP3 inflammasome in recipient non-hematopoietic cells via ERK phosphorylation. Importantly, GPR43 did not seem to play a role for host and donor hematopoietic cells, since absence of GPR43 did not lead to systemic activation of donor T cells or inflammation (50). While previous reports have already demonstrated a critical role of NLRP3 inflammasome activation in host APCs for the full manifestation of GVHD (63), this study shows that it has the opposite effect in non-hematopoietic IECs. Hence, these data shed new light on the function of NLRP3 in the pathogenesis of GVHD, implicating that the NLRP3 inflammasome can either mitigate or exacerbate GVHD, depending on the involved cell type.

In a recently published study, Varelias et al. demonstrated that recipient mucosal-associated invariant T (MAIT) cells are present in acute GVHD target organs in murine models, including the intestinal lamina propria (56). These cells express semiinvariant T cell receptor repertoires recognizing and responding to microbial riboflavin metabolites. During acute GVHD, MAIT cells generated large amounts of IL-17A, enhanced intestinal barrier function and reduced GI GVHD severity (56). Given the known ability of IL-17 to maintain epithelial cell barrier function (64, 65), the authors conclude that riboflavin metabolite sensing MAIT cells regulate acute GVHD at least partially via IL-17A secretion.

STRATEGIES AIMING TO PROTECT OR REGENERATE ISCS AND PANETH CELLS

Severe GI GVHD remains a major issue after allo-HCT, since it is difficult to treat and involvement of the GI tract is reported in almost all fatal acute GVHD cases (5). Therefore, new therapy approaches aiming for tissue regeneration in GVHD target organs, as opposed to systemic immunosuppression, are promising.

R-Spondin1 (R-Spo1)

Intestinal stem cells (ISCs) represent important players for both physiological renewal of intestinal cells and tissue regeneration after injury. In a murine model, it was shown that ISCs are damaged during the conditioning regimen and the development of GVHD (66). This could be rescued by treatment with the Wnt agonist R-spondin1 (R-Spo1), which protected against ISC damage after allo-HCT, thereby ameliorating GVHD. In a subsequent study the authors extended their findings, demonstrating that R-Spo1 induces differentiation of ISCs into Paneth cells (67). Paneth cells are known to produce anti-microbial peptides, most importantly alpha defensins, which shape the intestinal antimicrobial flora by mostly targeting non-commensals. Targeting and destruction of Paneth cells during GVHD has already been reported (29, 68, 69). Consistently, by inducing ISC differentiation into Paneth cells, R-Spo1 treatment augmented secretion of alpha defensins, and prevented GVHD-mediated dysbiosis, restoring intestinal homeostasis. The authors suggest that R-Spo1 could inhibit the growth of pathogenic non-commensals, while protecting favorable symbiotic bacteria (67). These findings represent a potential novel strategy to physiologically shape the intestinal microbiome.

IL-22

IL-22 is a member of the IL-10 cytokine family that plays opposing roles in different autoimmune diseases, being able to drive or regulate disease (70–73). Similarly, both protective and pro-inflammatory roles for IL-22 in GVHD models have been described. On the one hand, IL-22 was recently shown

to exacerbate acute GVHD by reducing regulatory T cells in a parent-to-F1 mouse model (74) and to promote cutaneous chronic GVHD (75).

In contrast, Hanash et al. demonstrated that recipient innate lymphoid cell (ILC)-derived IL-22 was able to enhance regeneration of IL-22R-expressing ISCs in a more acute major mismatch GVHD model (76). Host deficiency of IL-22 caused increased epithelial damage, loss of ISCs, and loss of epithelial integrity, resulting in GVHD exacerbation. In a subsequent study, the Hanash group further elucidated the underlying mechanisms, showing that IL-22 increased the growth of murine and human organoids by inducing STAT3 phosphorylation in $Lgr5^{+}$ ISCs (77). Moreover, the authors used two different *in vivo* treatment approaches and showed the feasibility of both recombinant IL-22 and a recombinant human IL-22-dimer/Fc-fusion protein (F-652) for the treatment of murine GVHD. Based on the results from these preclinical studies, a Phase IIa clinical trial investigating F-652 in combination with systemic corticosteroids in patients with grade II-IV lower GI acute GVHD is currently recruiting (NCT0240665).

The described conflicting data of both a regulatory and inflammatory role for IL-22 in GVHD might result from many factors differing between the studies, including kinetics of the model, source of IL-22, presence or absence of other cytokines and pathophysiological differences between acute and chronic GVHD models.

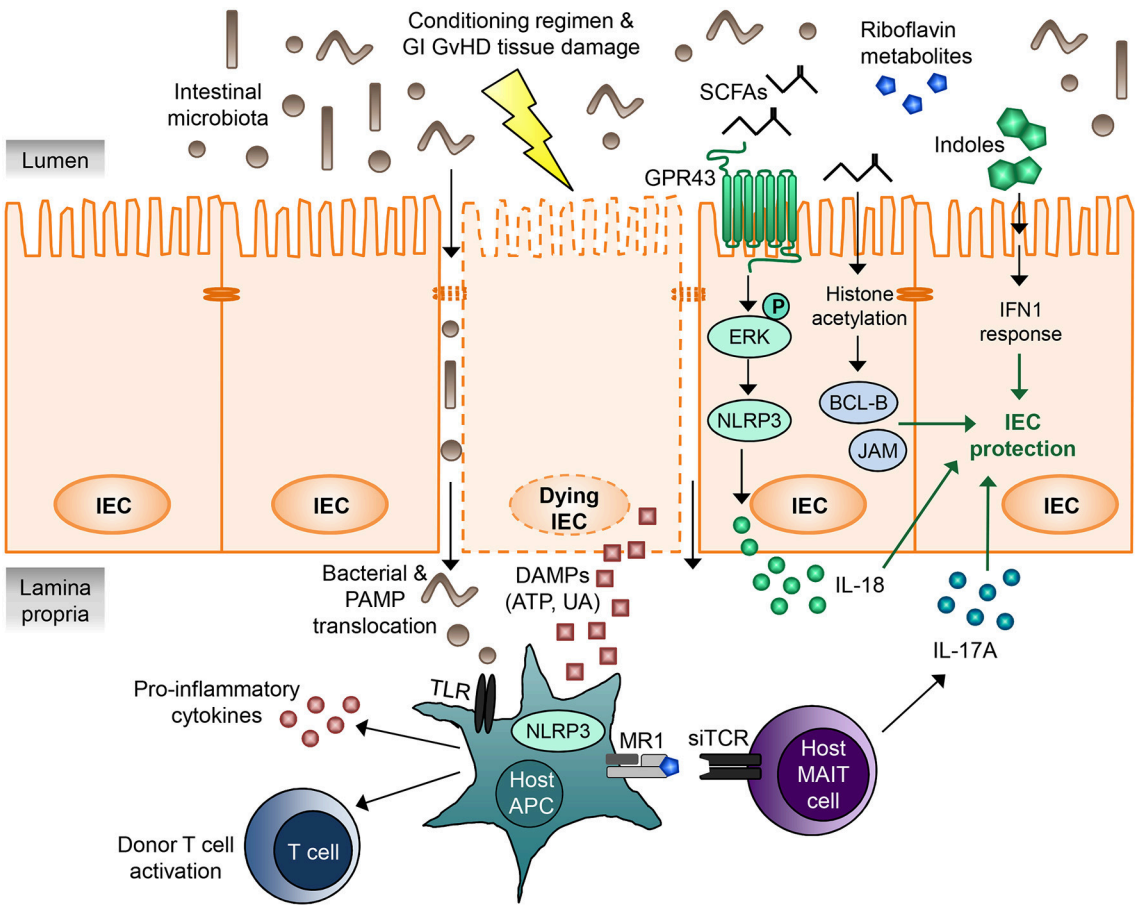

FIGURE 1 | Microbial metabolites regulating gastrointestinal GVHD. IECs are damaged by the cytotoxic conditioning regimen as well as by GI GVHD, leading to disruption of the intestinal barrier. DAMPs released by the dying IECs as well as translocating bacteria and PAMPs activate host APCs via TLRs and the NLRP3 inflammasome, resulting in pro-inflammatory cytokine release, donor T cell activation, and GVHD. Microbial metabolites derived from intestinal microbiota can regulate IEC damage and mitigate GVHD. SCFAs mediate IEC protection via at least two different mechanisms. Firstly, binding of SCFAs to the G-protein-coupled receptor GPR43 on IECs leads to ERK phosphorylation and subsequent NLRP3 inflammasome activation, which promotes IEC integrity and repair by increasing IL-18 secretion. Secondly, the SCFA butyrate acts as a histone deacetylase inhibitor, thereby increasing expression of many different target genes, including anti-apoptotic BCL-B and the junctional protein JAM. This results in decreased IEC apoptosis and increased junctional integrity and hence IEC protection. MAIT cells located in the lamina propria respond to riboflavin metabolite antigens presented on the MHC class I-like molecule MR-1 to secrete large amounts of IL-17A, which enhances intestinal barrier integrity. Indoles and indole derivatives act via type I IFN signaling to protect and repair the mucosal barrier from damage and ameliorate GVHD. The exact molecular mechanisms and involved proteins remain to be elucidated. APC, antigen-presenting cell; DAMP, danger-associated molecular pattern; GI, gastrointestinal; GVHD, graft-vs.-host disease; IEC, intestinal epithelial cell; IFN, interferon; JAM, junctional adhesion molecule; MAIT cell, mucosal-associated invariant T cell; PAMP, pathogen-associated molecular pattern; SCFA, short chain fatty acid; siTCR, semiinvariant T cell receptor; TLR, Toll-like receptor; UA, uric acid.

TABLE 2 | Summary of studies investigating how microbial metabolites affect intestinal epithelial regeneration.

Metabolite	Effect on intestinal epithelium	References
AHR LIGANDS		
AhR ligands	In the intestine, AhR deficiency or lack of AhR ligands reduced intraepithelial lymphocyte numbers and the control of the microbial load, resulting in increased immune activation, and increased vulnerability to epithelial damage.	Li et al. (46)
Indole-3-aldehyde	*Lactobacillus*-derived indole-3-aldehyde induced AhR-dependent transcription of the epithelial cell regenerative factor IL-22 in mice.	Zelante et al. (47)
	Administration of indole-3-aldehyde reduced disease severity, damage of the intestinal epithelium, and transepithelial bacterial translocation in a GVHD mouse model. The effects were mediated through an increased type I interferon response.	Swimm et al. (48)
SHORT CHAIN FATTY ACIDS (SCFAS)		
Butyrate	Butyrate added to colonocytes from germfree mice rescued their deficit in mitochondrial respiration and prevented them from undergoing autophagy by acting as an energy source.	Donohoe et al. (49)
	Administration of butyrate improved IEC junctional integrity and reduced clinical scores and mortality in different murine GVHD models.	Mathewson et al. (34)
Butyrate and propionate	Sensing of SCFAs by GPR43 reduced GVHD severity and mortality in mouse models by activating the NLRP3 inflammasome in recipient non-hematopoietic cells via ERK phosphorylation.	Fujiwara et al. (50)
Acetate, butyrate and propionate	SCFAs were important for maintaining intestinal barrier integrity by stimulating MUC-2 production in goblet cells.	Willemsen et al. (51)
	Treatment with SCFAs enhanced IEC migration and promoted wound healing by promoting production of milk fat globulin E8 in mouse and rat IECs *in vitro*.	Bilotta et al. (52)
Acetate	Acetate produced by bifidobacteria protected mice from enteropathogenic infection by improving intestinal defense mediated by IECs.	Fukuda et al. (53)
BILE ACIDS AND POLYAMINES		
Bile acids	Lack of the farnesoid X receptor, a receptor for bile acids, caused reduced epithelial barrier function, and increased bacterial translocation in the distal small intestine.	Inagaki et al. (54)
Polyamines	*In vitro* study showing that polyamines enhanced E-cadherin transcription by activating c-Myc, thereby promoting epithelial barrier function.	Liu et al. (55)
RIBOFLAVIN METABOLITES		
Riboflavin metabolites	Intestinal MAIT cells responding to microbial riboflavin metabolites produced IL-17A, promoted GI tract integrity and ameliorated intestinal GVHD.	Varelias et al. (56)

Regenerating Islet-Derived Protein 3 Alpha (REG3α)

A recent study nicely demonstrated the role of the Paneth cell derived protein Regenerating islet-derived protein 3 alpha (REG3α) to regulate GI acute GVHD (78). REG3α blood levels were strongly upregulated in patients developing severe GI GVHD, which had been shown before (79), and increased blood levels of REG3α in patients with GVHD inversely correlated with Paneth cell numbers. In murine models, the authors demonstrated that absence of REG3γ, which is the mouse homolog of REG3α, exacerbated GVHD without altering the microbial composition in the intestine (78). IL-22 is a known regulator of REG3γ expression (80, 81). In agreement with this, IL-22 administration restored REG3γ production and intestinal epithelial integrity by preventing ISC and Paneth cell apoptosis, resulting in amelioration of GVHD. In *Reg3γ* deficient mice this protection was completely abrogated, emphasizing the important role of REG3γ for gastrointestinal crypt homeostasis (78).

Since host genetics can shape the microbiome, single nucleotide polymorphisms (SNPs) in the genes encoding two of the most abundant Paneth cell antimicrobial peptides REG3α and HD5 were studied (82). Interestingly, SNPs in the gene for HD5 modulated the risk for acute GVHD severity in allo-HCT recipients, potentially by affecting intestinal expression of HD5, which leads to dysbiosis. In contrast, *REG3A* SNPs were not associated with acute GVHD severity in this retrospective study including 350 patients (82).

Keratinocyte Growth Factor (KGF)

Keratinocyte growth factor (KGF) is a member of the fibroblast growth factor family, regulating differentiation, and proliferation of epithelial cells, including IECs, as well as intestinal stem cells (83, 84). Almost 20 years ago, KGF was already shown to ameliorate GVHD-induced tissue damage in murine models, while preserving GvL effects (85, 86). Based on these preclinical findings two placebo-controlled randomized clinical trials evaluated the efficacy of human recombinant KGF (palifermin) to decrease acute GVHD. Severity of mucositis was reduced by palifermin in a subgroup of patients conditioned with cyclophosphamide and fractionated total-body irradiation in one trial (87). However, in contrast to the findings in murine models, GVHD incidence, severity, and survival were not significantly improved by KGF as compared to placebo in both trials (87, 88). Consistently, a more recent meta-analysis of several

clinical studies found no statistically significant difference in oral mucositis and acute GVHD severity in palifermin treated patients compared with those receiving placebo (89).

CONCLUSIONS AND FUTURE PERSPECTIVES

In the last 10 years it has become evident that a loss of diversity of the intestinal microbiota flora due to reduced food intake, chemotherapy-related damage, and antibiotics promotes the development of GVHD, which changed the dogma that antibiotics-based decontamination of the intestinal tract improves outcome post-allo-HCT. With the discovery that indoles and butyrate promote intestinal homeostasis the connection between antibiotics treatment and unfavorable outcome post-allo-HCT could be explained. Beyond bacterial species that were connected to GVHD, recent studies have also identified fungi and viruses that occurred more frequently in patients with severe GVHD. Based on this improved understanding on how the microbiome and the intestinal tract interact, novel strategies have been developed such as FMT that hold promise to overcome acute GVHD in a majority of patients. Ongoing clinical studies will provide important information on the role of FMT and regenerative strategies.

AUTHOR CONTRIBUTIONS

NK and RZ collected literature, discussed the studies, and wrote the manuscript.

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GM-CSF Promotes the Expansion and Differentiation of Cord Blood Myeloid-Derived Suppressor Cells, Which Attenuate Xenogeneic Graft-vs.-Host Disease

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Myeloid-derived suppressor cells (MDSCs) are increased in tumor patients. Studies have shown generation of MDSCs from human peripheral blood mononuclear cells (PBMCs) by various cytokine combinations. However, large scale expansion of human MDSCs has not been demonstrated or applied in clinic settings. We investigated which cytokine combinations among GM-CSF/SCF, G-CSF/SCF, or M-CSF/SCF efficiently expand and differentiate human MDSCs following culture $CD34^+$ cells of umbilical cord blood (CB). GM-CSF/SCF showed the greatest expansion of MDSCs. Up to 10^8 MDSCs ($HLA\text{-}DR^{low}CD11b^+CD33^+$) could be produced from 1 unit of CB following 6 weeks of continuous culture. MDSCs produced from culture of $CD34^+$ cells with GM-CSF/SCF for 6 weeks had the greatest suppressive function of T cell proliferation and had the highest expression of immunosuppressive molecules including iNOS, arginase 1 and IDO compared to those differentiated with G-CSF/SCF or M-CSF/SCF. MDSCs secreted IL-10, TGB-β, and VEGF. The infusion of expanded MDSCs significantly prolonged the survival and decreased the GVHD score in a NSG xenogeneic model of GVHD. Injected MDSCs increased IL-10 and TGF-β but decreased the level of TNF-α and IL-6 in the serum of treated mice. Notably, FoxP3 expressing regulatory T (Treg) cells were increased while IFN-γ (Th1) and IL-17 (Th17) producing T cells were decreased in the spleen of MDSC treated mice compared to untreated GVHD mice. Our results demonstrate that human MDSCs are generated from CB $CD34^+$ cells using GM-CSF/SCF. These MDSCs exhibited potent immunosuppressive function, suggesting that they are useable as a treatment for inflammatory diseases such as GVHD.

Keywords: myeloid-derived suppressor cells (MDSCs), umbilical cord blood (CB), $CD34^+$ cells, recombinant GM-CSF and SCF cytokine combinations (GM-CSF/SCF), immunosuppressive function, xenogeneic graft-vs.-host disease (GVHD), $FoxP3^+$ regulatory T cells (Treg)

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a potentially curative procedure for a variety of malignancies and benign hematological disorders (1). The therapeutic benefit of allo-HSC is mediated by T cells of donor origin in blood that kill the leukemia, known as graft vs. leukemia response (2). However, the widespread use of allo-HSCT is limited by risks such as graft rejection and graft-vs.-host disease (3, 4). GVHD remains the major cause of mortality and morbidity after the allo-HSCT and is caused by a reaction of allo-reactive donor T cells to incompatible human leukocyte antigens (HLA) of the recipient. The ensuing proliferation/activation of other immune cells lead to a variety of injuries to host tissues caused by the release of inflammatory cytokines. The immunosuppressing drugs (ISDs) such as cyclosporine, tacrolimus, and steroids are used to modulate the adaptive immune system through blocking T cell activation or depletion of T cells. Further, all ISDs carry the risk of serious infections. Depletion of T cells from donor allograft can improve the survival rates for patients and decrease the incidence of acute GVHD. However, this comes at the risk of graft failure, reduced GVL activity, and increased incidence of leukemic relapse. Therefore, a new therapeutic approach is needed to optimize GVL and minimize GVHD. Recent strategies have introduced the infusion of newly identified immune suppressor cells such as mesenchymal stromal cells (MSCs) (5–7), regulatory T (Treg) cells (8–10), and myeloid-derived suppressor cells (MDSCs) (11–13) to take advantage of their immunoregulatory properties in order to prevent GVHD.

Umbilical cord blood (CB) is considered the most plentiful reservoir of regenerative cells for a large number of clinical applications (14, 15). CB has been used as a source to generate regulatory immune cells. Use of CB-derived MSCs had been investigated *in vitro*, in animal models, and in early stage clinical trials for cardiovascular diseases (16), as well as liver diseases, neurological deficits, immune system diseases, lung, and kidney injury (17). The use of CB-derived, *ex vivo*-expanded Treg or MSCs is currently being evaluated as one strategy to prevent GVHD. Their adoptive transfer has been associated with improved survival in mice (5, 8). Recently, others have reported that the fibrocytic MDSCs from CB expressed indoleamine dioxygenase (IDO), and promoted tolerance via Treg-cell expansion (18).

MDSCs are known to accumulate in the peripheral blood, lymphoid organ, spleen, and tumor sites in cancer, infection, chronic inflammation, transplantation, and autoimmunity (19, 20). These cells inhibit T cell proliferation following stimulation with allo-antigens. Importantly, MDSCs mediate the chemotaxis and activation of Treg cells *in vivo* (21, 22). The $CD14^{+}HLA\text{-}DR^{low/neg}$ monocytic MDSCs are significantly expanded in the peripheral blood of acute GVHD patients who received allo-HSCT, resulting in T cells dysfunction and GVHD inhibition (23, 24). The factors triggering MDSC expansion and activation are well-studied in tumor models, including cytokines such as IL-1β, IL-6, IL-10, and IL-13, growth factor such as SCF, VEGF, GM-CSF, G-CSF, and M-CSF, as well as calcium binding pro-inflammatory proteins such as S100A8, S100A9, cyclooxygenase-2, and prostaglandin E2 (25, 26). However, it is not known how to expand human MDSCs to a large scale enough to make their use feasible for clinical applications.

Here, we demonstrate that the combination of GM-CSF/SCF is the most potent enhancer to expand and differentiate functional MDSCs from human cord blood compared to G-CSF/SCF or M-CSF/SCF. We further show that adoptive transfer of CB-derived MDSCs ameliorate GVHD in a xenogeneic NSG mouse model.

MATERIALS AND METHODS

Subjects and Isolation of Cells With the MACS System

The use of human peripheral blood mononuclear cells (PBMCs) and human umbilical cord blood (CB) were approved by the institutional review board of the College of Medicine, Catholic University of Korea, Seoul, Republic of Korea, respectively (permit No. MC16SNSI0001, MC15TISE0023, MC17TNSI0002). Human peripheral blood samples were obtained from healthy donors, and mononuclear cells were isolated by Ficoll-Hypaque (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) density gradient centrifugation. After density separation, $CD14^{+}$ monocytes and $CD4^{+}$ T cells were isolated with the magnetic cell-sorting (MACS) system (Miltenyi Biotec, Bergisch Gladbach, Germany), using anti-CD14 and anti-CD4 antibodies, respectively, conjugated to magnetic MicroBeads (Miltenyi Biotec) according to the manufacturer's instructions.

Generation of Human MDSCs

Human CB was provided from the Catholic Hematopoietic Stem Cell Bank after written informed consent given by normal full-term pregnant women. For MDSCs generation, isolated $CD34^{+}$ cells (Miltenyi Biotec, Bergisch Gladbach, Germany) were cultured in a 48-well plate (BD Falcon, Bedford, MA) at 1×10^5 cells/ well with 1 ml of IMDM containing 10% FBS (Gibco, Grand Island, NY, United States), 10% penicillin–streptomycin (100 U/ml; Lonza Walkersville, MD, United States), 2 mM L-glutamine (Lonza Walkersville) (10% complete medium), 100 ng/ml human GM-CSF (300–03, PeproTech, Rocky Hill, NJ, United States), 100 ng/ml human G-CSF (300–23, PeproTech), or 100 ng/ml human M-CSF (300–25, PeproTech) and 50 ng/ml human SCF (300–07, PeproTech). After incubation for 7 days, the cells were removed from the 48 well plate and centrifuged at 1,300 rpm for 5 min. After one wash with serum free IMDM, the cells were cultured for 2 weeks and media was changed every 7 days. From weeks 4–6, the cells were cultured at a higher density (5×10^5 cells/well). Media was changed every 7 days throughout 6 weeks of the culture.

Production of HCMV pp65 mRNA by *in vitro* Transcription

The sequences encoding full-length pp65 were cloned into the pcDNA3 vector (Invitrogen, Grand Island, NY, United States). The pcDNA3-pp65 were linearized with Sma I restriction enzyme and purified using phenol/chloroform extraction and ethanol precipitation. In *vitro* transcription of recombinant pp65 from the linearized plasmids was conducted by using T7 RNA

polymerase of Ambion mRNA T7 Ultra Kit (Life Technologies) according to the manufacturer's instructions.

Generation of Monocyte Derived DCs and pp65 mRNA Electroporation

Immature DCs (iDCs) were generated from $CD14^+$ monocytes of human PBMCs by culturing them with the $CD14^+$ cells were cultured with human GM-CSF (100 ng/ml; PeproTech) and IL-4 (100 ng/ml; PeproTech) in 10% complete RPMI 1640 medium (Lonza Walkersville) for 6 days. Media was changed every 2 days. On day 6, 5×10^6 iDCs were resuspended with 200 μl OptiMEM without phenol red (Invitrogen Life Technologies, Grand Island, NY). The cells were transfected with 20 μg pp65 mRNA to a 2 mm cuvette with a single wave pulse (300 V and 500 μs) by using a Gene pulser (BTX, San Diego, CA). The electroporated iDCs were matured at 1×10^6 cells/ml in 37°C, 5% CO_2 for 24 h using a maturation cocktail containing 100 ng/mL IL-4, 100 ng/mL GM-CSF, 10 ng/mL TNF-α (PeproTech), 10 ng/mL IL-6 (PeproTech), and 10 ng/mL IL-1β (PeproTech).

Flow Cytometric Analysis

All samples were incubated with anti-CD16/CD32 to block Fc receptor binding on ice for 20 min and then stained with the indicated anti-human antibodies. The expression of MDSCs was assessed by staining with monoclonal antibodies specific for surface markers including CD33, CD11b, HLA-DR, CD14, CD15, CD11c, CD13, HLA-ABC, CD45, CD40, CD80, CD86, CD83, PDL-1, CCR2, CCR5, CCR7, CD62L, E-Cadherin, CXCR4, and ICAM-1 (**Supplemental Table 1**)

For intracellular staining of pStat, pAkt and pmTOR, the MDSCs were lysed and fixed using BD Phosflow Lyse/Fix buffer for 10 min at 37°C. Cells were then permeabilized in BD Phosflow Perm Buffer III on ice for 30 min. For intracellular staining of iNOS, IDO, arginase 1, MPO, and FoxP3 (For Treg analysis, anti-human CD4 PerCP-Cy5.5 and anti-human CD25 APC were already stained), the MDSCs were fixed in BD Cytofix buffer and then permeabilized in BD Cytoperm buffer. Cells were then washed twice in the stain buffer and stained on ice for 30 min with monoclonal antibodies specific for pStat1, pStat3, pStat6, pAkt, pmTOR, iNOS, IDO, arginase 1, MPO, FoxP3 (**Supplemental Table 1**). The compensation bead (UltraComp eBeads Catalog No. 01-2222, Invitrogen) were used to avoid the spillover of fluorescence conjugated antibodies. All samples were acquired on a BD LSR Fortessa and then analyzed using FlowJo 9.2.1.

Cell Sorting

Cells cultured with GM-CSF/SCF were harvested at 3 weeks post culture and stained with anti-human CD33-FITC antibody and anti-human CD11b-PE antibody. $CD33^+$ $CD11b^+$ and $CD33^+$ $CD11b^-$ were sorted by a FACS Aria sorter (BD Biosciences).

Suppression Assay

To evaluate suppressive activity of MDSCs, PBMCs were labeled with 5 μmol/ml CFSE (Invitrogen, Cat.No.C34554) and activated with Dynabead Human T-Activator anti-CD3 and anti-CD28 (0.5 μg/mL, Gibco, Cat.No.11131D) microbeads. MDSCs were added to parallel cultures at ratio of 1:1, 1:0.5, and 1:0.25 (PBMCs: MDSCs) ratio. After 6 days of co-culture, cells were harvested and stained with anti-human CD3 PE-Cy7, CD4 APC, and CD8 efluor450 antibodies. The cells were analyzed on a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA). Data were analyzed using ModFit LT software (Verity Software House Inc., Topsham, ME, United States).

Treg Induction by MDSCs

The $CD4^+$ T cells were isolated from healthy adult PBMCs. 1×10^6 $CD4^+$ T cells were stimulated with Dynabead Human T-Activator anti-CD3 and anti-CD28 (0.5 μg/mL) microbeads in the presence or absence of 2×10^6 MDSCs at a 1:2 ($CD4^+$ T cells: MDSCs) ratio in 12 well plates for 3 days. After 3 days of co-culture, the cells were harvested and Treg induction was determined by flow cytometric analysis.

3H-Thymidine Mixed Lymphocytes Reaction (MLR) Assay

Mature DCs (1×10^4) were mixed with responder $CD4^+$ T cells (1×10^5) in 96 well plates with or without MDSCs (1×10^4). After 5 days culture, cell were pulsed with 1 μCi [^{3}H]thymidine/well for 18 hrs and harvested using a Packard filtermate cell harvester (Packard Instruments, Meriden, CT). Specific [^{3}H]thymidine incorporation into genomic DNA was determined using a Packard TopCount NXT.

ELISPOT Assay

To measure the effect of MDSCs on antigen-specific T cell responses, ELISPOT assay was performed using DCs electroporated with mRNA encoding human cytomegalovirus (HCMV) pp65 antigen according to the manufacturer's instructions (BD ELISPOT assay kit; BD Biosciences) as described previously (27). Because HCMV infects between 60 and 90% of adults and then it remains latent under control of immune system, T cells specific for various HCMV antigens are present in a high frquency, mainly in response to the HCMV pp65 antigen. Therefore, we used mononuclear cells from a healthy volunteer donor that has been shown to have a high T cell immune response to the HCMV pp65 antigen. It has been established in our previous study that DCs electroporated with mRNA encoding HCMV pp65 antigens present antigen to T cells through HLA class I and class II molecules after antigens processing, so T cell responses to whole antigens are measured rather than specific HLA-restricted antigen epitopes (28).

Briefly, anti-human IFN-γ antibody was pre-coated in a 96 well-microplates for 24 h at 37°C. pp65 RNA-electroporated mature DCs were added to a 96-well-microplates at a concentration of 1×10^4 cells/well in 10% complete RPMI 1640. 1×10^5 autologuous $CD4^+$ T cells were then added to the well as a stimulator in the presence or absence MDSCs (1×10^4). After incubation for 24 h, the cells were removed and the plates were washed three times with the wash buffer and the PBS-Tween buffer, respectively. The wells were added with the biotinylated antibody for human IFN-γ and incubated for 2 h at room temperature. The plates were washed with PBS-Tween buffer, and then incubated with streptavidin-HRP for 1 h

at room temperature. The washing of well was repeated, and then the 100 μl AEC substrate and H_2O_2 were added to the well. After development of the spots, the reaction was stopped with addition of distilled water. Plates were dried for overnight. The spots number of IFN-γ-secreting cells was quantitated with an automatic an AID-ELISPOT reader (AID Diagnostika GmbH, Strassberg, Germany).

Assessment of Pro-inflammatory Cytokine and Anti-inflammatory Cytokine by ELISA

ELISA was performed according to the manufacturer's instructions (R&D Systems). Human IL-17, IFN-γ, transforming growth factor (TGF)-β, IL-10, and vascular endothelial growth factor (VEGF) were measured in the cell culture supernatant. Whole blood collected from mouse was centrifuged at 400 × g for 10 min, and the serum was analyzed for the levels of human IL-10, TGF-β, tumor necrosis factor (TNF)-α, and IL-6. The plates were then read using an ELISA microtiter plate autoreader at 450 nm (Molecular Devices, Sunnyvale, CA, United States).

Xenogeneic GVHD Mouse Model

NSG (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ, 6 weeks old) male mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and acclimated in pathogen-free animal facilities for 2 weeks before the experiments. All animal work was approved in advance by the Institutional Animal Care and Use Committee, College of Medicine, Catholic University of Korea. Recipient (8 weeks old) mice were irradiated with 200 cGy by Mevatron MXE-2 instrument (Siemens, New York, NY, United States) at day −1 and received 1×10^6 human PBMCs intravenously (IV) at day 0 to induce xenogeneic GVHD mouse models. The control did not receive human PBMCs or irradiation. To examine the effect of MDSCs in a xenogenic GVHD model, MDSCs (1, 2.5 and 5×10^6 cells) were infused into the tail vein on days 21 and 24. Survival after PBMCs transplantation was observed daily, and the grade of clinical GVHD was recorded every other day using a scoring system on the basis of weight loss, posture, physical activity, fur texture, and skin integrity. We evaluated GVHD according to overall survival rate until day 200. Each group was consisted of total eight mice and three mice were sacrificed at day 60 for FACS analysis and ELISA, respectively.

Multi-Cytokine Membrane Array

Serum collected from GVHD mice or MDSCs mice was analyzed using the human cytokine array (Proteome Profiler Array Human Cytokine, R&D Systems, ARY005). The array analysis was performed according to the manufacturer's instructions. Those cytokine levels were captured by exposure to the X-ray films and quantified by densitometry using Image J software.

Statistical Analysis

Data were analyzed for statistical significance using Prism version 6.0 (GraphPad, San Diego, CA). Student's *t*-test or ANOVA was used to calculate the significance between groups. Differences in animal survival (Kaplan Meier curves) were analyzed by log-rank test. Results were considered statistically significant if $p \leq 0.05$.

RESULTS

Expansion of Cord Blood CD34$^+$ Cells and Differentiation Into MDSCs

To generate MDSCs from cord blood, 1×10^5 CD34$^+$ umbilical cord blood cells were plated in 48 well plates. Recombinant human cytokines such as GM-CSF/SCF, G-CSF/ SCF or M-CSF/SCF were added weekly and cultured for an additional 3 weeks. After 3 weeks, the 5×10^5 cells per well were plated in 48 well plates for 3 weeks (**Figure 1A**). The cells were most effectively expanded by the combination of GM-CSF/SCF (MDSCs). Up to 10^8 MDSCs could be generated from 1 unit of cord blood. This is more than a thousand folds (1,104 ± 70.30) increase. The expansion of cells cultured with G-CSF/SCF or M-CSF/SCF were 500 (514 ± 42.62) and 300 (317 ± 39.27) fold, respectively (**Figure 1B**). After 3 weeks of culture, expansion of CD34$^+$ cells reached stagnation in the three different groups. CD34$^+$ cells were continuously differentiated until 6 weeks (**Figure 1D**). MDSCs from GM-CSF/SCF or M-CSF/SCF expressed CD11b$^+$CD33$^+$ (90% of cells positive for these markers) at 6 weeks. Only 10% of cells from G-CSF/SCF cultures expressed CD11b$^+$CD33$^+$ at 3 weeks. The CD11b$^+$CD33$^+$ almost disappeared by 6weeks following G-CSF/SCF cultures (**Table 1**).

To further define the differentiation of MDSCs from GM-CSF/SCF, CD11b$^+$CD33$^+$ and CD11b$^-$CD33$^+$ cells at 3 weeks were sorted via FACS, and then cultured with GM-CSF/SCF for a further 1 week (**Figure 1E**). The sorted CD11b$^-$CD33$^+$ cells changed to a phenotype that expressed more than 69.92 (69.92 ± 0.92)% of CD11b$^+$ CD33$^+$ and the phenotype of the sorted CD11b$^+$ CD33$^+$ cells remained unchanged. Therefore, this result demonstrates that GM-CSF/SCF induces differentiation from the CD11b$^-$CD33$^+$ cells to the CD11b$^+$CD33$^+$ cells. Next, we evaluated the expression of HLA-DR, CD14, and CD15 in cells cultured with GM-CSF/SCF, G-CSF/SCF, or M-CSF/SCF at 3 weeks and 6 weeks (**Figure 1F**). The expression of HLA-DR in the cells cultured with GM-CSF/SCF or G-CSF/SCF was gradually decreased and became low, whereas cells cultured with M-CSF/SCF highly expressed HLA-DR at 3 and 6 weeks, respectively. From 3 to 6 weeks, the expression of CD14 were increased in the cells cultured with GM-CSF/SCF or M-CSF/SCF whereas that of CD15 were decreased (**Figure 1F**, **Table 1**). At 6 weeks of culture, cells cultured with GM-CSF/SCF showed the phenotypic markers of monocytic MDSCs; HLA-DR^{low}, CD11b$^+$CD33$^+$, CD14$^+$, CD15$^-$. These results suggest that GM-CSF/SCF among cytokine combinations most efficiently induces the cell expansion during 3 weeks of culturing with at low cell concentration, and the differentiation to MDSC progresses during the remaining 3 weeks of culturing at 5 fold higher cell concentration.

Lineage Markers and Immunophenotypes of MDSCs

We performed immunophenotypic characterization of MDSCs following 6 weeks of GM-CSF/SCF, G-CSF/SCF, and M-CSF/SCF culture. Expression of surface markers was examined using flow cytometry and adult monocyte derived mature DCs (mature

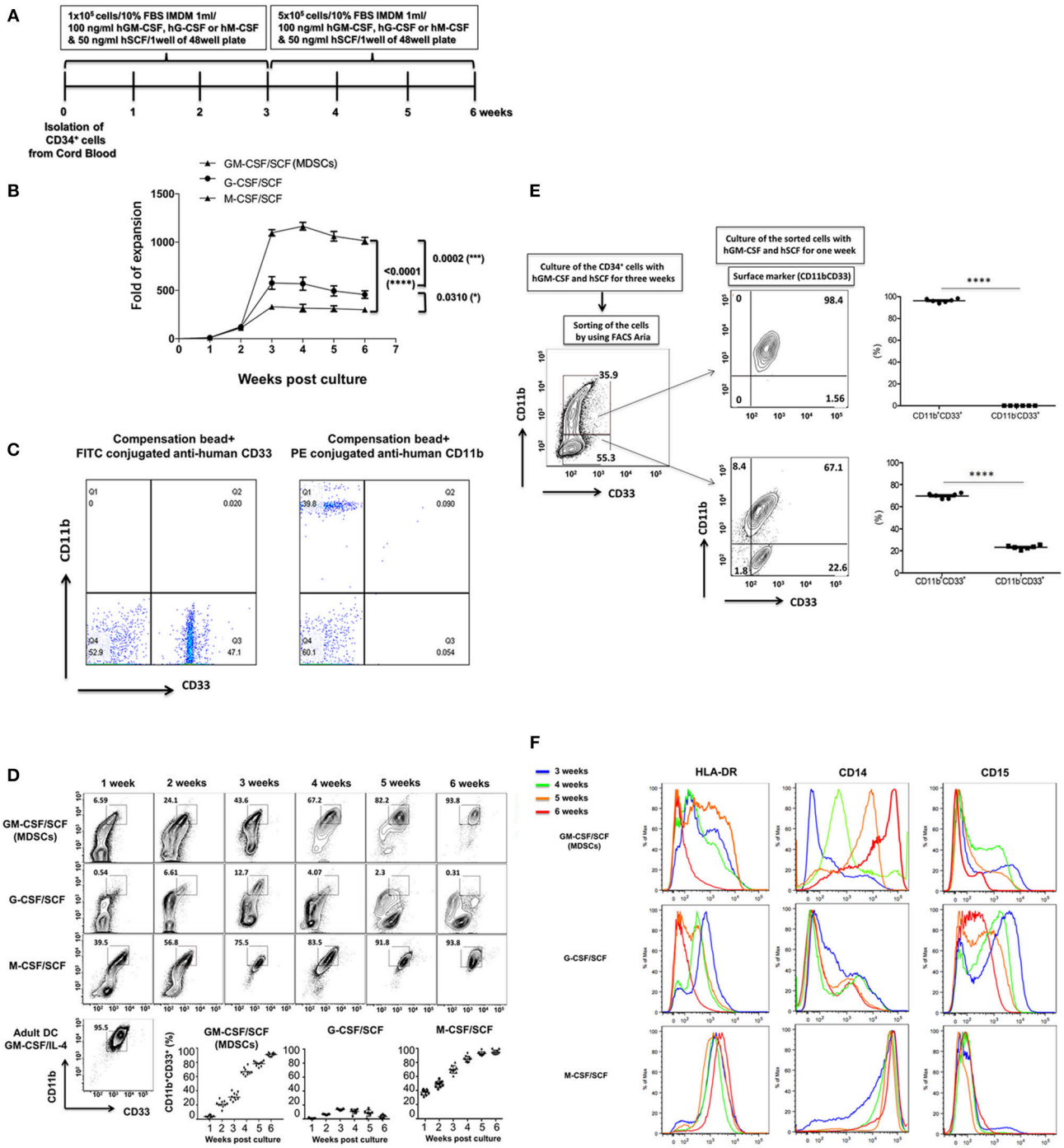

FIGURE 1 | Expansion of CB CD34$^+$ cells according to cytokine combinations and differentiation into MDSCs. **(A)** The scheme of cell expansion or differentiation from CB CD34$^+$ cells. **(B)** Fold expansion by different cytokine combinations. The culture medium was replaced with each cytokine combinations every week. **(C)** The FITC conjugated anti-human CD33 were compensated with compensation bead to avoid spillover with PE conjugated anti-human CD11b. **(D)** Expression of CD11b$^+$CD33 $^+$. The cells were stained with fluorochrome conjugated anti-human CD33 (FITC) and aniti-human CD11b (PE) every week. **(E)** The CD34$^+$ cells were cultured with human GM-CSF (100 ng/ml) and human SCF (50 ng/ml) for 3 weeks and the cells were stained like **(D)**. The stained cells were sorted by FACS Aria and the sorted cells (CD11b$^+$CD33b$^+$ vs. CD11b$^-$CD33$^+$) were cultured with human GM-CSF (100ng/ml) and human SCF (50ng/ml) for a further 1 week. After one week, the sorted cells were stained like **(D)** and analyzed by flow cytometry. **(F)** Expression of HLA-DR, CD14 and CD15. The cells were stained with efluor450-conjugated anti-human HLA-DR, PE-Cy7 anti-human CD14 and APC anti-human CD15 from 3 to 6 weeks, respectively and analyzed by flow cytometry. These experiments were reproduced in 10 individuals (from **B** to **D** and **F**) and 6 individuals **(E)**.

TABLE 1 | Characterization of cultured cells for 6 weeks.

	GM-CSF/SCF	G-CSF/SCF	M-CSF/SCF	
Level of HLA-DR	low	low	high	**Figure 1F**
$CD11b^{+}CD33^{+}$	high	dim	high	**Figure 1D**
Monocyte (CD14):Granulocytes(CD15) ratio	9: 1 (M-MDSCs)	2: 8	10:0	**Figure 1F**
Level of Arginase 1 and IDO	+++	+	++	**Figure 3A**
Suppressive activity	+++	++	+	**Figure 4A**
Treg induction	+++	++	++	**Figure 4B**

Typical nomenclature of human myeloid derived suppressor cells (MDSCs): HLA-DR$^{negative\ or\ low}$, $CD11b^{+}CD33^{+}$, $CD14^{+}$: Monocytic MDSCs (M-MDSCs). HLA-DR$^{negative\ or\ low}$, $CD11b^{+}CD33^{+}$, $CD15^{+}$: Granulocytic MDSCs (G-MDSCs).

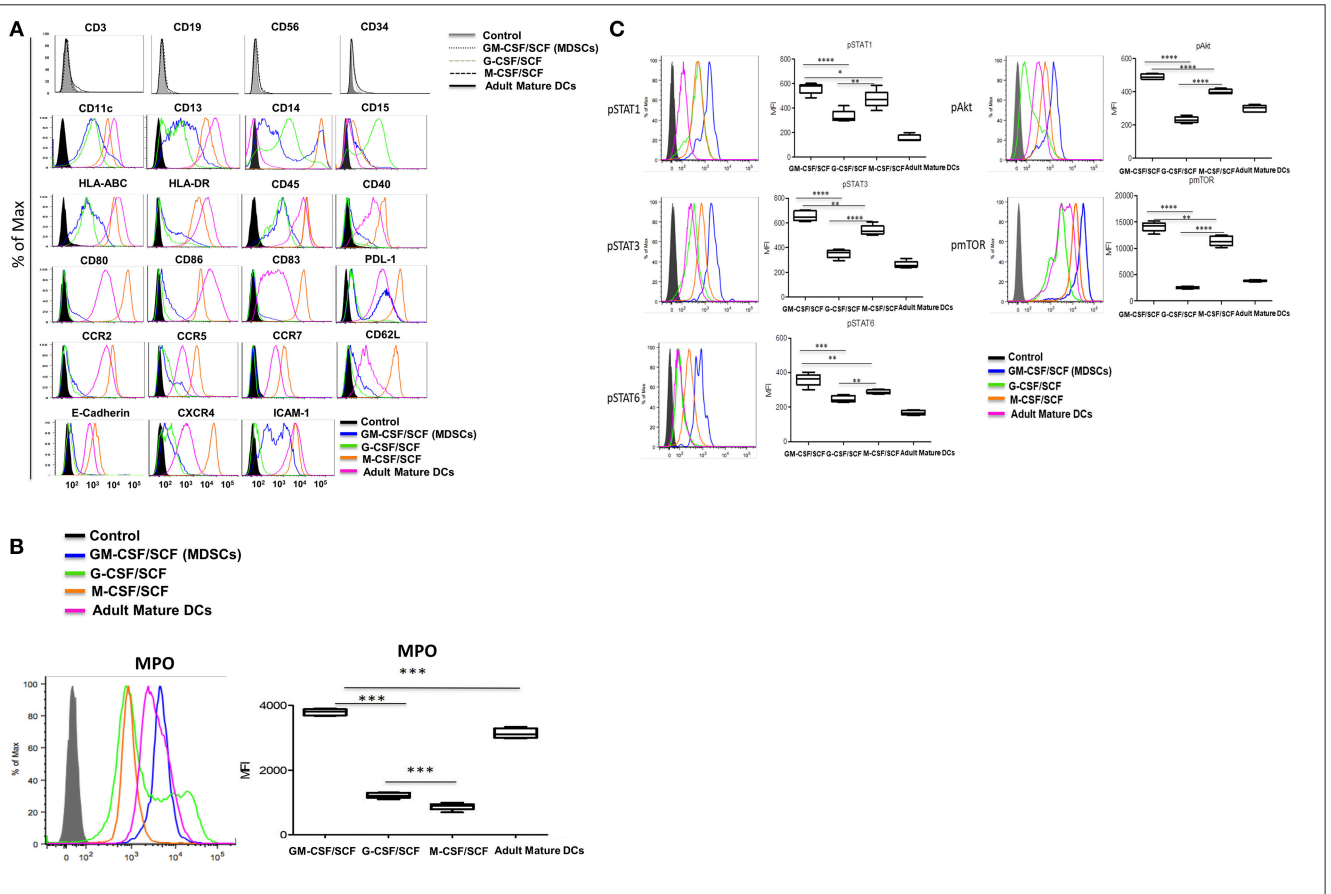

FIGURE 2 | Characterization of cultured MDSCs. **(A)** FACS profiles of cells cultured with human GM-CSF, G-CSF, or M-CSF (100 ng/ml) and human SCF (50 ng/ml) for 6 weeks. Blue histogram lines represent the GM-CSCF/SCF (MDSCs), light green histogram lines the cells cultured G-CSF/SCF, orange histogram lines the cells cultured M-CSF/SCF and pink histogram lines represent the adult mature DCs. **(B)** The cells were intracellular stained with anti-human MPO antibody and analyzed by flow cytometry. **(C)** The cells were lysed and fixed in a single step using Phosflow Lyse/Fix buffer for 10 min at 37°C. Cells were then permeabilized in Phosflow Perm Buffer for 30 min on ice. Cells were then washed twice in the stain Buffer and stained with fluorochrome conjugated anti-Stat (AF647), anti-Stat3 (PE), anti-Stat6 (PerCP-Cy5.5), anti-Akt (PE) and anti-mTOR (PerCP-efluor718) antibodies for 30 min on ice. Cells were washed twice in the stain buffer. All samples were acquired on a BD LSR Fortessa and then analyzed using FlowJo 9.2.1. Data are mean ± S.E.M. of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

DCs) were compared using as controls, since a variety of immune molecules are strongly expressed. The lineage and differentiation markers showed that the cells were lineage-negative for CD3, CD19, and CD56 but were uniformly positive for myeloid associate marker for CD11b (**Figure 1C**), CD11c, and CD13 (**Figure 2A**).

Cell surface markers associated with immune functions are shown in **Figure 2A** (**Table 2**). The expression of HLA-ABC was positive in all cells cultured with the three different cytokine combinations. In contrast, the expression of HLA-DR was negative in G-CSF/SCF, expressed as low level in GM-CSF/SCF and high in M-CSF/SCF cultured cells (**Table 1**). The expression

TABLE 2 | Immunophenotypes of cultured cells for 6 weeks (MFI).

	GM-CSF/SCF (MDSCs)	G-CSF/SCF	M-CSF/SCF	Adult mature DCs
CD11c	10^3	10^3	10^4	10^4
CD13	10^3	10^3	10^4	10^4
CD14	10^5	10^3	10^5	10^2
CD15	10^2	10^3	10^2	10^2
HLA-ABC	10^3	10^3	10^4	10^4
HLA-DR	10^2	10^2	10^4	10^4
CD40	10^1	10^1	10^3	10^3
CD80	10^1	10^1	10^4	10^3
CD86	10^1	10^1	10^3	10^4
CD83	10^1	10^1	10^4	10^3
PDL-1	10^3	10^2	10^4	10^1
CCR2	10^1	10^1	10^4	10^4
CCR5	10^2	10^2	10^4	10^3
CCR7	10^1	10^1	10^4	10^3
CD62L	10^2	10^1	10^4	10^3
E-Cadherin	10^1	10^1	10^3	10^3
CXCR4	10^2	10^2	10^4	10^3
ICAM-1	10^3	10^2	10^4	10^4

of costimulatory molecules CD86 and CD40 were low and CD80 and CD83 were negative in GM-CSF/SCF cultured cells. The chemokine receptors CCR2, CCR5, CD62L, and CXCR4 were expressed at low levels in both GM-CSF/SCF and G-CSF/SCF cultured cells. However, the M-CSF/SCF cultured cells showed the high expression of chemokine receptors. The cells cultured with M-CSF/SCF expressed E-cadherin (mediated cell–cell adhesion) at high levels, but the expression on cells cultured with GM-CSF/SCF and G-CSF/SCF were negative. ICAM-1 was not expressed in G-CSF/SCF cultured cells. The GM-CSF/SCF and M-CSF/SCF cultured cells showed the high expression for PDL-1. PDL-1 expression on G-CSF/SCF cultured cells were low. Myeloperoxidase (MPO) is a heme protein synthesized during myeloid differentiation. GM-CSF/SCF cultured cells showed a significantly higher level of MPO activity than G-CSF/SCF and M-CSF/SCF (**Figure 2B**).

Signal Molecules of MDSCs

We next tested for expression of molecules (phosphorylated-(p) Stat1, pStat3, pStat6, pmTOR, and pAkt) related to MDSCs signaling pathways (**Figure 2C**). The GM-CSF/SCF cultured cells showed the highest expression levels of pStat1, pStat3, pStat6, pmTOR, and pAkt protein. The G-CSF/SCF showed the lowest expression in these signaling except pStat1 which showed similar expression level compared with M-CSF/SCF. These results demonstrate that cells cultured with GM-CSF/SCF expressed signaling molecules associated with MDSCs. Taken together, our phenotypic data suggests that CB $CD34^+$ cells cultured with GM-CSF/SCF or G-CSF/SCF were consistent with MDSCs. Cells cultured with M-CSF/SCF were consistent with monocytes.

Immune Suppressive Molecules in MDSCs

Expression of inhibitory molecules such as Arginase, IDO, and inducible nitric oxide synthase (iNOS) in MDSCs was investigated by intracellular stain. Expression of arginase 1 and IDO was significantly higher in MDSCs generated by GM-CSF/SCF culture compared to those from M-CSF/SCF culture (**Table 1**). The expression of iNOS in GM-CSF/SCF culture was significantly lower following culture with M-CSF/SCF. The expression of these inhibitory molecules was lowest in G-CSF/SCF culture. In addition, the expression of arginase 1 and IDO was negative in the mature DCs but iNOS were intermediately expressed in the mature DCs (**Figure 3A**). The results showed that the level of arginase 1, iNOS, and IDO gradually increased from 4 to 6 weeks of culture with GM-CSF/SCF (**Figure 3B**).

The sustained and induced secretion of immunosuppressive cytokines such as human VEGF, TGF-β and IL-10 was measured in culture supernatants (3 weeks through 6 weeks) of CB $CD34^+$ cells differentiated with GM-CSF/SCF to become MDSCs (**Figure 3C**). Stimulation of GM-CSF/SCF derived MDSCs (GM-CSF/SCF MDSCs) with anti-CD40 antibody resulted in markedly elevated secretions of IL-10 and VEGF. The TGF-β was produced to high levels regardless of stimulation. The expressions of these inhibitory molecules were indicative of the immunosuppressive functions of GM-CSF/SCF derived MDSCs.

Immune Suppressive Functions on T Cells of MDSCs *in vitro*

To define whether the MDSCs exert suppressive function on T cells *in vitro*, adult human PBMCs were labeled with CFSE and then stimulated with anti-CD3 and anti-CD28 microbeads in the presence or absence of MDSCs. After 6 days of co-culture, T-cell proliferation was assessed by measuring CFSE intensity within $CD4^+$ and $CD8^+$ subsets (**Figure 4A**). GM-CSF/SCF MDSCs showed the strongest suppressive effect on both $CD4^+$ T cells (6.3% [range, 3.3–9.2%]), and $CD8^+$ T cells (15.3% [range, 9.4–20.0%]) at 1:1 (PBMC: MDSC) ratio (**Table 1**). M-CSF/SCF generated cells exhibited the weakest suppressive ability.

To determine if cytokine generated MDSCs could alter the polarization of helper T cells, the cells were co-cultured with adult naïve $CD4^+$ T cells for 3 days. We then measured the expression of FoxP3 (**Figure 4B**) in $CD4^+$ gated Treg cells. We also measured the expression of IL-17 and IFN-γ (**Figure 4C**) in culture supernatants. The GM-CSF/SCF MDSCs resulted in a highest frequency of $FoxP3^+$ Treg cells (58.5 ± 3.3 %) compared with G-CSF/SCF (39.4 ± 5.2 %) and M-CSF/SCF (45.7 ± 2.1 %) (**Table 1**). By contrast, MDSCs from all groups did not secrete the IL-17 (**Figure 4B**). The GM-CSF/SCF MDSCs mediated the most potent suppression of human IL-17 and IFN-γ production. The G-CSF/SCF exhibited decreased production of human IFN-γ but human IL-17 was not significant between G-CSF/SCF and M-CSF/SCF (**Figure 4C**). The GM-CSF/SCF MDSCs most effectively

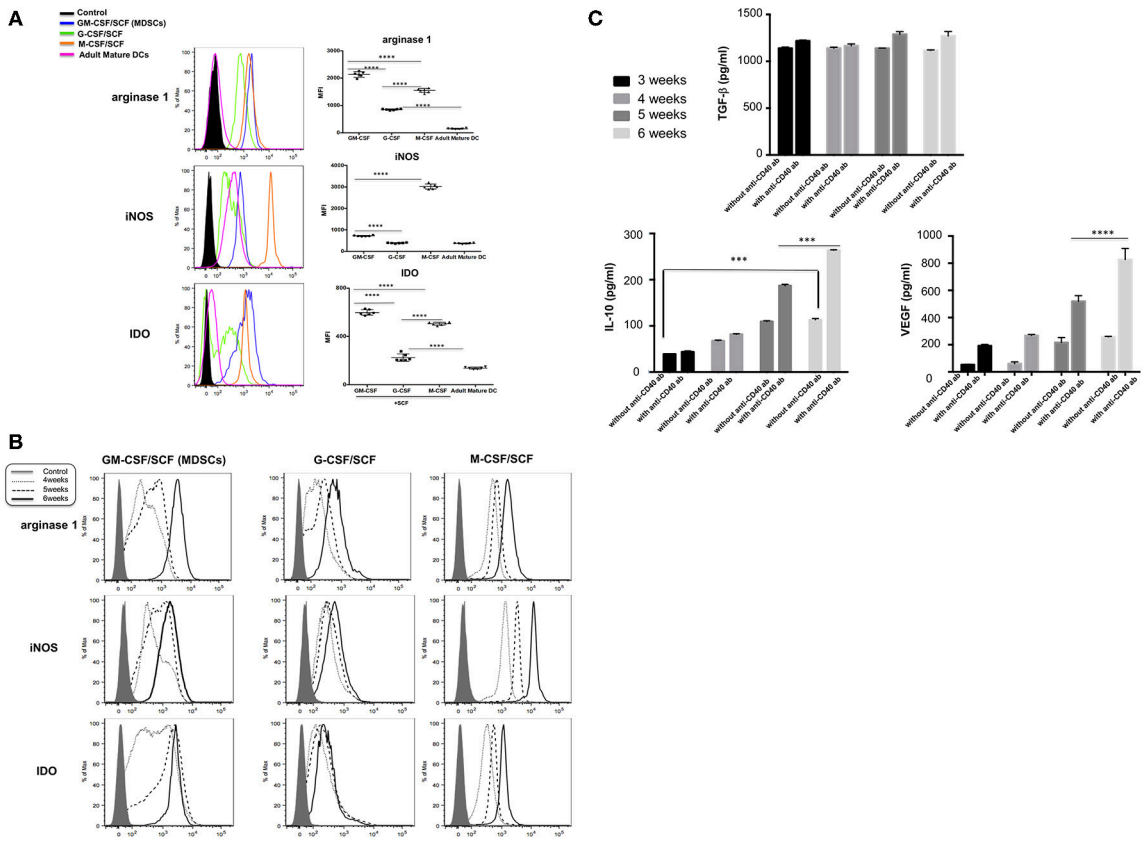

FIGURE 3 | MDSCs express immune suppressive molecules. **(A)** The cells cultured with each cytokine combinations for 6 weeks or **(B)** The cells cultured with each cytokine combinations from 4 to 6 weeks were fixed in Cytofix buffer for 10 min at 37°C and then permeabilized in Cytoperm buffer for 30 min on ice. Cells were then washed twice in the stain buffer and stained with FITC anti-iNOS antibody, PE anti-IDO antibody, and PerCP-Cy5.5 anti-arginase 1 antibody 30 min on ice. Cells were washed twice in the stain buffer and acquired on a BD LSR Fortessa. **(C)** The cells cultured with GM-CSF/SCF were stimulated with or without anti-CD40 antibody for 48 h and human VEGF, TGF-β and IL-10 were compared in culture supernatants between 3 and 6weeks. Data are mean ± S.E.M. of three independent experiments, each performed in triplicate. ***$p < 0.001$, ****$p < 0.0001$.

inhibited T cell proliferation and production of inflammatory cytokines and led to the greatest production of FoxP3$^+$ Treg cells.

Inhibition of T Cells Proliferation and Antigen-Specific T Cell Responses by MDSCs

GM-CSF/SCF derived MDSCs was chosen for further study because they exhibited the greatest expression of immune suppressive molecules (**Figures 3A,C**) and greatest immune suppressive function (**Figure 4A**). To address the suppressive capacity of T cells proliferation by MDSCs *in vitro*, the mature DCs were co-cultured with adult naïve CD4$^+$ T cells in the presence or absence GM-CSF/SCF MDSCs. The reactivity of T cells was assessed based on the incorporation of [3H]thymidine. GM-CSF/SCF MDSCs profoundly reduced the ability to prime responses of T cells (**Figure 5A**). To examine the inhibition of antigen-specific T cell responses by MDSCs, the pp65 mRNA were electroporated into adult monocyte derived immature DCs. The DCs were matured and co-cultured with adult naïve CD4$^+$ T cells in the presence or absence GM-CSF/SCF MDSCs. The MDSCs markedly inhibited (7-fold decreases) the number of pp65 specific IFN-γ secreting cells (**Figure 5B**).

Inhibition of GVHD by MDSCs in the Xenogeneic Mouse Model

Lethal irradiated NSG mice were given human PBMCs to induce the fatal GVHD. In some mice, three doses of GM-CSF/SCF MDSCs were injected intravenously on days 21 and 24 (**Figure 6A**). The GVHD mice not given MDSCs showed hunched back on day 35 and loss of fur on day 70 (**Figure 6B**). Infusion of MDSCs significantly decreased GVHD scores in a dose dependent manner (**Figure 6D**) and inhibited the rapid decrease in body weight (**Figure 6E**) in comparison to control GVHD mice. The MDSCs significantly prolonged survival compared to control GVHD mice (GVHD vs. MDSCs $1 \times 10^6 = 0.0070$, GVHD vs. MDSCs $2.5 \times 10^6 = 0.0156$, GVHD

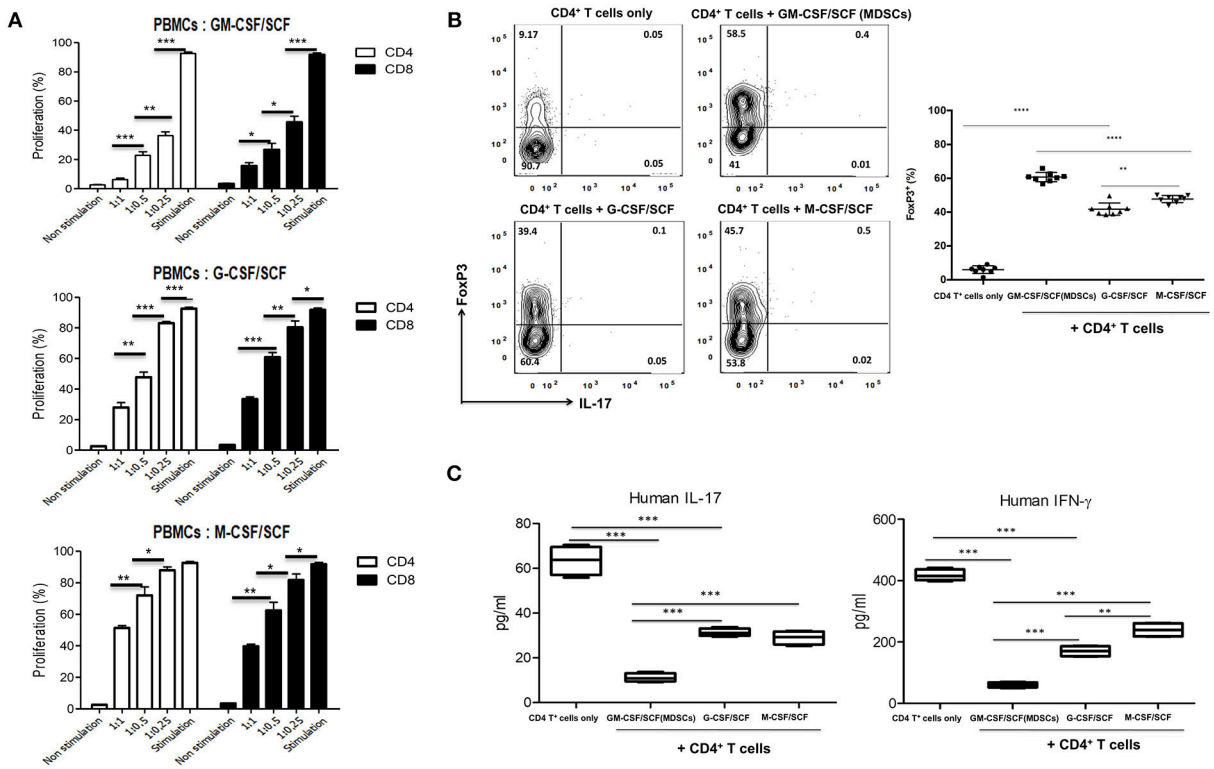

FIGURE 4 | MDSCs have suppressive effect for T cells and induce a polarization of helper T cells. **(A)** Healthy adult PBMCs were labeled with CFSE and stimulated with Dynabead Human T-Activator CD3 and CD28 in the presence of MDSCs at a 1:1, 1:0.5, and 1:0.25 (PBMCs: MDSCs) ratio. After 6 days, cells were then harvested, stained with anti-human CD3 (PE-Cy7), anti-human CD4 (APC) and anti-human CD8 (efluor450) antibodies. The cells were analyzed by FACSCanto II device. **(B)** $CD4^+$ T cells were isolated from healthy adult PBMCs and 1×10^6 $CD4^+$ T cells were stimulated with 0.5 μg/mL Dynabead Human T-Activator CD3 and CD28 in the presence or absence of 2×10^6 MDSCs at a 1:2 ($CD4^+$ T cells: MDSCs) ratio in 12 well plates for 3 days. After 3 days, the cells were stained with fluorochrome-conjugated anti-human CD3 (PE-Cy7), anti-human CD4 (FITC), anti-human CD25 (APC), anti-human IL-17 (PerCP-Cy5.5) and anti-human FoxP3 (PE) antibodies. **(C)** Human IL-17 and IFN-γ was measured in the culture supernatants of **(B)**. Data are mean ± S.E.M. of three independent experiments, each performed in triplicate. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

vs. MDSCs 5×10^6 = 0.0059) (**Figure 6C**). On day 100, all GVHD control mice died, but more than 50 % of the mice given MDSCs survived. Increased MDSCs mediated survival was not cell dose dependent. These results show that CB GM-CSF/SCF derived MDSCs can ameliorate fatal GVHD in the xenogeneic mouse model.

Change in Anti-inflammatory and Pro-inflammatory Cytokines and Induction of FoxP3$^+$ Treg Cells via MDSCs Infusion in the Xenogeneic Mouse Model

Mice were bled at 60 days post human PBMCs transplantation. Cytokine levels in the serum were measured by ELISA (**Figure 7A**). Serum levels of IL-6 and TNF-α were significantly increased in control GVHD mice, whereas IL-10 and TGF-β were similarly low in control GVHD mice compared to normal NSG mice. In contrast, serum levels of IL-10 and TGF-β in mice given 1×10^6 GM-CSF/SCF MDSCs were significantly increased compared to those in normal NSG or control GVHD mice. IL-6 and TNF-α were reduced in mice given 1×10^6 MDSCs compared to control GVHD mice but similar to normal NSG mice. Cytokine levels were further estimated semi-quantitatively via a multi-cytokine membrane array (**Figure 7F**). Mice given 1×10^6 MDSCs showed lower serum levels of C reactive protein (CRP), IL-1β, IL-6, TNF-α, IL-17, macrophage inflammatory protein-3β (MIP-3β), matrix metallopeptidase 9 (MMP9), Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES) and stromal cell-derived factor-1α (SDF-1α) compared to control GVHD mice.

To explore the mechanism of reduction of GVHD severity caused by Th subsets, the expression of FoxP3 and CD25 for Treg cells (**Figure 7B**), IFN-γ for Th1 cells, IL-4 for Th2 cells and IL-17 for Th17 cells were measured by intracellular staining assay (**Figure 7C**). Splenocytes from mice given 1×10^6 MDSCs showed increased frequencies (11.8 % ± 0.44 %) of human FoxP3$^+$ Treg cells and decreased frequencies of IL-17 (0.88 % ± 0.075 %) and IFN-γ (0.58 % ± 0.061 %) producing cells compared with those of control GVHD mice. The frequencies of $CD4^+$ T cells expressing human IL-4 were very low and

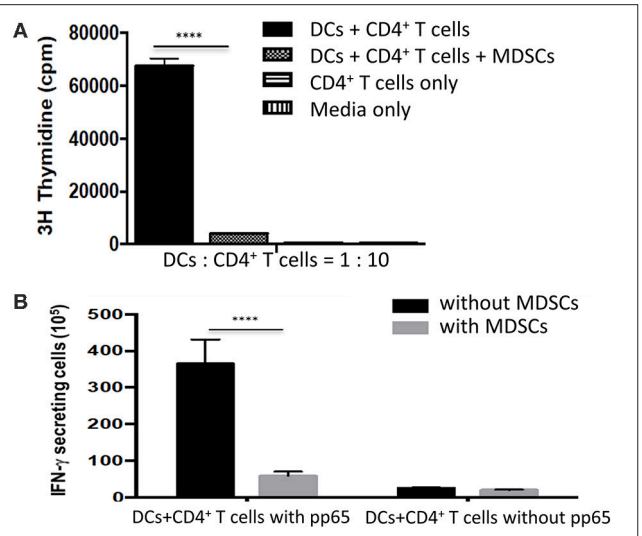

FIGURE 5 | MDSCs effectively suppress the proliferation of $CD4^+$ T cells and antigen-specific $CD4^+$ T cells responses. **(A)** $CD4^+$ T cells isolated from healthy adult PBMCs were used as stimulators from three different donors. Adult monocyte derived mature DCs (1×10^4 cells) were mixed with the autologous $CD4^+$ T cells (1×10^5 cells) in the presence or absence 1×10^4 MDSCs. The cells were cultured for 5 days and 1 μCi [^{3}H]thymidine was added to the wells for 18 hr before harvesting. The incorporation of [^{3}H]thymidine was determined using a liquid β-scintillation counter. **(B)** The HCMV pp65 RNA-electroporated mature DCs were added to a 96-well microplate at a concentration of 1 x 10^4 cells/well in 10% complete RPMI 1640. 1×10^5 autologous $CD4^+$ T cells were then added to the well as a stimulator in the presence or absence MDSCs (1×10^4). An ELISPOT assay for human IFN-γ was conducted according to the manufacturer's protocol. Data are mean ± S.E.M. of three independent experiments, each performed in triplicate. ****$p < 0.0001$.

there was no difference between control GVHD mice and mice given 1×10^6 MDSCs. The Treg/Th17 ratio (**Figure 7E**) was significantly higher and Th1/Th2 ratio (**Figure 7D**) was significantly lower in mice given 1×10^6 MDSCs compared to control GVHD mice. There was no significant difference in anti-inflammatory and pro-inflammatory cytokines observed between the cell number of MDSCs administered (**Figures 7A–C**; data not shown).

These results show that MDSCs had a protective effect against GVHD by increasing $FoxP3^+$ Treg cells *in vivo*, altering the balance among Th1, Th2, and Th17 cells and inhibiting the inflammatory responses.

DISCUSSION

Although efforts have been undertaken to expand MDSCs, the number of cells were limited to 3×10^7 in mice (29–33). MDSCs are not typically present in healthy individuals but soluble immune modulatory factors induce the expansion of MDSCs from normal human peripheral blood mononuclear cells (34). Others have shown that $CD33^+$ MDSCs with potent suppressive capacity can be generated *in vitro* by GM-CSF and IL-6, and secondarily by GM-CSF + IL-1β, PGE2, TNF-α, or VEGF. CB $CD34^+$ cells cultured with GM-CSF and G-CSF for 4 days were differentiated into fibrocytic MDSCs. The cells expressed the phenotypic markers of MDSCs, DCs, and fibrocyte, and induced Treg cells by increasing of IDO expression (18). In this study, MDSCs cultured with GM-CSF/SCF expanded up to 10^8 cells/1 unit of cord blood (CB) and revealed the most potent influence of expansion among three different cytokine combinations. It has been reported that the concentration of GM-CSF was related to regulation between immune suppression of MDSCs and immune stimulation of mature DCs (35). SCF plays an important role in MDSCs expansion of tumor bearing mice and suppression of tumor-infiltrating T cells. In addition, SCF receptor (ckit)–SCF interaction promoted a development of tumor and Treg (32).

Here, we characterized human MDSCs generated over 6 weeks from CB $CD34^+$ cells using GM-CSF/SCF. The MDSCs showed phenotypic markers of human monocytic MDSCs; HLA-DR^{low}, $CD11b^+$ $CD33^+$, $CD14^+$. CD80, CD83, and CD86 were not expressed in GM-CSF/SCF MDSCs or cells cultured with G-CSF/SCF (G-CSF/SCF). It has been reported that low levels of GM-CSF promote myeloid cell viability in culture and expanded $CD33^+$ cells (34, 36). GM-CSF/SCF have been used for generation of tolerogenic dendritic cells from $CD34^+$ cells of cord blood which were expanded up to 10^8 that expressed $CD11C^+$ $CD11b^+$ $CD13^+$, $CD80^{low}$ $CD86^{low}$, and CD83 ^{low} (37). In our study, culture of CB CD34 cells with G-CSF/SCF led to expansion of cells by low expression of HLA-DR, small increase (10%) of $CD11b^+$ $CD33^+$. GM-CSF/SCF differentiated cells expressed MPO. These results corroborate that MDSCs were successfully generated by coculture of CB $CD34^+$ cells with GM-CSF/SCF (38).

GM-CSF/SCF MDSCs had the strongest suppressive capacity to inhibit proliferation of T cells. The MDSCs increased the frequencies of $FoxP3^+$ Treg cells and remarkably inhibited the generation of Th1 and Th17 cells compared with myeloid cells from G-CSF/SCF or M-CSF/SCF cultures. Furthermore, GM-CSF/SCF MDSCs inhibited the proliferation of $CD4^+$ T cells and the secretion of IFN-γ by antigen-specific T cells. Bone marrow MDSCs prevent GVHD in an arginase 1–dependent manner that is up-regulated by addition of interleukin-13 (11). Under GVHD inflammatory condition, MDSCs rapidly lose their suppressive function and their potential to inhibit GVHD lethality (12). Indeed, the infusion of GM-CSF/SCF MDSCs augmented the survival and reduced GVHD lethality such as recovery of weight and GVHD score. In the mice given MDSCs, serum concentrations of most inflammatory cytokines were decreased, while IL-10 and TGF-β were increased. GM-CSF/SCF MDSCs showed an inhibitory effect on Th1 and Th17 polarization and led to increase human $Foxp3^+$ Treg cells. These *in vitro* and *in vivo* results demonstrate the mechanism by which MDSCs are immunosuppressive. Consistent with these immunosuppressive functions, the expression of immunosuppressive molecules such as arginase 1, IDO, and iNOS and the secretion of immunosuppressive cytokines such as TGF-β, IL-10, and VEGF were increased in GM-CSF/SCF MDSCs in our studies. However, the pathophysiological

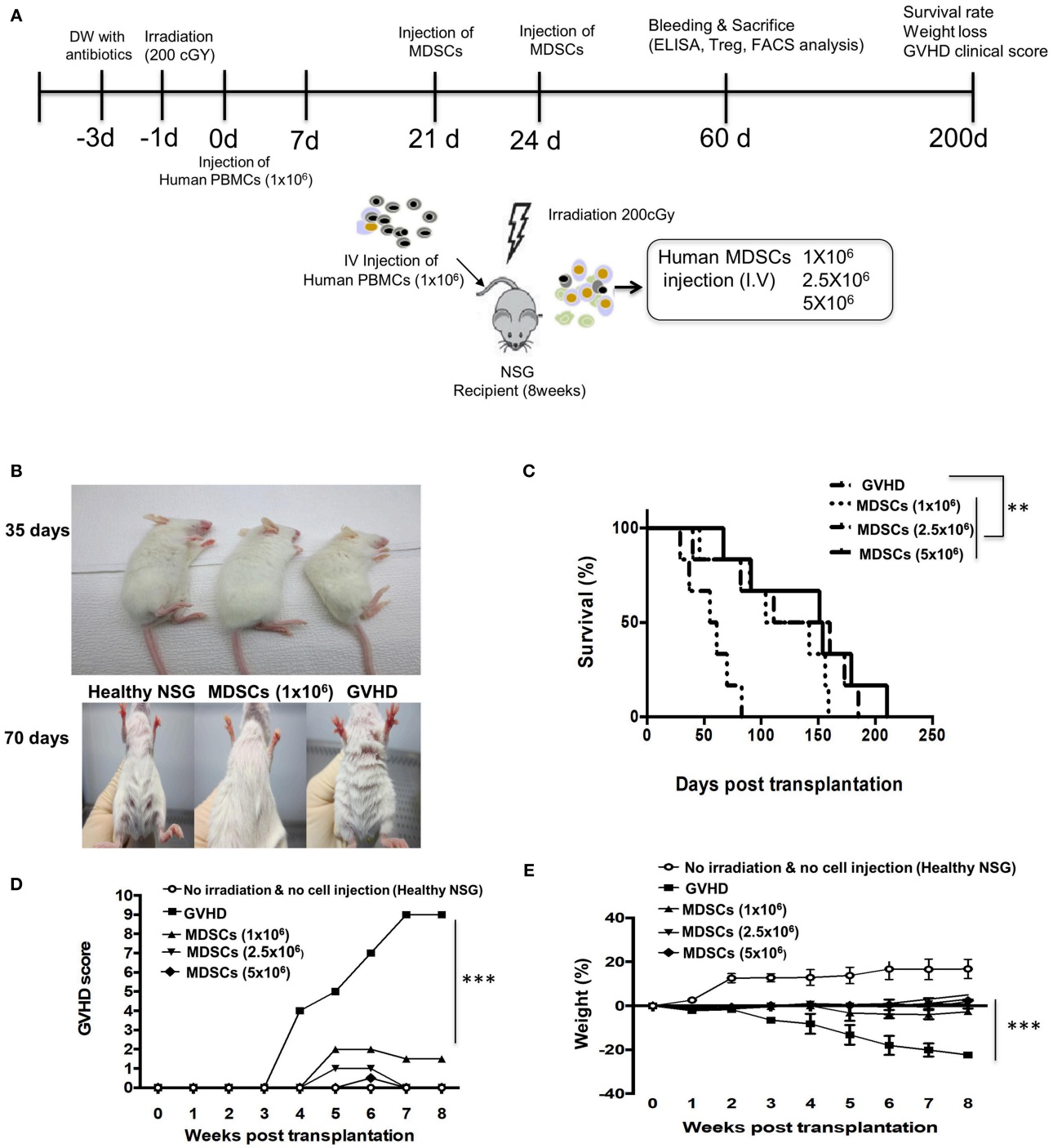

FIGURE 6 | MDSCs infusion ameliorates xenogeneic GVHD. **(A)** Experimetnal scheme. NSG mice were administered with/without sublethal irradiation (200 cGy) at day−1 and 1 × 10^6 human PBMCs were infused at day 0. The NSG mice were administered either 1 × 10^6, 2.5 × 10^6, 5 × 10^6 MDSCs (MDSCs mice) or PBMCs only (GVHD mice) at day 21 and 24. **(B)** Condition of fur: MDSCs mice vs. GVHD mice at day 35 and 70. **(C)** Overall survival. At a median of 100 days after PBMCs infusion, MDSCs mice were > 60% as compared with < 0% for GVHD mice (GVHD vs. MDSCs 1 × 10^6 =0.0070, GVHD vs. MDSCs 2.5 × 10^6 = 0.0156, GVHD vs. MDSCs 5 × 10^6 = 0.0059; log-rank test; $n = 5$ mice/group). Data represent one experiment of two independent experiments (MDSCs vs. GVHD $^{**}p < 0.01$; log-rank test; n = 5 mice/group). **(D)** The degree of GVHD was recorded daily using a scoring system that summed changes in five clinical parameters: weight loss, posture, physical activity, fur texture, and skin integrity. The MDSCs mice showed lower GVHD score (MDSCs vs. GVHD $^{***}p < 0.001$). **(E)** The weight was measured every other day and the MDSCs treatment attenuates a weight loss (MDSCs vs. GVHD $^{***}p < 0.001$). Data are mean ± S.E.M. of two independent experiments.

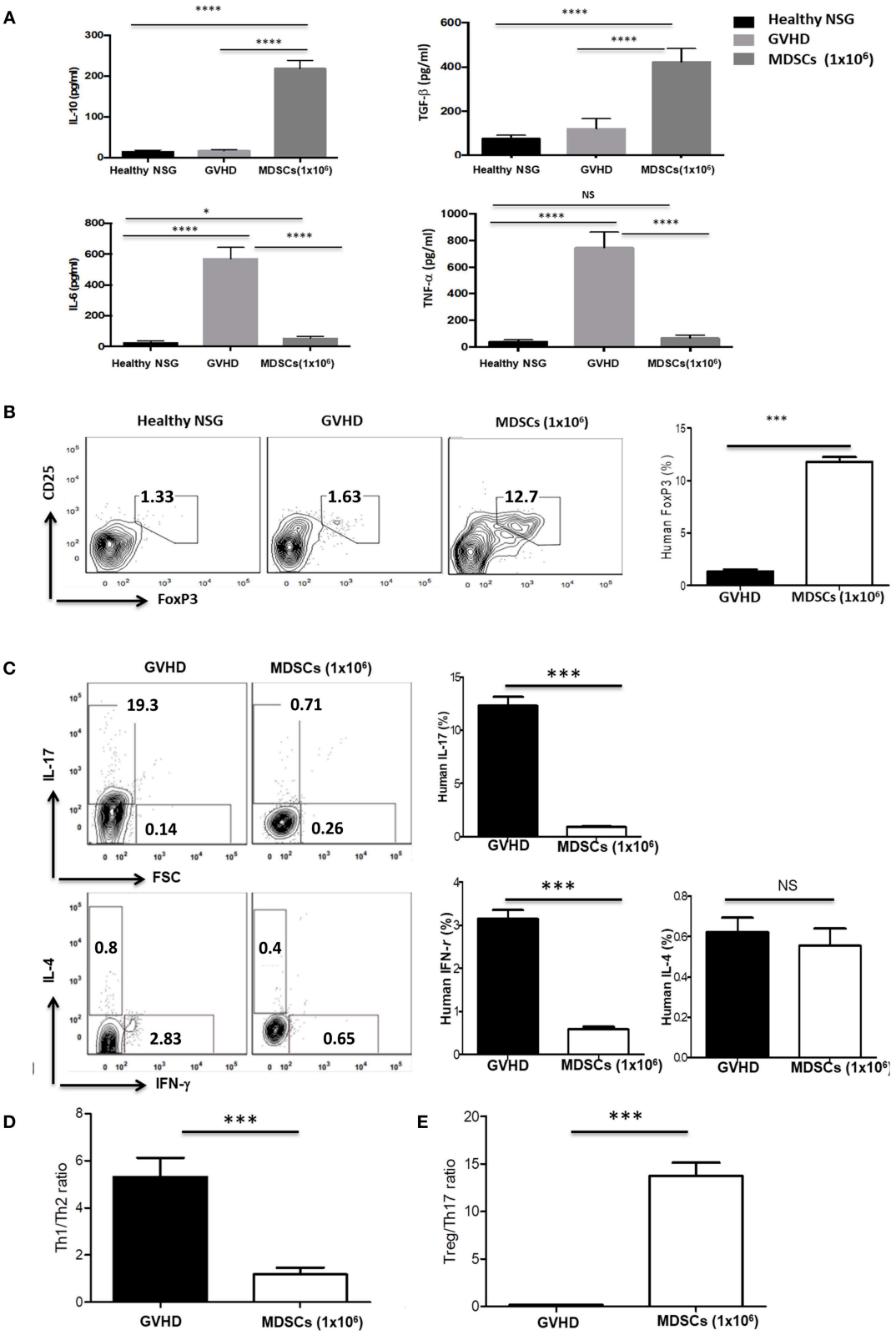

FIGURE 7 | Continued.

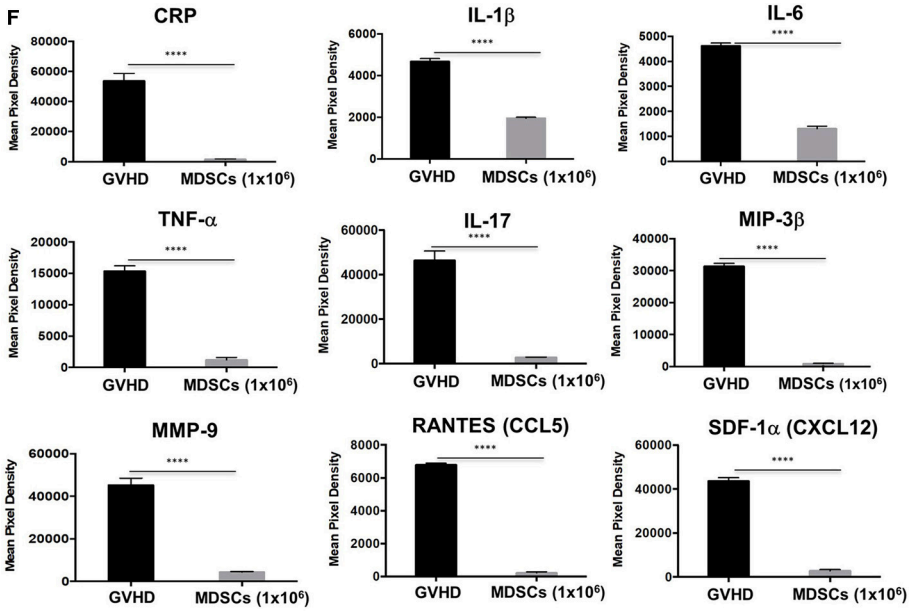

FIGURE 7 | MDSCs infusion increases induction of FoxP3$^+$ Treg cells and anti-inflammatory cytokine and decreases pro-inflammatory cytokine production in xenogeneic GVHD model. The mice were bled and sacrificed at day 60 post human PBMCs infusion and **(A)** the human cytokine levels in the serum were measured by ELISA kits. **(B-C)** Splenocytes were stained with fluorochrome-conjugated anti-human CD3 (PE-Cy7), anti-human CD4 (FITC) and anti-human CD25 (APC) and then intracellular stained with anti-human Foxp3 (PE), anti-human IL-17 (PerCP-Cy5.5), anti-human IFN-γ (Pacific Blue) and anti-human IL-4 (APC-Cy7) antibodies using fixation and permeabilization buffer. CD3$^+$CD4$^+$ T cells gated contour plots were presented. **(D)** The ratio of Th1/Th2 on CD3$^+$CD4$^+$ T cells. The ratio was calculated as the ratio of IL-4$^+$ CD3$^+$CD4$^+$ T cells divided by the percentage of IFN-γ$^+$ CD3$^+$CD4$^+$ T cells. **(E)** The ratio of Treg/Th17 on CD3$^+$CD4$^+$ T cells. The ratio was calculated as the ratio of IL-17$^+$ CD3$^+$CD4$^+$ T cells divided by the percentage of CD25$^+$FoxP3$^+$CD3$^+$CD4$^+$ T cells. **(F)** Serum cytokine levels were estimated semi-quantitatively with a multi-cytokine (human) membrane array. The data were presented a decrease in the pro-inflammatory cytokine levels, including C reactive protein (CRP), IL-1β, IL-6, TNF-α, IL-17, macrophage inflammatory protein-3β (MIP-3β), matrix metallopeptidase 9 (MMP9), Regulated on Activation Normal T cell Expressed and Secreted (RANTES) and stromal cell-derived factor-1α (SDF-1α) in the MDSCs mice compared with GVHD mice ($n = 3$ mice/group). Data are mean ± S.E.M. of three independent experiments, each performed in triplicate. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$.

and migration mechanisms of human MDSCs need to be further clarified to use a new therapeutic strategy for immune regulation.

Cells cultured at high cell concentration from 3 to 6 weeks stopped proliferating and progressed to differentiation instead. The level of arginase 1 and IDO were significantly higher in 6 weeks cultured cells with GM-CSF/SCF compared to those of 4 weeks. At a high cell concentration, low oxygen tensions may induce striking increase in iNOS and arginase 1 enzyme levels, suggesting a role of HIF-1a-dependent hypoxic regulation in myeloid cell-mediated T cell suppression and the differentiation of MDSCs (39). Autophagy induced by starvation or various stresses may affect the differentiation of myeloid cells (40, 41). Future studies should further investigate factors that influence this *in vitro* differentiation in order to increase understanding on the generation of MDSC in various disease status.

Preclinical studies using adoptive transfer of MDSCs have been conducted in various experimental animal models in order to treat autoimmune diseases and to inhibit the graft rejection or GVHD in organ and hematopoietic stem cell transplantation, and successful treatment effects and mechanism of action have been reported (11–13). However, clinical applications have been difficult due to the numerical limitations of MDSC. Therefore, MDSCs expanded from CB provides the possibility of being used in clinical studies to investigate the safety and therapeutic effects of adoptive transfer in these diseases.

In conclusion, the three different cytokine combinations had an obviously different influence on the differentiation and immunosuppressive functions of human MDSCs. Moreover, the GM-CSF/SCF combinations revealed to be most efficient for the generation of functionally MDSCs from CD34$^+$ cells of cord blood. The human MDSCs could provide a useful strategy for the treatment of inflammatory diseases such as GVHD in the clinics.

ETHICS STATEMENT

All animal experiments were performed according to the investigator's protocol approved in advance by the Institutional Animal Care and Use Committee, College of Medicine, Catholic University of Korea. This study involving human subjects was carried out in accordance with the recommendations of the Declaration of Helsinki. The protocol was approved by the institutional review board of the College of Medicine, Catholic University of Korea, Seoul, Republic of Korea (permit No. MC16SNSI0001, MC15TISE0023, MC17TNSI0002). All subjects gave written informed consent

for sample donation in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

M-YP designed research, performed experiments, analyzed data, and wrote the manuscript. B-GL performed and analyzed proliferation experiments using CFSE. S-YK performed isolation of mononuclear cells from cord blood. H-JS provided human PBMCs and reviewed the data. SK performed proliferation experiments using CFSE and edited the manuscript. T-GK designed and organized research and edited the manuscript.

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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.2019.00183/full#supplementary-material

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REVIEW
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Achievement of Tolerance Induction to Prevent Acute Graft-vs.-Host Disease

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Acute graft-vs.-host disease (GVHD) limits the efficacy of allogeneic hematopoietic stem cell transplantation (allo-HSCT), a main therapy to treat various hematological disorders. Despite rapid progress in understanding GVHD pathogenesis, broad immunosuppressive agents are most often used to prevent and remain the first line of therapy to treat GVHD. Strategies enhancing immune tolerance in allo-HSCT would permit reductions in immunosuppressant use and their associated undesirable side effects. In this review, we discuss the mechanisms responsible for GVHD and advancement in strategies to achieve immune balance and tolerance thereby avoiding GVHD and its complications.

Keywords: graft-vs.-host disease, immune tolerance, alpha-1 antitrypsin, allogeneic hematopoietic stem cell transplantation, T regulatory cells

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INTRODUCTION

Immunological tolerance is a self-regulatory mechanism of the immune system to protect the host from a wide variety of foreign antigens without causing immunopathology such as autoimmunity (1, 2). The mechanisms of immunological tolerance can be divided into central and peripheral tolerance. Central tolerance involves the clonal deletion of self-reactive lymphocytes in the primary lymphoid organs, namely the thymus and bone marrow. Despite its high efficiency, central tolerance often is incomplete due to the escape of self-reactive lymphocytes into the periphery. Hence, there is need of an additional layer of tolerance in the periphery to suppress self-reactive lymphocytes. Peripheral tolerance mechanisms consist of deletion, anergy, ignorance and immune regulation (2, 3).

Although significant progress has been made toward immunological tolerance induction in experimental animal models, translation to the clinic for allogeneic hematopoietic stem cell transplantation (allo-HSCT) remains challenging. One manifestation of tolerance induction failure in allo-HSCT is graft-vs.-host disease (GVHD), a life-threatening complication due to donor T cell recognition of host alloantigens. During GVHD, conditioning regimen induced tissue injury drives proinflammatory processes that support the priming of donor anti-host alloreactive T cells via T cell receptor (TCR) engagement, co-stimulation and cytokine signaling. These inflammatory events are counteracted by anti-inflammatory processes often augmented by proinflammatory cytokines; however, for those that develop GVHD, it is clear that anti-inflammatory compensatory mechanisms are overwhelmed and hence unable to control T-cell activation, differentiation and expansion (4, 5). This review will focus on acute GVHD pathogenic and tolerance mechanisms including as available clinical trial results and conclude with the concept of tissue tolerance. Since GVHD acquisition is a sign of failed tolerance induction, we will not discuss GVHD therapy.

OVERVIEW OF ALLOREACTIVE T-CELL ACTIVATION, AMPLIFICATION AND MIGRATION

In allo-HSCT, donor CD4+ and CD8+ T cells can receive TCR signals engagement of peptide- major histocompatibility complex (MHC) (termed signal 1) that occurs as a result of major or minor histocompatibility antigen disparities between donor and host. Studies from mouse models revealed that donor CD4+ T cells play a central role in GVHD induction by exhibiting cytolytic activity, producing effector cytokines and helping donor CD8+ T cells to proliferate via IL-2 production (6).

Upon alloantigen activation, CD4+ T cells differentiate into T helper (Th) cell subsets including, most relevant to this review, Th1 (secreting IL-2, IFN-γ), Th2 (secreting IL-4, IL-5, IL-10, IL-13) and Th17 (secreting IL-17A, IL-17F, IL-21, IL-22, TNF) (6). Our group and others have previously provided evidence against the assumption that GVHD is strictly a Th1 driven process (7–9). In our previous study, deletion of IFN-γ in donor inoculum accelerated GVHD lethality, while deletion of IL-4 resulted in reduced GVHD lethality (7). In other studies, Th2 and Th17 subsets were shown to contribute to GVHD severity with different GVHD target organs (8–10). Recently a subset of CD4+ T cells was found to produce GM-CSF that was linked to the support of GVHD pathology by licensing myeloid cells to produce IL-1 and reactive oxygen species (11).

Similar to CD4+ T cells, CD8+ T cells have been implicated as contributing to GVHD in both major and minor histocompatibility models, the former typically in conjunction with CD4+ T cells and contributing to tissue injury, whereas in the latter, CD8+ T cells alone can be sufficient to cause GVHD (12–15). Similar to CD4+ T cells, CD8+ T cells can also differentiate to cytokine producing subsets including Tc1, Tc2, and Tc17 subsets. These CD8+ subsets possess variable capacities to induce acute or chronic GVHD (cGVHD) (16, 17).

A second or co-stimulatory signal (termed signal 2) then is required for full CD4+ and CD8+ T cell activation, expansion, differentiation, survival, and metabolic fitness. Previous studies (18–22) delineated the role of co-stimulatory molecules including CD28 (18), ICOS (CD278) (19), CD40L (CD154), OX40 (CD134) (20), and 4-1BB (CD137) (21). Co-inhibitory molecules can counterbalance co-stimulatory molecules. Cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4; CD152) (23), programmed death−1 (PD-1; CD279) and its ligand (PD-L1; CD274) (24, 25), B and T lymphocyte attenuator (CD272) (26), and B7-H3 (CD276) (27) have been shown to attenuate GVHD lethality. A third signal provided by inflammatory cytokines such as IL-12 or type 1 interferon is required for optimal CD8+ T cell function (28, 29).

An amplifying component of the immune response is ascribed to conditioning-related tissue damage releases damage-associated molecular pattern (DAMPs) and pathogen-associated molecular pattern (PAMPs) molecules (5). These molecules initiate immune responses during the early phases of GVHD and also provide a source of inflammatory cytokines that drive T cell responses. The role of DAMPs in accelerating GVHD lethality was illustrated by the binding of extracellular ATP and subsequent signaling of the purinergic P2X7 and P2Y2 receptors in host antigen presenting cells (APCs) bolstering donor T cell priming and alloreactive responses (30, 31). Conversely, ecto-nucleotidases such as CD39 and CD73, which regulate extra-cellular ATP levels, play suppressive roles in controlling GVHD (32–34). Other DAMPs such as uric acid, IL-33, heparan sulfate, high-mobility group box 1 protein, sialic acid–binding immunoglobulin-type lectins, mitochondrial components, and biglycans fuel GVHD responses (5).

The role of bacterial components in activating APCs and promoting GVHD via PAMPs is well established (35). For example, lipopolysaccharides are toll-like receptor 4 ligand and are implicated in marshaling innate immunity reactions, NF-κB activation, and transcription of pro-inflammatory cytokines genes (35). DAMPs and PAMPs not only contribute to GVHD initiation but also may augment later allogeneic T cell activation, differentiation, and expansion. Priming of allo-reactive donor T cells most often occurs in secondary lymphoid organs through interaction of the TCR with allo-peptide and MHC antigens expressed on host (termed direct allorecognition) or less often, on donor (termed indirect allorecognition) APCs. Both hematopoietic cells and non-hematopoietic cells are involved in alloantigen presentation that promotes and amplifies GVHD responses (36, 37). Recently, neutrophils have also been shown to exacerbate GVHD lethality by releasing reactive oxygen species in the gastrointestinal (GI) tract and surprisingly up-regulating MHC class II antigens (38, 39).

Chemokines guiding the migration of T cells toward GVHD target organs (40) wherein activated T cells mediate targeted tissue cell death via FAS ligand, perforin/granzymes, and releasing pro-inflammatory mediators mainly tumor necrosis factor (TNF-α), interferon (IFN-γ) (5, 41, 42). Other cytokines such as IL-7, IL-15, and IL-6 directly or indirectly support the expansion or activation of the innate and adaptive immune system and have been implicated in exacerbating GVHD lethality (43, 44). To achieve long term tolerance in allo-HSCT settings, strategies to control T cell activation, differentiation, expansion, and homing are critical to allow anti-inflammatory and central and peripheral regulatory events to be dominant over pro-inflammatory mechanisms. The following sections discuss approaches to blunt the distinct stages of GVHD induction (**Table 1**).

REDUCING DONOR ANTI-HOST ALLOREACTIVE T CELL BURDEN

In vitro or *in vivo* T Cell Depletion

In allo-HSCT, the cellular composition of the graft includes hematopoietic stem cells (HSCs) and a wide variety of cells, which influence engraftment. HSCs restore hematopoietic function, whereas other cell types such as mature T cells promote engraftment by inhibiting graft rejection mediated by recipient immune responses. Although T cells play a central role in the pathogenesis of GVHD, depletion of T cells increases the risk of infection and also of leukemia relapse (88, 89). Donor

TABLE 1 | Approaches to blunt the distinct stages of GVHD induction.

Strategies (agent or cell)	Mechanism of action	Predominant clinical indication	References
REDUCING DONOR ANTI-HOST ALLOREACTIVE T CELLS			
Anti-thymocyte globulin	Depletion of donor T cells	Prophylaxis and therapeutics	(45, 46)
Alemtuzumab	Depletion of CD52+ mature lymphocytes	Prophylaxis and therapeutics	(47–49)
Post-transplant cyclophosphamide	Depletion of rapidly proliferating alloreactive donorT cells	Prophylaxis	(50–54)
Exvivo depletion of CD45+ naïve T cells	Depletion of naive T cells	Depletion of naive T cells	(55)
BLUNTING TCR SIGNALS (Standard approaches usually in combinations)			
Tacrolimus and Cyclosporine	Calcineurin inhibitors; blocks T cell proliferation and IL-2 transcription	Prophylaxis	(56, 57)
Methotrexate	Folate antagonist; inhibits T cell proliferation	Prophylaxis	(56, 58, 59)
Mycophenolate mofetil	Blocks *de novo* synthesis of purine metabolism; inhibits T cell proliferation	Prophylaxis	(56, 58, 59)
Sirolimus	mTOR inhibitors; block T cell activation	Prophylaxis	(56, 60, 61)
INHIBITING CO-STIMULATORY SIGNALS			
CTLA-4 Ig	Inhibits CD28 mediated T cell activation	Prophylaxis	(62)
IMPAIRING ACTIVATING AND INFLAMMATORY CYTOKINE SIGNALS DRIVEN GVHD INJURY			
Ruxolitinib, Pacritinib	JAK inhibitors; Block T cell activation, cytokine production, and proliferation	Therapeutics	(63–65)
Alpha-1-antitrypsin (AAT)	Reduces pro-inflammatory cytokine secretion, expands Treg numbers, Inhibits neutophil elastase, decreases CD8+ effector memory cells	Therapeutics	(66–68)
REGULATING HISTONE DEACETYLASE			
Histone deacetylase inhibitors (vorinostat)	Reduce pro-inflammatory cytokine secretion, increase Treg numbers, modulate the function of APCs, upregulate IDO expression in DCs	Prophylaxis	(69–71)
BLOCKING T CELL CHEMOKINE RECEPTOR DIRECTED MIGRATION INTO GVHD ORGANS			
CCR5 inhibitor (Maraviroc)	Prevents T cell infiltration into GVHD tissues	Prophylaxis	(72, 73)
α4β7 (Natalizumab, Vedolizumab)	Prevents T cell infiltration into intestines	Prophylaxis	(72, 73)
CELLULAR THERAPY			
Mixed hematopoietic chimerism	Promotes immune tolerance	Prophylaxis	(74–76)
nTregs	Promotes immune tolerance	Prophylaxis and Therapeutics	(77–79)
iTregs	Promotes immune tolerance	Prophylaxis	(80–82)
Tr1	Promotes immune tolerance	Prophylaxis	(83–85)
MSCs	Immunomodultaor, Tissue repair	Therapuetics	(86, 87)

T cell depletion may be accomplished by *in vitro* or *in vivo* strategies. Pan-T cell depletion of the donor grafts can be highly effective but is associated with increased susceptibility to infections and malignancy recurrence due to the relatively long period of time required to reconstitute the immune system (90). *In vivo* administration of anti-T cell globulin (45, 46) or anti-CD52 mAb, CAMPATH-1 (47–49), reduce the donor T cell burden, while resulting in a state of T cell deficiency.

T cells are broadly classified as naïve vs. antigen experienced memory T cells (TM) (91). Stage of T cell differentiation is a critical factor in determining the capacity of T cells to induce GVHD. For instance, unlike naïve T cells, alloreactive effector and central TM cells failed to induce GVHD in pre-clinical models (92–94). The reduced ability of TM cells to induce GVHD is attributed to their reduced survival, expansion and alloreactivity (95). In a first-in-human trial, depletion of CD45RA+ naïve T cells from peripheral blood stem cells did

not reduce the incidence of GVHD (55). Nonetheless, all patients with GVHD uniformly responded to corticosteroids (55). A recent clinical trial (NCT01523223) used a final infusate of highly purified (>94%) CD8+ TM cells to treat relapse after allo-HSCT patients (96). Consistent with the results of pre-clinical models, CD8+ TM infusions are associated with low incidence of GVHD (1 of 15 patients, grade II liver GVHD). Altogether, strategies employing T cell grafts depleted of Tnaive cells may facilitate immune tolerance in allo-HSCT settings by hampering pro-inflammatory responses.

In vivo Post-transplant Cyclophosphamide Induced Alloreactive T Cell Depletion

In a recent approach, cyclophosphamide (Cy) that has both anti-neoplastic and immune modulatory effects, has been used to deplete alloreactive donor T cells and thereby prevent GVHD (50–52). Post-transplant cyclophosphamide (PTCy), typically given for 2 consecutive daily doses between days 3–5 post-transplant in combination with calcineurin inhibitors (CNI) and mycophenolate mofetil (53, 97, 98) or as a single agent (99, 100). Cy, a cytotoxic alkylating agent, specifically targets rapidly proliferating alloreactive T cells because of their impaired ability to replicate their damaged DNA (100–102). On the other hand, Tregs are relatively resistant to PTCy through increased expression of aldehyde dehydrogenase enzyme (103), which converts active to inactive Cy metabolites. The expansion and induction of Tregs promotes peripheral tolerance by suppressing remaining allo-reactive T cells and also hastens immune reconstitution. The final step for achieving long-term tolerance induced by PTCy is mediated by the later stage intrathymic deletion of immature alloreactive donor T cells. In clinical trials, PTCy reduced GVHD in both HLA-matched and partially HLA-mismatched allo-HSCT patients (53, 54). There are multiple ongoing clinical trials (NCT01028716, NCT01349101, NCT01860170, NCT02053545, NCT02065154, NCT02167958, NCT02169791) to investigate the effects of PTCy in conjunction with other agents to prevent GVHD. Overall results of clinical trials have shown a reduction in acute GVHD with a pronounced reduction in cGVHD albeit with organ toxicity, carcinogenicity and increased rates of infections.

BLUNTING TCR SIGNALS

Standard pharmacological regimens to prevent acute GVHD involve calcineurin inhibitors (CNI), mammalian target of rapamycin (mTOR) inhibitors, and anti-metabolites (5, 56). Calcineurin inhibitors such as tacrolimus or cyclosporine inhibit IL-2 production and subsequently clonal expansion of activated T cells (57). Sirolimus, a lipophilic macrocytic lactone, which binds to FKBP12, and inhibits the mTOR kinase activity, reducing cytokine responses and regulating cell proliferation, survival and metabolism by integrating information from environmental cues including stress signals such as nutrient deprivation (60). TCR, IL-2, CD28, sphingosine-1-phosphate receptor and leptin signals up-regulate the mTORC1 complex. Unlike CNI, sirolimus preferentially supports Tregs generation as Teffectors (Teffs) are mTOR-dependent whereas *in vitro* or *in vivo* induced peripheral Tregs and FoxP3 expression are favored by mTORC1 complex inhibition by sirolimus (61). Antimetabolites predominantly methotrexate, a folic acid antagonist and mycophenolate mofetil, an inhibitor of the *de novo* purine metabolism are being used with other immunosuppressants in allo-HSCT patients (56, 58, 59).

INHIBITING CO-STIMULATORY SIGNALS

It is well established that the fine-tuned balance between co-stimulation and inhibitory signals dictates immune responses (104, 105). Numerous co-stimulatory and co-inhibitory molecules have been identified and targeted to prevent and reduce various inflammatory diseases including GVHD. Preclinical studies of co-stimulatory and co-inhibitor pathway blockade for GVHD prevention have been comprehensively reviewed recently; the reader is referred to (22). Here we will focus on clinical trial results to prevent GVHD using CTLA4-Ig to block B7/CD28 co-stimulation.

The two-signal model of T cell activation required that both antigen and secondary stimuli are essential for optimal T cell activation (106, 107). The co-stimulatory CD28, identified as a receptor for B7-1 (CD80) ligand and B7-2 (CD86), is constitutively expressed on T cells (108–112). CD28 signals support T cell growth and survival. The co-inhibitory receptor CTLA-4, which also binds to B7-1 and B7-2, serves to temper T cell responses in part by down-regulating CD28 expression.

Linsley and coworkers developed CTLA-4 Ig, consisting of the extracellular CTLA-4 domain, and an immunoglobulin Fc fragment fusion partner to prolong its half-life, as a therapeutic agent that binds and sequesters B7 ligands from CD28 engagement (22). Earlier studies from the 1990s, including from our group, reported the efficacy of CTLA-4 Ig in the prevention of autoimmunity, solid organ allograft rejections and GVHD lethality (113–115) in murine models. The results from these studies laid the foundation for the first clinical trial (NCT01012492) in GVHD using abatacept (humanized CTLA4-Ig fusion protein) that showed a reduced GVHD incidence (62). Phase II studies (NCT01743131) testing the efficacy of abatacept against standard GVHD prophylaxis has been completed for in 7–8/8 HLA matched related or unrelated donor transplants.

The immunomodulatory effect of abatacept was associated with increased expression of PD-1 on T cells of the clinical responders. The role of the PD-1 pathway in inducing immune tolerance and controlling acute GVHD has been well established (24, 116). Although the beneficial effect of abatacept depends on blocking CD28 co-stimulation, it can also interfere with the endogenous CTLA-4 co-inhibition pathway and can lead to unwanted immune responses (117). The advent of fusion proteins or antibodies that block only the CD28 pathway without interfering with CTLA-4 may have an edge over abatacept due to their specificity. Belatacept, a 2 amino acid derivative of abatacept, was developed as a selective co-stimulatory pathway blocker, that has favorable results in renal transplant rejection compared to cyclosporine prophylaxis (118). A CD28 antagonistic antibody, FR104, has been tested in various pre-clinical models (119–121). More recently, in a non-human primate (NHP) GVHD model,

compared to CTLA4-Ig or CTLA4-Ig/sirolimus prophylaxis, FR104 or combined FR104/sirolimus prophylaxis delayed the onset of GVHD by controlling T cell activation and proliferation (122). However, there were non-GVHD-related deaths in the FR104/sirolimus-treated NHP due to sepsis. Detailed immunological analysis revealed that T cells from those primates failed to produce IFN-γ. The results from this study still highlight FR104/sirolimus combination as a promising therapy to treat GVHD in human patients due to better infection control compared to NHP.

IMPAIRING ACTIVATING AND INFLAMMATORY CYTOKINE SIGNALS DRIVEN GVHD INJURY

Immune Activating Cytokines Contributing to GVHD

JAKs are intracellular tyrosine kinases and act as downstream of cytokines, growth factors and hormone signaling. The JAK family members comprises JAK1, JAK2, JAK3, and TYK2 (123). JAK signaling supports the development, proliferation, and activation of T- and B- cells, DCs, macrophages, and neutrophils, all implicated in GVHD pathogenesis.

Ruxolitinib, a selective inhibitor of JAK1 and JAK2 reduced GVHD, associated with decreased proinflammatory cytokine production, Th1 differentiation and increased Tregs proportions (124, 125). Although ruxolitinib has been primarily reported as a treatment for steroid refractory or resistant GVHD (63), a recent study in myelofibrosis patient reported that ruxolitinib, given during peritransplant period, can reduce GVHD (64). Overall, 1 out of 12 patients developed severe (grade III) GVHD without major events during conditioning. However, CMV reactivation was seen in 4 of 6 CMV positive patients and 2 had cytopenias requiring ruxolitinib discontinuation (64). In other studies (126), baricitinib, a best-in-class Jak1/2 inhibitor, blunted IFNγR and IL6R signaling, resulting in complete protection from GVHD lethality as well as the reversal of active GVHD prevents GVHD with 100% survival, and reverses ongoing GVHD with dramatically increased Tregs along with decreased Th1 and Th2 differentiation, MHC class II and B7 ligand expression on APCs (126). Pacritinib is a potent JAK2 inhibitor that can reduce GVHD by sparing iTregs and polarizing T cells toward Th2 differentiation (65). A phase I/II trial (NCT02891603) combining pacritinib with standard immune suppression to prevent GVHD is currently being investigated.

Tofacitinib, a first generation JAK1/JAK3 inhibitor, reduced murine GVHD lethality (127). Antibodies directed to the IL2R common gamma chain that signals via JAK3 and STAT5 reduce proinflammatory cytokine production, CD8+ T cell granzyme B expression and severe GVHD lethality (128). Indeed JAK3 knockout T cells were unable to cause GVHD mortality in sublethally irradiated MHC class II disparate recipients. Pharmacological JAK3 inhibition with WHI-P131 given as prophylaxis ameliorated GVHD severity with a prolonged survival when compared to control mice (129). As many of these reagents are in the clinic including for GVHD prevention they may become part of an *in vivo* approach to achieve tolerance.

Alpha-1-antitrypsin to Reduce Pro-inflammatory Responses Post-transplant

Alpha-1-antitrypsin (AAT) is an acute phase secretory protein and a serine proteinase inhibitor, elevated during inflammation due to its predominant synthesis in hepatocytes (130, 131). Numerous lines of evidence demonstrated the anti-inflammatory properties of AAT. Studies have shown that the deficiency of AAT aggravated the severity of inflammatory disease, whereas addition of AAT to LPS-stimulated monocytes or mononuclear cells inhibited the release of pro-inflammatory cytokines (132–134). In GVHD patients, there was a negative correlation between AAT levels in donor plasma and occurrence of GVHD (135). Indeed, AAT treatment attenuated the lethality of GVHD in pre-clinical murine models by both increasing IL-10 levels and numbers of Tregs, and reducing the levels of pro-inflammatory cytokines such as IL1-β, TNF-α, and IL-6 (66, 135, 136). This tolerogenic effect of AAT, which induced Tregs expansion, was mediated by an increase in the numbers of $CD8^+$ $CD205^+$ DCs (135). AAT strongly inhibits neutrophil elastase and that may also contribute to reduced GVHD lethality due to the pathogenic role of neutrophils in GVHD (38). In clinical trials (NCT01523821 and NCT01700036), AAT treatment increased the proportion of Tregs and reduced GVHD manifestations (67), while decreasing numbers of $CD8^+$ TM cells (68) in steroid refractory (SR) GVHD patients without clinical toxicity.

Regulating Histone Deacetylase

Histone acetylation epigenetically regulates cell function by modulating gene expression. Acetylation is often associated with transcription activation, while deacetylation is associated with repression. The interplay between histone acetyltransferases (HATs) and histone deacetylases (HDAC) influences histone acetylation to impact numerous cellular functions, including cell differentiation, and apoptosis (56). HDAC inhibitors (HDACi) function an anti-inflammatory agents in autoimmune and inflammatory disorders (137). HDACi, namely vorinostat (SAHA), romidepsin (Istodax) and panobinostat (LBH589), are FDA-approved agents to treat cancers. HDACi treatment ameliorated murine GVHD through upregulation of indoleamine 2,3-dioxygenase (IDO) in DCs, in a STAT-3-dependent pathway (138, 139). Trytophan depletion and/or the generation of tryptophan catabolites has proven to be immune suppressive for murine GVHD (140, 141) as discussed in detail below. A completed phase I/II clinical trial (NCT00810602) of vorinostat with standard GVHD prophylaxis in patients who received matched related donor allo-HSCT reported reduced GVHD with lower levels of plasma IL-1β, TNF-α, IL-6, and IL-8 (69–71). Furthermore, HDACi treatment increased Treg cell numbers and enhanced their function in those patients (71). Extending this treatment to unrelated donor HCT (NCT01790568) also showed vorinostat to be result in a low rate of GVHD (70).

BLOCKING T CELL CHEMOKINE RECEPTOR DIRECTED MIGRATION INTO GVHD ORGANS

Chemokine receptors control the trafficking of T cells into tissues, where they may be primed, re-stimulated in the case of memory T cells, or cause cytolysis and tissue destruction. Chemokines produced by tissues injured by the conditioning regimen or GVHD itself may result in the elaboration of chemokines that direct the recruitment of specific innate and adaptive immune cells. Chemokine and chemokine receptor interactions that can influence GVHD pathogenesis have been reviewed (142). For example, during tissue damage, the up-regulation of CCR5 directs lymphocyte homing to the inflamed intestine and liver tissues (143–146). In mouse GVHD models, the efficacy of CCR5 blockade was dependent upon the degree of conditioning regimen injury. Whereas, anti-CCR5 mAb prevented T cell homing to Peyer's patches in the absence of conditioning (146), GVHD was accelerated with lethal radiation conditioning due to increased T cell expansion, IFN-γ and TNF-α production, and infiltration into the liver and lung (144, 146). In patients, reduced CCR5 expression correlated with lower GVHD (147, 148). Short-term addition of CCR5 antagonist, maraviroc added to standard GVHD prophylaxis resulted in reduced GI and liver GVHD in allo-HSCT patients given reduced intensity conditioning (72). Compared to this short-term treatment of 1 month (72), the extended course of maraviroc (3 months) was also safe and resulted in a significantly improved survival and higher GVHD-free (73). The relationship between conditioning regimen intensity and efficacy of CCR5 antagonism in allo-HSCT patients is unknown and warrants investigation.

Studies have demonstrated that the expression of gut-homing molecules, including α4β7-integrin and chemokine receptor CCR9, by T cells is required for homing to the intestines. GI injury due to conditioning is a key trigger for GVHD pathogenesis and results in the homing of donor T cells to the injured GI tract. Natalizumab is a potential drug of interest to mitigate GI GVHD due to its selective inhibition against α4 integrins of α4β7. Natalizumab and vedolizumab, a specific anti-α4β7 integrin monoclonal antibody, have been used in for GVHD treatment but not prevention, which have distinct cellular infiltrates and pathophysiologies (149, 150). Homing receptor blockade may potentiate tolerance induction in allo-HSCT as GVHD by precluding immune cell recruitment into GVHD organs and amplification of tissue injury.

REGULATING GVHD BY EXPLOITING CELLULAR METABOLISM MECHANISMS

Intrinsic T Cell Metabolic Energy Sources Required for GVHD

One way to tailor immune tolerance is to change the metabolic fitness. Immune cells require considerable bioenergy to generate and sustain immune responses against pathogens, allografts, and tumor cells. To accomplish these effector responses, immune cells utilize multiple metabolic pathways. The major metabolic pathways involved in cellular growth and proliferation are tricarboxylic acid (TCA) cycle, glycolysis, amino acids, pentose phosphate, fatty acid synthesis and oxidation (151, 152). Despite their diverse end products, these pathways are interdependent as biosynthesis of one pathway depends on the intermediate products of other pathways.

The TCA cycle takes place in the mitochondria to generate energy through oxidation of acetyl CoA, which is derived from sources such as glucose, fatty acids (FA) and glutamine (151, 152). The end products of the TCA cycle, namely NADH and FADH2 contribute electrons into the electron transport chain (ETC). The ETC is involved in highly efficient ATP generation by supporting oxidative phosphorylation (OXPHOS). Metabolically quiescent cells, like naive T cells, generate energy via OXPHOS by fueling TCA cycle with the available nutrients. However, upon cognate antigen encounter, T cells undergo a metabolic switch from OXPHOS to glycolysis to meet their energy needs (151–153). In glycolysis, extracellular glucose enters the cell through glucose transporters followed by the sequential conversion of glucose to pyruvate and other products by different enzymes. The availability of oxygen in the cell influences the fate of pyruvate. In the case of hypoxia, pyruvate is converted to lactate and NAD+. However, in normoxia, pyruvate is oxidized through the TCA cycle. Glycolysis plays a crucial role in cellular metabolism by providing precursors to other metabolic pathways. For example, cytoplasmic acetyl-CoA, a metabolite of glycolysis, promotes lipid synthesis by generating cholesterol and fatty acids. In a preclinical model, donor T cells shown to increase oxidative phosphorylation in both syngeneic and allogeneic recipients (153, 154). Glycolytic activity was only higher in donor T cells of allogeneic recipients than those of acute GVHD controls or syngeneic BMT recipients, indicating that Teffector cells causing GVHD are more dependent upon glycolysis (154–156). Pharmacological inhibition of mTORC1 or a phosphofructosekinase-2 isoform PFKB3 reduced GVHD lethality (154). Moreover, mice given T cell deficient in the glucose transport glut-1 were unable to induce GVHD (157).

Apart from glycolysis, glucose can also be metabolized via the pentose phosphate pathway (PPP) and glycogen synthesis (151). PPP is comprised of oxidative and non-oxidative branches. The oxidative branch of PPP maintains the cellular redox environment by generating reducing equivalents of NADPH. Whereas, the non-oxidative branch supports cell proliferation by generating required nucleotide and amino acid precursors (151). During GVHD, PPP activity of alloreactive T cells was increased (154).

Fatty acid oxidation (FAO) generates energy by converting FA to acetyl CoA, which enters into the TCA cycle (151, 153). In addition, FAO supports the ETC production of ATP by generating NADH and FADH2. Short and medium chain FA passively diffuse into the mitochondria, whereas the carnitine palmitoyl transferase (CPT) system regulates long chain FA (C14 to C18) metabolism (158). Discordant results have been reported about the activity of FAO in alloreactive T cells with some studies reporting increased FAO (155, 159), while a recent one demonstrated diminished FAO (154). FA synthesis plays a crucial role in sustaining T cell proliferation by generating lipids

through utilization of products derived from other metabolic pathways (151, 153). Lipid synthesis is regulated by enzymes such as acetyl-CoA carboxylases (ACC 1 and 2) and fatty acid synthase (151, 153, 154). Deficiency of ACC1 in donor T cells ameliorated GVHD due to impaired *de novo* FA synthesis (160). Sphingolipids are major components of eukaryotic cell membranes and play a crucial role in cellular survival, proliferation, differentiation and growth arrest. A recent study reported that ceramide, a metabolite of sphingolipids, modulate GVHD lethality (161). Targeting ceramide synthase 6, a ceramide biosynthetic enzyme, by either genetic deletion in donor T cells or pharmacological inhibition ameliorated GVHD due to reduced donor T cell proliferation and Th1 differentiation (161).

Glutamine, a key amino acid and readily available resource in serum, is required for T cell activation (5). Glutamine is involved in nucleotide synthesis and its metabolite glutamate also fosters the TCA cycle, glutathione and amino synthesis (151, 153). In allo-HSCT, donor T cells upregulated glutamine transport channels namely glutaminase 2, phosphoribosyl pyrophosphate amidotransferase, and glutamine-fructose-6-phosphate transaminase to increase the uptake of glutamine (25, 154). Furthermore, only donor T cells from allo-HSCT had increased levels of glutamate products aspartate and ornithine, which indicate that donor T cells can restore the exhausted intermediates of TCA cycle by increasing glutaminolysis (153, 154). Based on these studies, strategies inhibiting glycolysis, fatty acid oxidation, oxidative phosphorylation, or glutaminolysis may be an area of great potential to control GVHD (5).

Extrinsic Regulation of Cellular Metabolism in GVHD

A defense mechanism against GVHD lethality can be conferred by essential amino acid depletion results in a state of metabolic starvation. For example, high host tissue expression of IDO that catabolizes and hence depletes L-tryptophan was critical to reduce colonic GVHD (140). Donor T cell-derived IFN-γ upregulated the expression of IDO in colonic epithelial cells which in turn, diminished T cell proliferation and inflammation (141). Similarly, IDO expression was upregulated in the duodenal epithelial cells of GVHD patients and may be involved in the control of GI GVHD (162). The metabolic products of tryptophan catabolism has been shown to be immune suppressive. Whereas, combined administration of three tryptophan metabolites suppressed GVHD, kynurenines given in this way did not appear to be tolerogenic since GVHD was controlled only during the continuous administration period (141). In other studies, arginine depletion by myeloid-derived suppressor cell production of arginase I or infusion of pegylated L-arginase I was shown to reduce the vigor of the GVHD lethality response (163).

Mammalian hosts harbor a large number and a wide variety of commensal bacteria on surfaces of the body, especially in the GI tract. Commensal bacterial density in the GI ranges from 10^{11} to 10^{14} per gram of luminal content (164). The interaction between GI commensals and host immune cells plays a critical role in the development of the immune system and the maintenance of intestinal immune homeostasis. For example, germ-free mice have impaired immune systems with smaller Peyer's patches, lower numbers of IgA-producing plasma cells and lower numbers of CD8+ intraepithelial cells (165, 166). Dysregulation of GI microbiota has been associated with various inflammatory diseases (167–169).

In addition to metabolizing host dietary components, microbes produce their own metabolites that can have substantial immune system effects (170–173). In GVHD mice (174, 175) and patients (176–178), the diversity of the intestinal microbiota is significantly altered, which can be associated with the lethality of the disease. For example, butyrate, a short chain fatty acid microbial metabolite is an HDACi serves as the main energy source of intestinal epithelial cells (IECs) (179, 180). In a mouse model of GVHD, the reduction in intestinal butyrate resulted in decreased histone acetylation within CD326+ IECs (181). Administration of exogenous butyrate mitigated GVHD by increasing both anti-apoptotic and junctional proteins of IECs. This beneficial effect was not found to be mediated by donor Tregs, however the role of host Tregs in this model remains to be explored. Similarly, intragastric gavage of 17 rationally-selected strains of high butyrate–producing *Clostridia* also reduced GVHD and improved survival (181). A clinical trial (NCT02763033), which aims to increase butyrate levels in the intestines using dietary supplements containing potato-based resistant starch, is ongoing. Overall, these results demonstrate that HDACi can mitigate GVHD lethality.

In addition to butyrate, a recent study (182) reported that allo-HSCT conditioning regimens reduced indole or indole derivatives due to altered intestinal microbiome. Importantly, either supplementation with exogenous indole derivative or colonization of bacteria that can deliver indole metabolites into intestines of allogeneic murine recipients ameliorated GVHD lethality with reduced mucosal damage and pro-inflammatory cytokines (182). Beyond GVHD amelioration, recipient-specific tolerance was developed in donor T cells of recipients that were administered with the tryptophan metabolite and indole derivative, indole-3-carboxaldehyde, found in foods such as collard greens and broccoli.

TISSUE TOLERANCE MECHANISMS

It is a known fact that the survival of host against infections depends on the capacity of host's immune system. Recently, the role of parenchymal tissues on reducing disease severity and protecting from immunopathology has been gaining attention as tissues can modulate immune responses (183, 184). In non-infectious disease settings like GVHD, tissue tolerance is defined as an intrinsic and protective mechanism of parenchymal tissue to ameliorate GVHD against ongoing alloimmune responses. Studies from our laboratory demonstrated that the expression of the co-inhibitory molecule by parenchymal tissues promoted tolerance and reduced the lethality of GVHD. Experimental evidence has demonstrated the increased expression of co-inhibitory molecules such as programmed death-1 ligands and B7-H3 on T cells in GVHD targeted tissues (27, 156). The absence

of these molecules accelerated GVHD lethality due to augmented T cell effector responses. Thus, co-inhibitory pathways induced during alloresponses serve to dampen alloreactive donor T cell responses and hence GVHD.

REPARATIVE PROCESSES

Emerging data suggest that tissue tolerance can also be mediated through the regeneration of damaged tissues. In a recent study, administration of the Wnt-agonist R-spondin1 mitigated GVHD by protecting intestinal stem cells (ISC) and facilitating repair of the intestinal epithelium (185). In line with this finding, IL-22, which has been shown to activate ISC, enhanced intestinal epithelial regeneration and ameliorated GVHD (186). A phase I/II clinical trial (NCT02406651) is currently investigating the safety and efficacy of use of IL-22 in combination with corticosteroids for the treatment of patients with newly diagnosed GI GVHD.

Tregs and innate lymphoid cells 2 (ILC2) aid tissue repair by secreting amphiregulin, an epidermal growth factor that promotes tissue repair under inflammatory conditions (187). Non-lymphoid cells, in particular, mesenchymal stem cells (MSCs), have also been shown to facilitate tissue repair by polarizing tissue macrophages to the anti-inflammatory phenotype (188). These macrophages help repair tissues through enhanced fibroblastic proliferation and also reduce donor T cell proliferation and so limit GVHD. Furthermore, MSCs promote tissue repair by increasing the proliferation of ILC3 and their subsequent IL-22 production (189). Overall, strategies harnessing tissue tolerance represent a novel and expanding area of research in GVHD.

CELLULAR THERAPIES

Infusions of tolerogenic cells are one of the most attractive strategies to achieve long-term immune tolerance in clinical studies due to the long-term persistence of those cells. There are numerous immunoregulatory cells that have been used to induce transplantation tolerance in clinical models, but herein we will focus on Tregs, invariant natural killer T (iNKT) cells (see also Dominik Schneidawind's chapter) and MSCs.

Mixed Hematopoietic Chimerism and Tolerance Induction

Mixed hematopoietic chimerism also has been shown to be to facilitate kidney and liver solid organ graft acceptance in mice and humans (190–193), with high levels causing central deletional tolerance albeit at the risk of GVHD and transient chimerism allowing for peripheral tolerance that begins with Treg mediated mechanisms and transitions into peripheral tolerance likely including deletion of donor alloreactive T cells (74, 194–196). While transient T cell chimerism in hematological malignancy patients can decrease GVHD (75), mixed donor T cell chimerism present on day 90 in allo-HSCT patients receiving a reduced intensity conditioning regimen did not preclude GVHD generation; however the incidence was significantly lower than those with full donor T cell chimerism (35 vs. 61%), providing a platform upon which to tolerance induction may be more likely to be achieved (76).

T Regulatory Cell Infusion for Tolerance Induction

Tregs play a crucial role in maintaining immune homeostasis and tolerance by preventing autoimmunity and immunopathology. Tregs may be derived from the thymus (thymic-derived or natural Tregs (tTregs or nTregs), peripherally derived Tregs (pTregs), and *in vitro* induced Tregs (iTregs) (197). In this review, we will focus on both basic and clinical studies using different subsets of Tregs for the prevention of GVHD and discuss their limitations.

Thymic-Derived Tregs

Phenotypic features of tTregs include the constitutive expression of CD25, the high- affinity IL-2 receptor, CTLA-4, and Forkhead box P3 (Foxp3), a lineage transcription factor. Adoptive transfer of tTregs has been demonstrated to control allograft rejection and GVHD by limiting alloimmune responses (198–200). Preclinical studies have shown a high efficacy of Treg infusion and GVHD prevention (201–203). In allo-HSCT patients, there was an inverse correlation between Treg frequency and risk of acute GVHD (204).

Translation to the clinic proved challenging due to the low frequency of tTregs (typically 2–3% of CD4+ T cells) in the peripheral blood (205). The phenotypic profile of human tTregs was not as readily demarcated in peripheral blood as in the spleen and lymph nodes of mice. Moreover, compared to non-Treg T cells, Tregs were found to be hyporesponsive resulting in poor expansile properties and a preponderance of contaminating non-Tregs even when the latter represented a minor proportion of input cells. The first acute GVHD prevention clinical studies were reported by two groups (77, 78). In our study (77), umbilical cord blood cells were used as a source of tTregs (NCT00602693). Advantages included ease of tTreg isolation as a result of higher frequency of CD4+CD25bright cells and reduced likelihood of CD25+ Teffs contamination due to fetal microenvironment that minimizes external antigen exposure. *Ex vivo* expansion permitted tTreg activation, maximizing expansion and suppressor function. GVHD was reduced but not eliminated at Treg:Teff ratios of $\leq$1:6 in patients receiving cyclosporine A or sirolimus and mycophenolate. In the second study by our group, tTreg expansion was dramatically increased by restimulating tTregs with cell-based artificial antigen presenting cells and when given to patients receiving sirolimus and mycophenolate mofetil, GVHD was virtually eliminated (79). In the study by Martelli and coworkers (78), tTregs were freshly isolated from peripheral blood and allowed to become activated and expanded *in vivo* prior to the infusion of haploidentical T cells and in the absence of post-transplant immune suppression. GVHD was very low considering the high T cell dose given. Since tTregs could not be detected in peripheral blood beyond ∼2 weeks post-transplant, these studies suggest that tTregs have tolerized the donor T cell graft. In other studies, antigen-specific tTregs have been

generated and expanded *in vitro* in rodents (206) and are being tested in the clinic for GVHD prevention.

Tregs rely on IL-2 for their generation, proliferation, lineage stability and survival; however, they are poor producers of IL-2 (207). In patients, ultra-low dose IL-2 given as GVHD prophylaxis days 7–30 resulted in Treg expansion *in vivo* and no instances of GVHD in 16 pediatric allo-HSCT recipients (208). In patients with cGVHD, low dose IL-2 administration ameliorated cGVHD lethality by preferentially allowing *in vivo* Treg expansion, increasing the Treg:Teff ratio and thus favoring tolerance (209, 210). Since both Tregs and activated Teffs respond to IL-2, it is currently unknown whether these studies can be extrapolated to the higher risk adult population, which may be benefitted by more selective Treg expansion approaches. For example, two recent studies employed novel approach to selectively target Treg expansion. In one of the studies (211), investigators engineered IL-2 cytokine-receptor orthogonal pairs that interact with one another but not with their natural cytokine and receptor counterparts. Introduction of a mutated IL-2Rβ into T cells that preferentially binds orthogonal but not natural IL-2 enabled the selective cellular targeting of engineered T cells *in vitro* and *in vivo*, with limited off-target effects and negligible toxicity, suggesting a clinical strategy to selectively target Tregs *in vivo* in patients. In a different study, the same group employed complexes of human IL-2 with a unique conformational structure that stabilized IL-2 and promoted preferential STAT5 phosphorylation and Treg expansion (212).

In a non-IL-2 based approach, investigators have used reagents that stimulate death receptor 3 (DR3, TNFRSF25), a member of the tumor necrosis factor (TNF) receptor superfamily primarily expressed on Tregs, lymphoid tissue inducer cells, and NKT cells (213). The natural ligand of DR3, TL1a, is expressed on endothelial cells and APCs (213). An agonistic αDR3 mAb significantly expanded Tregs *in vivo* and prevented the development of allergic lung inflammation (214) and cardiac allograft survival by increasing the proportion of Tregs (215). Treating donors with αDR3 preferentially allowed Tregs expansion with reduced Tcon activation and those donor T cells reduced GVHD (216). A key role of TNF binding to TNFR2 was discovered to be critical to Treg control of GVHD (217, 218). Collectively, strategies to increase the tTreg/Teffs *in vivo* represent a promising therapeutic option to reduce GVHD and remain an active area of research.

Inducible Tregs (iTregs)

Although tTreg cellular therapy has great potential in controlling GVHD, higher doses of Tregs are required and it has been challenging to achieve uniform and robust tTreg expansion in clinics. Generation of iTregs is an alternative strategy to overcome the obstacle of limited nTreg cell numbers. Previous studies have established the potency of iTregs in controlling various autoimmune disorders (219, 220). In an experimental GVHD study, antigen-specific iTregs were generated and they were able to reduce GVHD by inhibiting the activation, proliferation and migration of donor T cells (221). The methylation status of the Treg-specific demethylated region (TSDR) of the Foxp3 promoter determines the stability of Tregs by maintaining the stable expression of Foxp3 (222, 223). Unlike tTregs, iTregs are completely methylated at the TSDR and tend to be unstable in GVHD mice (80, 224). Hence, studies have attempted to use various agents such as rapamycin, retinoic acid, and IL-6 blockade to induce and maintain iTregs (224). However, only the usage of sirolimus both *in vitro* and *in vivo* was shown to improve CD4+ iTreg stability in a mouse model of GVHD (80). Given the role of iTregs in controlling GVHD, there is an ongoing phase-I trial (NCT01634217) initiated by our institute to test the safety of CD4+ iTregs, generated using sirolimus, TGF-β, and IL-2, when given as GVHD prophylaxis to matched sibling donors along with CNI and mycophenolate thereby reducing the inflammatory environment. Intriguingly, CD4+ tTregs and iTregs were shown to be synergistic in controlling colitis in mice (81). A previous study reported that CD8+ iTregs can be induced by activating CD8+ CD25- T cells with allogeneic CD11c+ DCs, IL-2, TGF-β and retinoic acid. Although CD8+ iTregs expressed higher levels of suppressive molecules like $CD39^{+}CD73^{+}$, CTLA-4, and granzyme than CD4+ iTregs, there was no difference observed between their *in vitro* suppressive functions (82). In contrast to their *in vitro* suppressive functions, CD8+ iTregs are less potent than CD4+ iTregs in controlling GVHD due to their pro-apoptotic phenotype and thus reduced survival but are more effective in eliminating leukemia cells (82, 225). Intriguingly, CD8+ iTreg expression of FoxP3 can be stabilized by JAK2 targeting (226).

Type 1 T Regulatory (Tr1) Cells

Type 1 T regulatory (Tr1) cells are a distinct pTreg subset discovered by Bacchetta and Roncarolo and colleagues in severe combined immune deficiency patients who did not develop GVHD but had anti-host reactive T cell clones that produced high IL-10 and low IL-2 protein (227, 228). Tr1 cells lack constitutive expression of Foxp3, and have been shown to exert immune tolerance mainly via production of cytokines such as IL-10 and TGF-β (229, 230) that can inhibit murine GVHD lethality (228).

Using novel transgenic mice, Hill's group recently reported that Tr1 cells are the dominant immunoregulatory cells after allo-HSCT due to defective tTreg homeostasis (231). Infusion of Tr1 cells reduced GVHD, while Tr1 deficiency aggravated GVHD lethality. Murine and human Tr1 cells are typically generated by alloantigenic stimulator cell exposure in the presence of high IL-10 (83, 228, 229, 232). As with antigen-specific tTregs, Tr1 cells may have a reduced capacity for global immunosuppression due to their allospecificity. A recently completed phase-I trial demonstrated the feasibility of host-specific donor Tr1 therapy in GVHD patients. Infusions of Tr1 cells reduced GVHD, enhanced immune reconstitution and promoted tolerance induction (84). There are ongoing clinical trials testing the efficacy of Tr1 cell therapy in controlling autoimmunity and other inflammatory disorders (230).

Invariant Natural Killer T (iNKT) Cells

iNKT cells are a rare lineage of immunomodulatory cells and they produce large quantities of anti-inflammatory cytokines such as IL-4 and IL-10 (85). Numerous lines of evidence have highlighted the potency of iNKT cells in promoting immune tolerance in GVHD (233). Studies from the early 2000s demonstrated that a combined regimen of fractionated total hematopoietic irradiation and depletion with anti-T cell antibodies reduced GVHD in rodent models (234, 235). The protective effect against GVHD was meditated by the expansion of host immunoregulatory iNKT cells, which secreted IL-4 and supported donor Treg proliferation (234–236). Pharmacological approaches to expand iNKT cells, using a synthetic iNKT TCR ligand, α-galactosylceramide (alphaGalCer), also attenuated GVHD (237). An important consideration in these studies was the usage of reduced conditioning regimens that may help in the survival of host iNKT cells and their expansion. However, using lethally irradiated GVHD mouse models, Negrin's group demonstrated that the lethality of GVHD could be mitigated by adoptive transfer of low numbers of recipient-type, donor-type, or third party iNKT cells (238–240). These studies shed light on the role of iNKT cells in expanding both donor Tregs and myeloid derived suppressor cells (MDSCs). Interestingly, the protective effects of iNKT cell and donor Treg expansions were dependent on MDSCs and thus, crosstalk between these distinct cell populations promoted immune tolerance in GVHD settings. Results from these experimental models led to the initiation of a phase-II trial (NCT01379209) in GVHD patients. This clinical trial used a single dose of RGI-2001, the liposomal formulation of α-GalCer to expand iNKT cells. While there was reduced GVHD and increased expansion of Tregs observed in some patients, iNKT cells were very low in number and difficult to detect in the peripheral blood (241). Clinical studies testing infusions of iNKT cells hold promise to control GVHD.

Mesenchymal Stem Cells (MSCs)

Therapeutic infusions of MSCs are one of the leading options to treat GVHD. Although MSCs are rare non-hematopoietic cells in bone marrow, these cells are easy to isolate and can be expanded rapidly *in vitro* due to their multipotent and self-renewable properties (205). Immunomodulatory effects of MSCs in attenuating GVHD are mediated by secretion of cytokines (IL-6, TGF-β), soluble receptors (PDL-1, PDL-2) and effector molecules (nitric oxide, PGE2). MSCs also downregulate a wide range of chemokine (CCL1, CCL3, CCL8, CCL17, CCL22) expressions on donor T cells to limit T cell effector migration into target tissues (86, 205). The suppressive capacity of MSCs is enhanced by IFN-γ produced during GVHD, which up-regulates PDL-1 and IDO expression on MSCs to control T cell activation (205, 242). In other studies, high host anti-donor cytotoxic T lymphocyte (CTL) activity serves to eliminate donor MSCs and at the same time induce IDO and immune suppression by perforin-dependent host CTL mediated donor MSC apoptosis (243). Additionally, MSCs participate in the reparative process of tissue by promoting angiogenesis, regeneration, and remodeling (205). These properties have led to multiple clinical trials (NCT03158896, NCT00284986, NCT00361049, NCT00366145, NCT02336230) exploring the use of MSC infusion as an adjunctive strategy for GVHD prevention (87).

CONCLUDING REMARKS

Recently, there have been significant advances in the field of allo-HSCT to treat GVHD. Early phase studies involving AAT, HDACi and co-stimulation blockade have shown promising results, although randomized clinical trials and longer follow-up will be required to validate these existing results. Adoptive cellular therapies are powerful strategies to achieve peripheral tolerance swiftly in allo-HSCT recipients by blunting the inflammatory component of GVHD. Clinical trials using tTregs have reported promising results, but the long-term effects of Tregs on immune responses against infections and tumors have yet to be determined. To reduce non-specific immunosuppression and increase potency of antigen-specific suppression, generation of antigen-specific Tregs by a variety of approaches including engineering Tregs using chimeric antigen receptors (CAR) or designated T-cell receptors reactive against antigens present in GVHD organs may be an attractive approach. The first clinical trial evaluating CAR Treg therapy in the prevention of organ transplant rejection is expected to start by next year. Gene augmentation and gene editing techniques may be employed to direct Tregs to particular GVHD organs such as the gut or to increase Treg stability under inflammatory conditions. Renewed efforts are required to gain insight into tolerance induction in allo-HSCT and to develop safe and effective strategies to combat GVHD.

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GT wrote the manuscript. BB assisted in the writing and editing of the manuscript.

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Naive and Stem Cell Memory T Cell Subset Recovery Reveals Opposing Reconstitution Patterns in CD4 and CD8 T Cells in Chronic Graft vs. Host Disease

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The success of allogeneic hematopoietic stem cell transplantation (allo-HSCT) in the treatment of hematological malignancies remains hampered by life-threatening chronic graft vs. host disease (cGVHD). Although multifactorial in nature, cGVHD has been associated with imbalances between effector and regulatory T cells (Treg). To further elucidate this issue, we performed a prospective analysis of patients undergoing unrelated donor allo-HSCT after a reduced intensity conditioning (RIC) regimen containing anti-thymocyte globulin (ATG) and the same GVHD prophylaxis, at a single institution. We studied T cell subset homeostasis over a 24-month follow-up after HSCT in a comparative analysis of patients with and without cGVHD. We also quantified naive and memory T cell subsets, proliferation and expression of the apoptosis-related proteins Bcl-2 and CD95. Finally, we assessed thymic function by T cell receptor excision circle (TREC) quantification and T cell receptor (TCR) diversity by TCRVβ spectratyping. While the total number of conventional CD4 (Tcon) and CD8 T cells was similar between patient groups, Treg were decreased in cGVHD patients. Interestingly, we also observed divergent patterns of Naive and Stem Cell Memory (SCM) subset recovery in Treg and Tcon compared to CD8. Patients with cGVHD showed impaired recovery of Naive and SCM Tcon and Treg, but significantly increased frequencies and absolute numbers of Naive and SCM were observed in the CD8 pool. Markedly increased EMRA CD8 T cells were also noted in cGVHD. Taken together, these results suggest that Naive, SCM and EMRA CD8 play a role in the emergence of cGHVD. Reduced Naive and recent thymic emigrant Tcon and Treg in cGVHD was likely due to impaired thymic output, as it was accompanied by decreased CD4 TREC and TCR diversity. On the other hand,

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CD8 TCR diversity was similar between patient groups. Furthermore, no correlation was observed between CD8 TREC content and Naive CD8 numbers, suggesting limited thymic production of Naive CD8 T cells in patients after transplant, especially in those developing cGVHD. The mechanisms behind the opposing patterns of CD4 and CD8 subset cell recovery in cGVHD remain elusive, but may be linked to thymic damage associated with the conditioning regimen and/or acute GVHD.

Keywords: chronic graft vs. host disease, hematopoietic stem cell transplantation, T lymphocyte, stem cell memory, Naive T cell, immune reconstitution

INTRODUCTION

Despite the recent advances with patient-tailored therapies, allogeneic hematopoietic stem cell transplantation (allo-HSCT) has been increasingly used in the USA and in Europe for the treatment of hematological malignancies (1, 2). However, this technique is not without risks and is frequently accompanied by serious complications such as graft vs. host disease (GVHD) and infections, which are major causes of morbidity and mortality post-transplant (3). GVHD results from the recognition of patient tissues by donor-derived effector cells. Acute GVHD (aGVHD) typically occurs early after transplant, has markedly inflammatory manifestations and is thought to be primarily mediated by mature T lymphocytes infused with the graft (4). On the other hand, chronic GVHD (cGVHD) usually occurs later after transplant and resembles an auto-immune disease (5), affecting specific target organs, primarily the eyes, mouth, gastrointestinal tract, liver, skin, lungs, musculoskeletal, and genitourinary systems (6). Chronic GVHD has a complex pathophysiology. Thymic damage resulting both from the conditioning regimen and acute GVHD likely has an impact on normal T cell development (7, 8), and self-reactive T cells are believed to play a pivotal role in the development of cGVHD. Moreover, there appears to be a deficit in the regulatory T cell pool, contributing to the loss of immunologic tolerance post-transplant. A pivotal role for autoreactive B cells and the production of self-reactive antibodies has also been clearly associated to cGVHD pathogenesis, whereby self-reactive B lymphocytes are activated due to increased levels of BAFF (9, 10). In the end, excessive macrophage activation leads to fibroblast proliferation and collagen deposition, which is a hallmark of cGVHD (11).

The imbalances in regulatory (Treg) and effector T cells (12) appear to be central to cGVHD pathogenesis. This has led to clinical trials investigating the effect of low-dose rhIL-2 in patients with cGVHD in order to induce Treg expansion *in vivo* (13, 14). Also with the aim of increasing the Treg pool, we and others are conducting clinical trials of donor Treg infusion in patients with moderate and severe cGVHD (www.tregeneration.eu).

The involvement of donor T cells in the pathophysiology of GVHD led to the development of *ex vivo* (T cell-depleted grafts) and *in vivo* (anti-thymocyte globulin; ATG) T cell depletion approaches that significantly reduce GVHD incidence (5). ATG also delays immune reconstitution post-transplant through the depletion and/or function modification of T, B and NK cells (15). However, ATG does not completely abrogate the emergence of cGVHD (16–18), which attests to the multifactorial nature of this condition. On the other hand, thymic ablation has been shown to prevent cGVHD (8), suggesting a significant role for *de novo* thymic-derived T cells in this pathology.

In this study, we aimed at further investigating the biology of cGVHD and its effects on T cell homeostasis. Given the role that T cell immunity plays in cGVHD, we prospectively evaluated T cell reconstitution and thymic function in a homogenous patient population undergoing allo-HSCT after a reduced intensity conditioning (RIC) regimen containing ATG. We assessed the kinetics of T cell reconstitution after allo-HSCT and performed a comparative analysis of patients developing cGVHD vs. those who did not.

MATERIALS AND METHODS

Patients and Sample Collection

We prospectively monitored 57 patients undergoing allo-HSCT at Hospital de Santa Maria (Centro Hospitalar Universitário Lisboa Norte) from unrelated donors after a RIC regimen containing fludarabine 30 mg/m^2/day for 5 days (D-8 to D-4), melphalan 70 mg/m^2/day for 2 days (D-3 and D-2), and ATG (thymoglobulin) 4–6 mg/Kg (total dose) divided in 2–3 days, according to HLA compatibility. GVHD prophylaxis consisted of cyclosporine A (CsA) plus mycophenolate mofetil (MMF) in all patients. CsA and MMF were initiated on D-1 with CsA at 3 mg/kg/day intravenously (*iv*) twice daily and MMF at 2 g/day (*iv* or *per os*). CsA blood levels were monitored to target levels of 200 ng/mL. In the absence of GVHD, immune prophylaxis was tapered and discontinued between months 6 and 9 post-transplant.

Our center has acquired a significant experience with the administration of thymoglobulin in unrelated donor allo-HSCT over the last decade and maintained the same protocol in the present patient cohort.

Abbreviations: aGVHD, acute GVHD; ALL, acute lymphoid leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; cGVHD, chronic GVHD; CMV, cytomegalovirus; F, female; G, grade; GVHD, Graft versus Host Disease; HL, Hodgkin lymphoma; HLA, human leukocyte antigen; M, male; MDS, myelodysplastic syndrome; MF, Myelofibrosis; MM, multiple myeloma; NHL, non-Hodgkin lymphoma; PBSC, peripheral blood stem cell.

Seventeen patients were excluded due to early disease relapse or death from either infection or aGVHD in the first 9 months post-transplant. Chronic GVHD diagnosis and staging were performed according to the 2014 NIH criteria (19). Patient and donor characteristics are shown in **Table 1**. Peripheral blood was collected on months 1, 2, 3, 6, 9, 12, 18, and 24 after HSCT, and from five healthy controls (HC). Up to 2 weeks of variance in sample collection was allowed for patient sample collection.

Written informed consent was obtained before sample collection in accordance with the Declaration of Helsinki. This study obtained approval from the Ethics Committee of Lisbon Academic Medical Center (ref. 459/13).

Flow Cytometry

Flow cytometry analysis was performed on fresh whole blood, at pre-determined fixed time-points as indicated above, using the Human Regulatory T Cell Whole Blood Staining Kit (eBioscience), according to the manufacturer's instructions and immediately acquired on a LSR Fortessa (BD Biosciences).

The following monoclonal antibodies were used: Bcl-2 (Bcl-2/100) and CD31 (WM-59) FITC; Ki-67 (20Raj1) and CD95 (DX2) PE; CD3 (OKT3) PerCPCY5.5, CD45RA (HI100) and CD25 (M-A251) PE-CY7; CD4 (RPAT4) APC; CD127 (eBioRDR5) and CD62L (DREG-56) APCeFlour780 and FoxP3 (PCH101) e450 (all from eBioscience, except for CD25 PE-CY7, Becton Dickinson). Daily flow cytometer quality control monitoring was performed using Cytometry Setup and Tracking Beads (Becton Dickinson). Eight peak calibration Rainbow Beads (Becton Dickinson) were used to ensure stable fluorescence measurements throughout the study. Data was analyzed without prior knowledge of cGVHD status, using FlowJo9®. For quantification of Ki-67, Bcl-2, and CD95 within T cell subsets a cutoff of 50 events was used as a valid data point for statistical analysis. Five healthy control samples were stained in the same conditions.

CD4 and CD8 T Cell Receptor (TCR) Repertoire Analysis

TCR Diversity was evaluated with a TCRβgene (TRB) complementarity-determining region 3 (CDR3) spectratyping assay, performed on patient samples from either early (month 3) or late (month 9 or 12) time-points post-HSCT, as previously described (20). Cryopreserved PBMC from a separate set of 8 healthy controls, 10 patients with, and 10 without cGVHD were thawed, stained for CD3, CD4 and CD8, and FACSorted (BD FACSAria III) into CD4 and CD8 T cells. Total RNA isolated from each cell fraction (AllPrep, Qiagen) was used for first-strand cDNA synthesis primed with an equivolume mixture of random hexamers and oligo (dT) (Invitrogen Superscript III). TCRβ transcripts were amplified with TRBV family specific primers and a common TRCB reverse primer (21, 22). A run-off reaction with a second TRCB FAM-labeled primer was used to extend these products. Each fluorescent TRBV-TRBC fragment was separated using capillary electrophoresis-based DNA automated sequencer. Data were analyzed with GeneMapper (Thermo Fischer Scientific) for size, peak count, and fluorescent intensity determination. Profiles of transcript TRB repertoires were classified based on peak count, distribution shape and relative fluorescence intensity (RFI) of each peak (% RFI = 100 × clonal peak area/total peak area) (23).

TABLE 1 | Patient/donor characteristics.

	cGVHD	No cGVHD
Patients	18	22
Gender	6 F; 12 M	12 F; 10 M
Age	51 (30–67)	46 (19–69)
DIAGNOSIS		
AML	7	10
ALL	0	4
CLL	1	0
CML	2	2
HL	1	0
NHL	4	3
MDS	2	0
MF	1	0
MM	0	3
CMV Positive	14	18
DONORS		
Gender	11 F; 7 M	9 F; 13 M
Age	35 (21–49)	32 (20–57)
Female Donor to Male Patient	6	4
CMV Pos	12	15
HLA MATCHING		
10/10	8	9
9/10	9 (3A, 1B, 4C, 1DQB1)	12 (8A, 1B, 2C, 1DQB1)
8/10	1 (DRB1+DQB1)	1 (A+DQB1)
SOURCE OF GRAFT		
BM	5	11
PBSC	13	11
aGVHD	17/18	9/22
GI	5	2
GII	6	4
GIII	6	3
GIV	–	–
cGVHD	18	–
Mild	7	–
Intermediate	4	–
Severe	7	–
Day of cGVHD Onset	230 (149–455)	–
Follow Up (days)	682 (286–1,051)	483 (90–840)

Signal-Joint TCR Excision Circle (sjTREC) Quantification in CD4 and CD8 T Cells

sjTREC sequences were analyzed with a multiplex qPCR assay (24) in CD4 and CD8 T cells using the same samples purified for the diversity studies. sjTREC sequence copy numbers were extrapolated from standard curves obtained by 10-fold serial dilutions of a triple-insert plasmid containing sjTREC and TRAC fragments in a 1:1:1 ratio (kind gift from L. Imberti, Spedali Civili of Brescia, Italy). Detected sjTREC copies were genome

normalized with the mean quantity of T cell receptor α-chain sequences. Results were expressed as sjTREC copies per 10^6 CD4 or CD8 T lymphocytes.

Statistical Analysis

The patients' clinical data shown in **Table 1** was compared using Fisher's exact test and the Mann–Whitney test when comparing continuous data. Data obtained for patients with and without cGVHD were compared at each time-point using the Mann-Whitney test on GraphPad Prism®. To analyze slopes of reconstitution early (up to 6 months) and late (from 6 months onwards) post-HSCT, we used linear mixed effects (LME) models over those two periods. In this approach, the data of all patients over time is analyzed together, with patient as the random effect, while time and cGVHD (yes/no) are covariates. In each case, we tested also for an interaction term of cGVHD with time (i.e., different slopes of reconstitution). Previous aGVHD, gender, and source of graft were further included as covariates. In all cases, statistical significance was assessed at $\alpha = 0.05$.

RESULTS

Patient Characteristics

All patients underwent allo-HSCT from an unrelated donor and received the same conditioning and GVHD prophylaxis regimens, as described in the methods section. Patient and donor characteristics are summarized in **Table 1**. The most frequent underlying disease within patients included in the analysis was AML (42.5%) and 60% of transplants used PBSC as the source of graft. After a 24-month follow-up post-HSCT, 18 patients developed cGVHD while 22 did not (No cGVHD). Median cGVHD onset day was 230. Patients without cGVHD had a shorter follow-up compared to cGVHD patients. Seventeen out of 18 cGVHD patients had previously developed aGVHD, in contrast to 9 out of 22 in the No cGVHD group. No significant differences were identified when comparing the patient characteristics between groups (**Table 1**), with the exception of the higher incidence of aGVHD in patients with cGVHD ($p = 0.0006$). Five healthy controls (HC), with a median age of 43 (range 36–45), were also studied.

Distinct Treg, Tcon, and CD8 Reconstitution Patterns After HSCT

Treg numbers were low in both patient groups up to month 6 after HSCT (**Figure 1A**). From months 9 to 18, Treg were decreased in cGVHD vs. No cGVHD patients. Analysis of *ex vivo* proliferation using intracellular Ki-67 staining revealed significantly decreased proliferation from months 3 to 18 in patients developing cGVHD as compared to No cGVHD, suggesting that reduced Treg numbers in cGVHD may be partly due to reduced homeostatic proliferation (**Figure 1B**).

Lower Treg numbers in cGVHD were not associated with increased susceptibility to apoptosis as assessed by Bcl-2 and CD95 expression levels, as these proteins were expressed at similar levels in both patient groups (**Figures 1C,D**). These data suggest that apoptosis mediated by these pathways did not play a major role in the low Treg numbers observed in cGVHD.

We further quantified conventional CD4 T (Tcon; Foxp3$^-$ CD4) and CD8 T cell numbers and found no major differences between patients with and without cGVHD (**Figure 1A**). We did observe significant reductions in Tcon and CD8 proliferation, as assessed by Ki-67 staining, in cGVHD compared to No cGVHD (**Figure 1B**). On the other hand, no clear trend for altered Bcl-2 or CD95 expression in cGVHD was observed (**Figures 1C,D**).

Impaired Naive and SCM Treg Reconstitution in cGVHD

Quantification of Naive/memory Treg subsets was performed by flow cytometry as described in the methods using CD45RA, CD62L, and CD95 surface markers. We identified Central Memory (CM) cells as CD45RA$^-$ CD62L$^+$, Effector Memory (EM) as CD45RA$^-$ CD62L$^-$ and CD45RA-expressing Terminal effectors (EMRA) as CD45RA$^+$ CD62L$^-$. We further used CD95 to distinguish Naive, CD45RA$^+$ CD62L$^+$CD95$^-$, from the Stem Cell Memory (SCM) subset, CD45RA$^+$ CD62L$^+$CD95$^+$, as previously described (25) (detailed gating strategy illustrated in **Supplementary Figure 1A**). The percentages of each subset within Treg from patients with and without cGVHD is summarized in **Figure 2A**. A detailed visualization of the overall data showing percentage and absolute counts for each subset in all time-points for both patient groups and healthy controls, as well as the corresponding significances, is displayed in **Supplementary Figure 1B**.

The most prevalent subset within Treg was CM, followed by EM, naïve, and SCM, while EMRA were an extremely rare subset (**Figure 2A**). We observed decreased Naive Treg percentages and absolute counts in cGVHD as compared to No cGVHD throughout the follow-up (**Figures 2A,C**). The only exception was at Month 1 when Naive Treg frequency and absolute counts were increased in cGVHD vs. No cGVHD patients (**Figures 2A,C**). Despite this, Naive Treg remained low in patients developing cGVHD while they increased in the absence of cGVHD. This trend, reflecting an inability of Naive Treg to recover in patients developing cGVHD, was confirmed in a LME analysis of the first 6 months post-HSCT. This analysis showed significantly different slopes estimated for the recovery of Naive percentages, whereby Naive Treg percentages only increased in the absence of cGVHD (**Figure 2B**). From month 9 onwards, Naive Treg percentages and counts were lower in patients developing cGVHD as compared to patients that remained free from cGVHD (**Figures 2A,C**).

Similar to Naive Treg, the recovery of the SCM Treg subset was impaired in patients developing cGVHD. This was the case for percentages and for absolute counts, which were significantly reduced in cGVHD from month 9 to 18 (**Figures 2A,D**). The relative proportion of the remaining memory subsets was largely unaltered by cGVHD (**Figure 2A**). CM and EM Treg absolute counts were either similar or reduced in cGVHD, whereas EMRA Treg were similar or increased, but no sustained statistical significances were noted (refer to **Supplementary Figures 1B,C** for details).

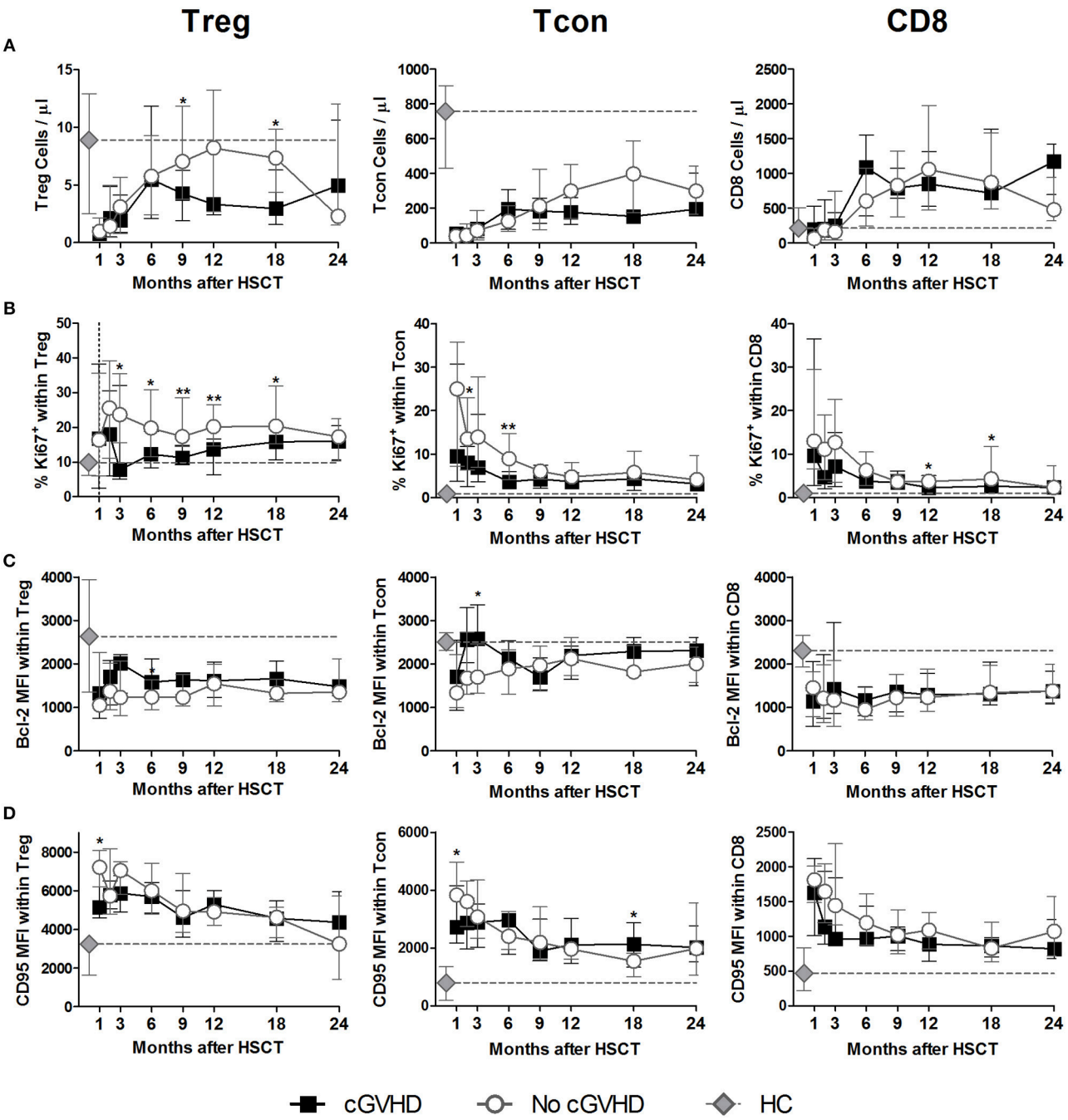

FIGURE 1 | Treg, Tcon and CD8 Homeostasis following HSCT. Patients were divided into No cGVHD (gray lines and open circles) and cGVHD (black lines and squares). Healthy controls (HC) are also shown (gray diamonds and dotted gray lines). **(A)** Absolute counts per microliter for Treg ($CD3^+$ $CD4^+$ $CD25^{bright}$ $Foxp3^+$ $CD127^{low}$), Tcon ($CD3^+$ $CD4^+$ $Foxp3^-$), and CD8 ($CD3^+$ $CD4^-$) T cells. **(B)** Frequency of Ki-67$^+$ cells within $CD3^+$ $CD4^+$ $Foxp3^+$ Treg, Tcon, and CD8 T cells. Median fluorescence intensity (MFI) for Bcl-2 **(C)** and CD95 **(D)** within $CD3^+CD4^+$ $Foxp3^+$ Treg, Tcon and CD8 T cells. Symbols represent median values and whiskers represent interquartile range (IQR). Asterisks denote statistically significant differences between patient groups (*$p = 0.01$ to 0.05; **$p = 0.001$ to 0.01).

In summary, cGVHD was associated with impaired Treg recovery, particularly of the Naive and SCM Treg subsets, and lower Treg proliferation as compared to the No cGVHD group.

Impaired Naive and SCM Tcon Reconstitution in cGVHD

Chronic GVHD was associated with a marked impairment in Naive Tcon recovery as compared to patients who did not develop

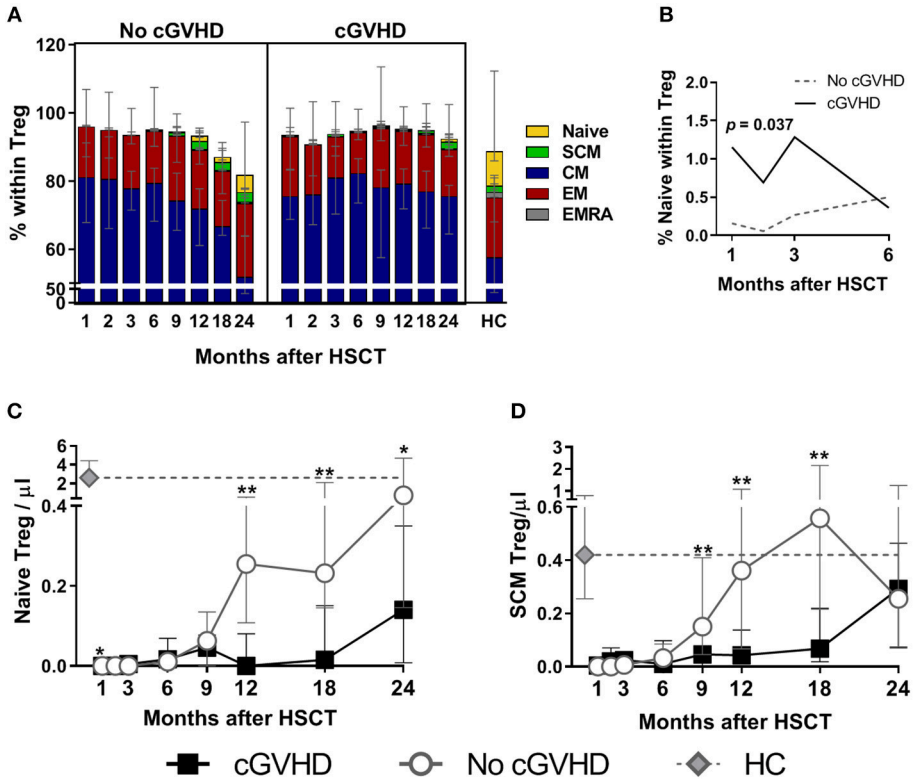

FIGURE 2 | Treg subset reconstitution in cGVHD. Treg were identified as $CD4^+$ $Foxp3^+$ within a $CD3^+$ lymphocyte gate. Naive and memory subsets were identified as shown in **Supplementary Figure 1**. **(A)** The median percentage for each subset within Treg is shown for patients with and without cGVHD, as well as for HC. **(B)** Naive Treg percentages in the first 6 months after HSCT in patients with cGVHD (black line) and No cGVHD (dotted gray line), illustrating the results of the LME analysis for this subset. Absolute counts of Naive **(C)** and SCM **(D)** Treg in patients without cGVHD (gray lines and open circles), with cGVHD (black lines and squares) and HC (gray diamonds and dotted gray lines). Symbols represent median values and whiskers represent IQR. Asterisks denote statistically significant differences between patient groups (*p = 0.01 to 0.05; **p = 0.001 to 0.01).

cGVHD. This became apparent from month 9 onwards in terms of both frequency and absolute counts (**Figures 3A,C**). Although differences between patient groups only reached significance after month 9, the LME analysis revealed that the percentage of Naive Tcon up to month 6 was better represented by a model with significantly different slopes for cGVHD and No cGVHD (p = 0.008) (**Figure 3B**). Hence, during the first 6 months after HSCT, Naive Tcon percentages increased in patients who did not subsequently develop cGVHD, while they remained stable in patients who develop cGVHD. This translated, at later time-points, into significant differences in Naive Tcon frequencies and numbers between patient groups from months 12 to 24 (**Figures 3A**,**C**; **Supplementary Figures 1B,C**). Impaired Naive Tcon recovery in cGVHD was mimicked by decreased SCM Tcon levels, reaching significance at month 18 for SCM Tcon counts (**Figures 3A,D**).

CM and EM were the most abundant Tcon subsets post-HSCT and were largely similar between patient groups, while EMRA showed a non-significant tendency to be increased in cGVHD (**Figure 3A**). Percentages and absolute counts for all Tcon subsets are detailed in **Supplementary Figures 1B,C**.

In summary, we did not observe significant disparities in whole Tcon recovery between patients developing cGVHD and those who did not. However, the composition of the Tcon pool after HSCT reveals an impairment in Naive and SCM subsets in patients who develop cGVHD, similar to what was observed in Treg.

Increased CD8 Naive, SCM, and EMRA in cGVHD

The reconstitution of Naive and memory subsets within the CD8 T cell pool differed greatly between patient groups (**Figure 4**) and had a strikingly different pattern to that observed in Tcon and Treg. This was particularly prominent not only for the least abundant Naive and SCM CD8 subsets, but also for the more abundant, terminally differentiated EMRA subset.

Interestingly, we observed significantly increased Naive CD8 percentages and counts in cGVHD vs. No cGVHD during the initial post-transplant period (**Figures 4A,C**). This statistical significance disappeared from month 12 onwards, as Naive CD8 increased in patients who did not develop cGVHD. Such accelerated Naive CD8 T cell recovery in the absence of cGVHD at later time-points was also revealed by the LME analysis of months 9 to 24 post-HSCT. This analysis showed a significant difference in the slopes for Naive CD8 percentages between the

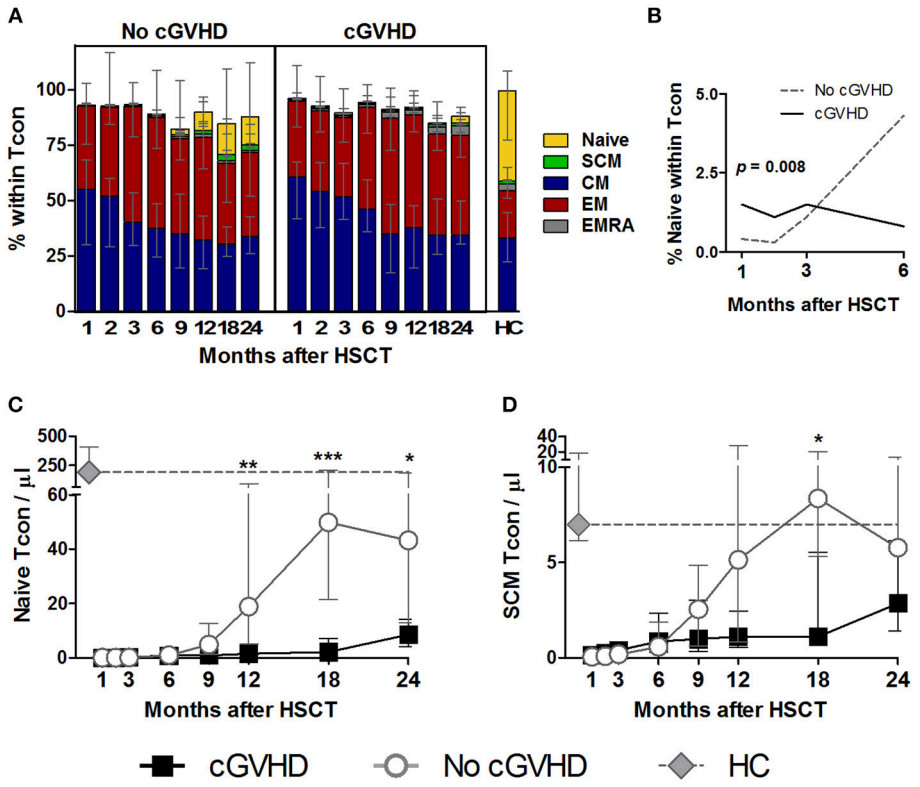

FIGURE 3 | Naive and memory Tcon reconstitution in cGVHD. Tcon were identified as $CD4^+$ $Foxp3^-$ within a $CD3^+$ lymphocyte gate. Naive and memory subsets were identified as shown in **Supplementary Figure 1**. **(A)** The percentage of each subset within Tcon cells is shown for patients with and without cGVHD, as well as HC. **(B)** Percentage of Naive within Tcon in the first 6 months after HSCT in patients with cGVHD (black line) and No cGVHD (dotted gray line), illustrating the results of the LME analysis for this subset. Naive **(C)** and SCM **(D)** Tcon absolute counts in patients without cGVHD (gray line and circles), patients with cGVHD (black line and squares) and HC (gray diamonds and dotted gray lines). Median values and IQR are shown. Asterisks denote statistically significant differences between patient groups (*$p = 0.01$ to 0.05; **$p = 0.001$ to 0.01; ***$p = 0.0001$ to 0.001).

two patient groups (**Figure 4B**). While in patients with cGVHD the percentage of Naive CD8 stabilized, in the absence of cGVHD the frequency of these cells increased over time.

Interestingly, the SCM CD8 subset was also increased in patients developing cGVHD vs. No cGVHD, starting from the early time points of the follow-up period, both in percentages and absolute counts (**Figures 4A,D**).

Despite such prominent differences in Naive and SCM subsets, these were always the least abundant populations. Within the most frequent populations the EMRA showed the most striking differences between patient groups. Hence, terminally differentiated EMRA CD8 cells were increased in percentage and counts from the very early post-transplant period in patients developing cGVHD (**Figures 4A,E**), while the remaining memory subsets, CM and EM, showed an opposite pattern to that observed in Naive, SCM and EMRA, with significant reductions in percentages being noted in patients developing cGVHD (**Figure 4A**; **Supplementary Figures 1B,C**).

In summary, CD8 T cell reconstitution in cGVHD was associated with persistently increased Naive, SCM and EMRA subsets. This became apparent very early after HSCT, suggesting a possible involvement of these subsets in disease development.

The Effects of Acute GVHD on SCM Tcon and CD8

In our patient cohort, all but one of the patients developing cGVHD had a history of previous aGVHD, while within the group of 22 patients who did not develop cGVHD, 9 had developed acute GVHD (aGVHD). In order to clarify the contribution of aGVHD and the associated therapies to differences in T cell reconstitution between patient groups, we tested the effect of adding aGVHD as a covariate in the LME analysis. This analysis showed that during the first 6 months after HSCT, SCM Tcon and CD8 percentages and counts were significantly affected by aGVHD ($p < 0.01$). No impact of aGVHD was observed from month 6 onwards.

To complement this analysis, we stratified the No cGVHD group into acute only (Ac GVHD, $n = 13$) and no GVHD at all (No GVHD, $n = 9$). The only patient in our cohort who did not have aGVHD prior to cGVHD was excluded from this analysis. Hence, the chronic GVHD group consisted of patients with acute and chronic GVHD (Ac & Ch GVHD, $n = 17$). Month 24 was excluded from this analysis due to insufficient number of data points.

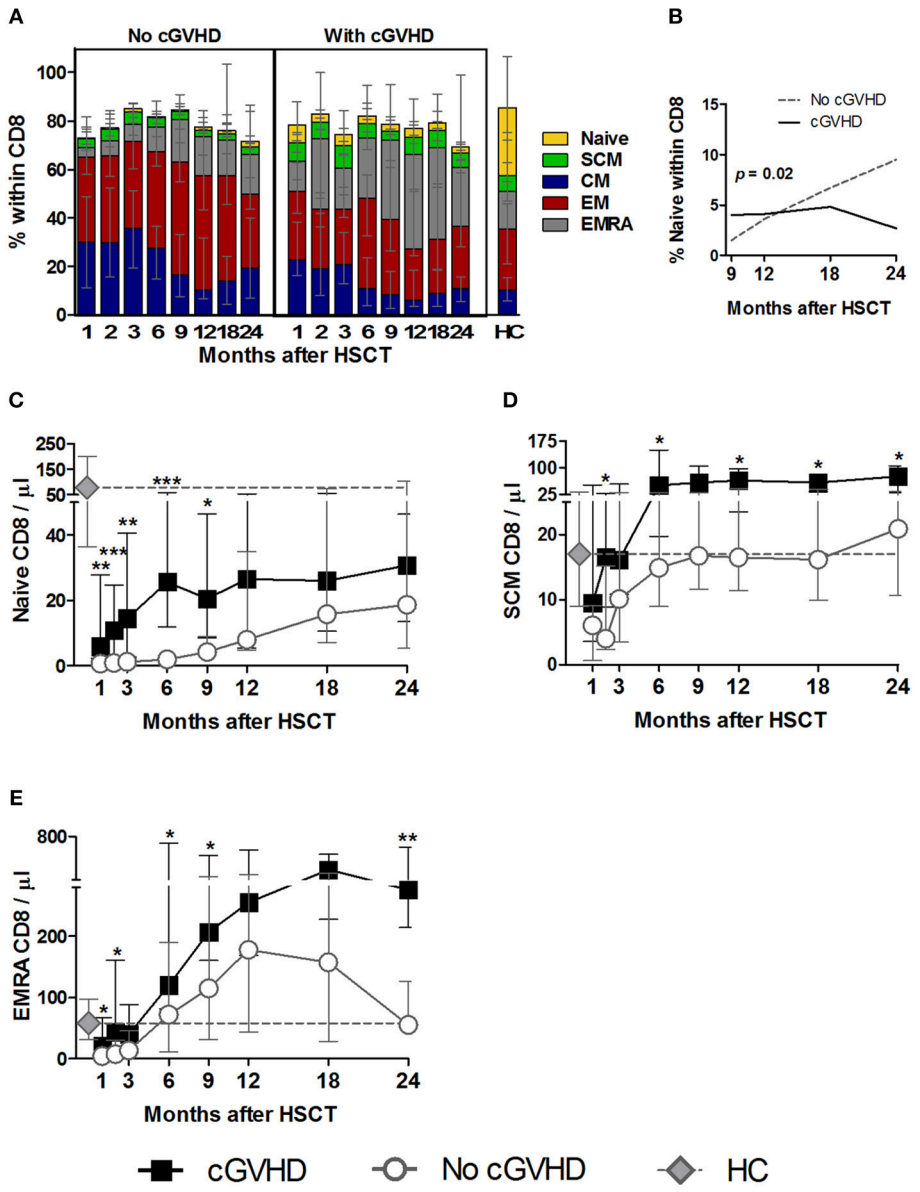

FIGURE 4 | Naive and memory CD8 reconstitution in cGVHD. CD8 T cells were identified as $CD3^{+}$ $CD4^{-}$ lymphocytes. Naive and memory subsets were identified as shown in **Supplementary Figure 1**. **(A)** Percentage of each subset within CD8 cells for patients with and without cGVHD, as well as HC. **(B)** Naive CD8 percentages from month 9 to 24 after HSCT in patients with cGVHD (black line) and no cGVHD (dotted gray line), illustrating the results of the LME analysis for this subset. Absolute counts of Naive **(C)**, SCM **(D),** and EMRA **(E)** CD8 cells in patients without cGVHD (gray circles and dotted lines), patients with cGVHD (black squares and lines) and HC (gray diamonds and dotted gray lines). Symbols represent median values and whiskers represent IQR. Asterisks denote statistically significant differences between patient groups (*$p = 0.01$ to 0.05; **$p = 0.001$ to 0.01; ***$p = 0.0001$ to 0.001).

Stem Cell Memory (SCM) Tcon numbers were significantly increased during the first 3 months in GVHD vs. No GVHD patients (**Figure 5A**). At later time-points, this pattern was reversed and while SCM Tcon reconstituted in the No GVHD and Ac GVHD groups, they became significantly reduced in the Ac & Ch GVHD patient group. A similar pattern was observed when analyzing SCM percentages within Tcon (data not shown). Overall, these data suggest that GVHD development is associated to early increases in SCM Tcon.

For CD8 T cells, initial time-points showed a similar reconstitution pattern to Tcon, whereby patients with GVHD showed significantly increased SCM as compared to No GVHD (**Figure 5B**). Interestingly, from month 6 onwards patients with aGVHD showed similar SCM CD8 counts as patients who did not develop any form of GVHD. On the other hand, patients who subsequently develop cGVHD appear to sustain a significant increase in this population throughout the follow-up period when compared to the other two groups of patients. A similar

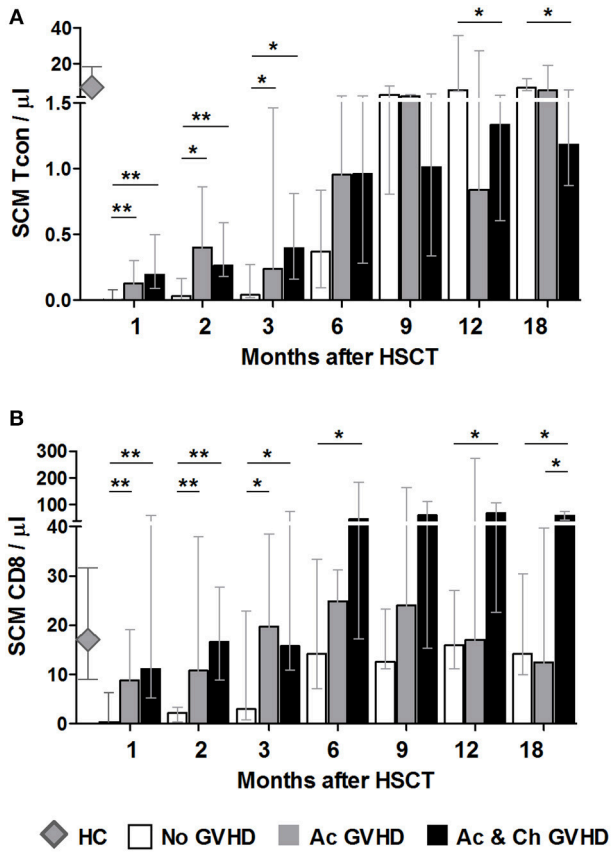

FIGURE 5 | The effect of acute GVHD on SCM Tcon and CD8 reconstitution. SCM absolute counts for Tcon **(A)** and CD8 **(B)** in patients without any form of GVHD (No GVHD, white bars), patients with acute GVHD only (Ac GVHD, gray bars), patients with acute and chronic GVHD (Ac & Ch GVHD, black bars) and healthy controls (HC, gray diamonds). Bar graphs shows median values and IQR. Asterisks denote statistically significant differences between patient groups (*$p = 0.01$ to 0.05; **$p = 0.001$ to 0.01).

pattern was observed when analyzing SCM percentages within CD8 (data not shown).

These data suggest that GVHD development is associated to early increase in the SCM subset after transplant. Furthermore, we observed a sustained increase in this CD8 subset in patients who subsequently develop cGVHD, further pointing to a possible role for these cells in disease development.

Chronic GVHD Is Associated With Reduced CD4 TCR Vβ Diversity

TCR repertoire diversity was analyzed in purified CD4 and CD8 T cells from patients at early (month 3) and late time-points (months 9 or 12) post-HSCT by TCRVB spectratyping (**Figure 6**).

Early post-HSCT, we observed no significant differences in TCR diversity between patient groups in either CD4 or CD8 cells (**Figures 6B,D**). There were few VB families displaying polyclonal distributions, while those with skewed, oligoclonal, and monoclonal distributions prevailed (**Figures 6A,C**). When early and late time-points were compared within each patient group, significant increases in TCR diversity were observed, suggesting *de novo* CD4 and CD8 T cell production during patient follow-up.

However, when patients developing cGVHD were compared to No cGVHD, a significant reduction in CD4 TCR diversity in cGVHD was observed at later time-points (**Figure 6B**). This was reflected in decreased prevalence of polyclonal Gaussian profiles and the appearance of numerous skewed profiles (**Figure 6A**), translating into decreased TCRVB peaks within CD4 T cells in cGVHD (**Figure 6B**).

Similarly to CD4, we observed an increase in TCR diversity from early to late time-points in CD8. However, the median number of CDR3 peak count within the VB families did not show statistically significant differences between patient groups (**Figure 6D**). This was likely the result of the observed high prevalence of skewed distributions in CD8 T cells from both patient groups (**Figure 6C**).

Chronic GVHD Negatively Impacts Thymic Function

We next analyzed sjTREC content as an estimate of thymic activity in purified CD4 and CD8 T cells at early (month 3) and late (months 9 or 12) time-points post-HSCT. Unfortunately, insufficient cell numbers precluded the analysis at month 3. sjTRECs were significantly reduced in CD4 (**Figure 7A**) and CD8 (**Figure 7C**) T cells in cGVHD vs. No cGVHD patients at late time-points.

Importantly, sjTREC content and Naive counts from all patients were positively correlated in CD4 (**Figure 7B**) but not in CD8 T cells (**Figure 7D**). Furthermore, Naive CD4 counts were positively correlated with sjTRECs in No cGVHD, but not in cGVHD (data not shown). In CD8 T cells, no significant correlation was observed between sjTREC content and Naive CD8 counts in either patient group (data not shown).

The presence of a significant correlation between CD4 TREC numbers and Naive CD4 counts at month 9 suggests that the Naive CD4 T cell reconstitution observed in patients who do not develop cGVHD is likely the result of *de novo* thymic production. On the other hand, the increase in Naive CD8 T cells observed in patients with cGVHD may depend more on T cell peripheral expansion.

Recent Thymic Emigrant (RTE) Tcon and Treg in GVHD

The expression of CD31 within Naive CD4 T cells has been reported to contain a population enriched in RTE (26). We therefore quantified this population in our patient cohort as an additional measure of thymic function. We found that patients developing cGVHD had significantly reduced RTE Treg (**Figure 7E**) and Tcon (**Figure 7F**) from month 9 onwards. Furthermore, LME analysis of the first 6 months revealed significantly different slopes for RTE Tcon absolute counts (**Figure 7H**) and percentages ($p = 0.0032$) (data not shown), whereby RTE Tcon increase in the absence of cGVHD with negligible recovery of this population in patients developing cGVHD.

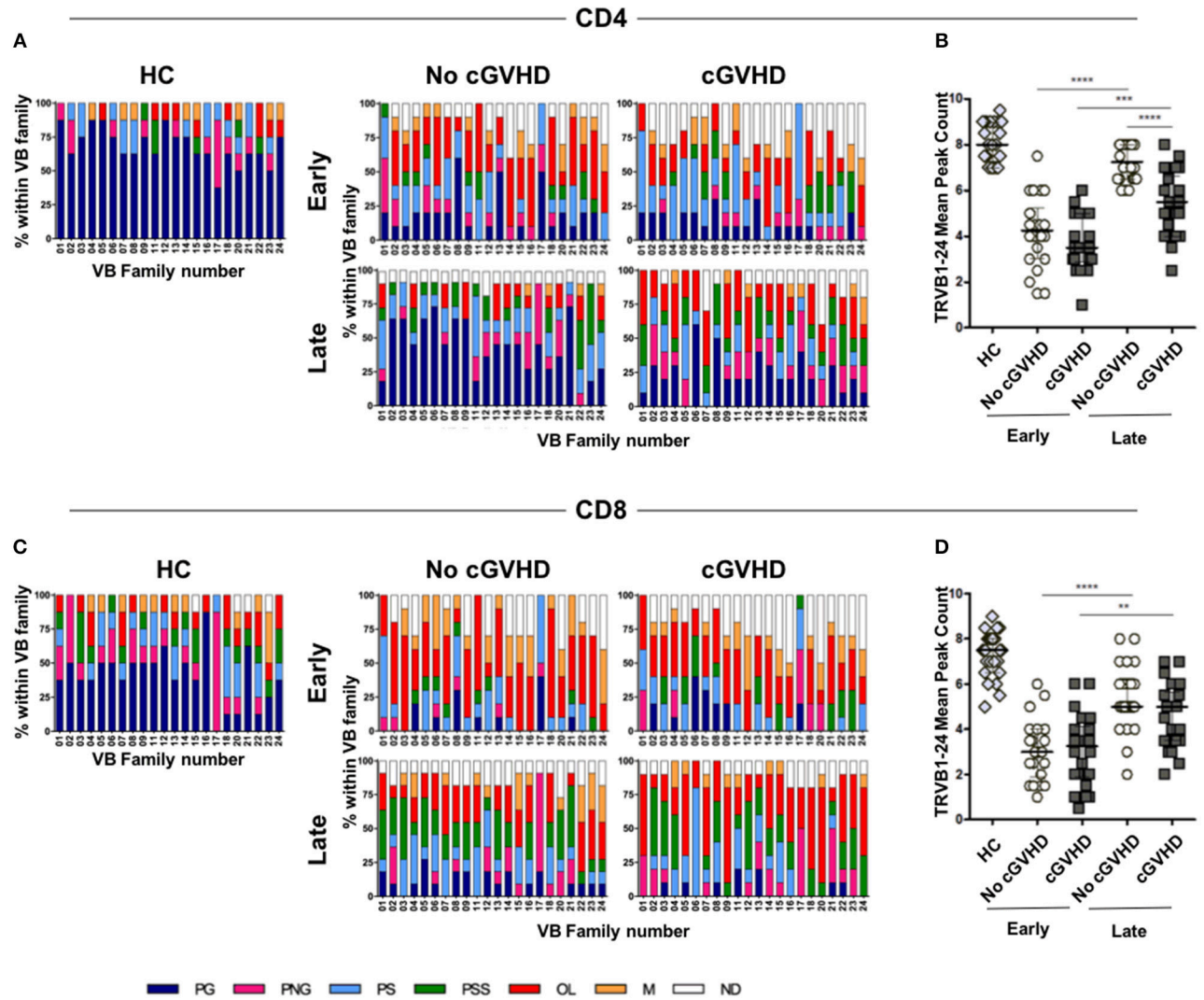

FIGURE 6 | TCR repertoire analysis in HSCT patients and controls. TCRVB diversity analysis was performed on purified CD4 and CD8 T cells from healthy controls (HC) and patients with (cGVHD) and without cGVHD (No cGVHD). Data shown represent values obtained from patient samples early (month 3) and late (months 9 or 12) after HSCT. Clonality analysis of TRB locus transcripts is displayed as profile frequencies of CDR3 spectratypes for TRVB1 to TRVB24 gene families. Profiles were classified on the basis of peak number, distribution shape and relative fluorescence intensity (RFI) as: polyclonal Gaussian (PG, ≥8 peaks with a Gaussian distribution), polyclonal non-Gaussian (PNG, ≥8 peaks with an uneven distribution), polyclonal skewed (PS, 6 peaks or 1 peak with RFI>40%), polyclonal severely skewed (PSS, 1 or 2 peaks with RFI>70%), oligoclonal (O, 2 to 3 peaks), monoclonal (M, 1 peak or a peak with RFI>90%), and not detected (ND). Color stacked columns represent the mean frequency for each of the clonality profiles in CD4 **(A)** and CD8 **(C)** in HC, as well as in patients with and without cGVHD early and late after HSCT. TRVB1-24 CDR3 median peak count in CD4 **(B)** and CD8 **(D)** T cells for HC and patient groups. Asterisks denote statistically significant differences between patient groups (**$p = 0.001$ to 0.01; ***$p = 0.0001$ to 0.001;****$p < 0.0001$).

The addition of aGVHD as a variable to the LME analysis of RTE Treg and Tcon showed that aGVHD was associated with increased RTE Tcon counts and percentages during the first 6 months post-HSCT ($p < 0.01$). No effect was observed at later time-points. Accordingly, when the No cGVHD patient group was split into No GVHD and aGVHD only as described earlier, RTE Tcon were significantly decreased in No GVHD vs. both GVHD patient groups at months 1, 2, and 3 (**Figure 7G**). After month 6, RTE Tcon counts increased in No GVHD and Ac GVHD, while Ac & Ch GVHD patients showed significantly reduced RTE Tcon numbers, suggesting that cGVHD may impact on RTE Tcon reconstitution.

DISCUSSION

Chronic GVHD remains a major hurdle in allo-HSCT. The significant role played by T lymphocytes in cGVHD pathophysiology has been highlighted in studies that identify graft T cells as major mediators in GVHD development, with Naive T cells playing a central role (27). This has led to

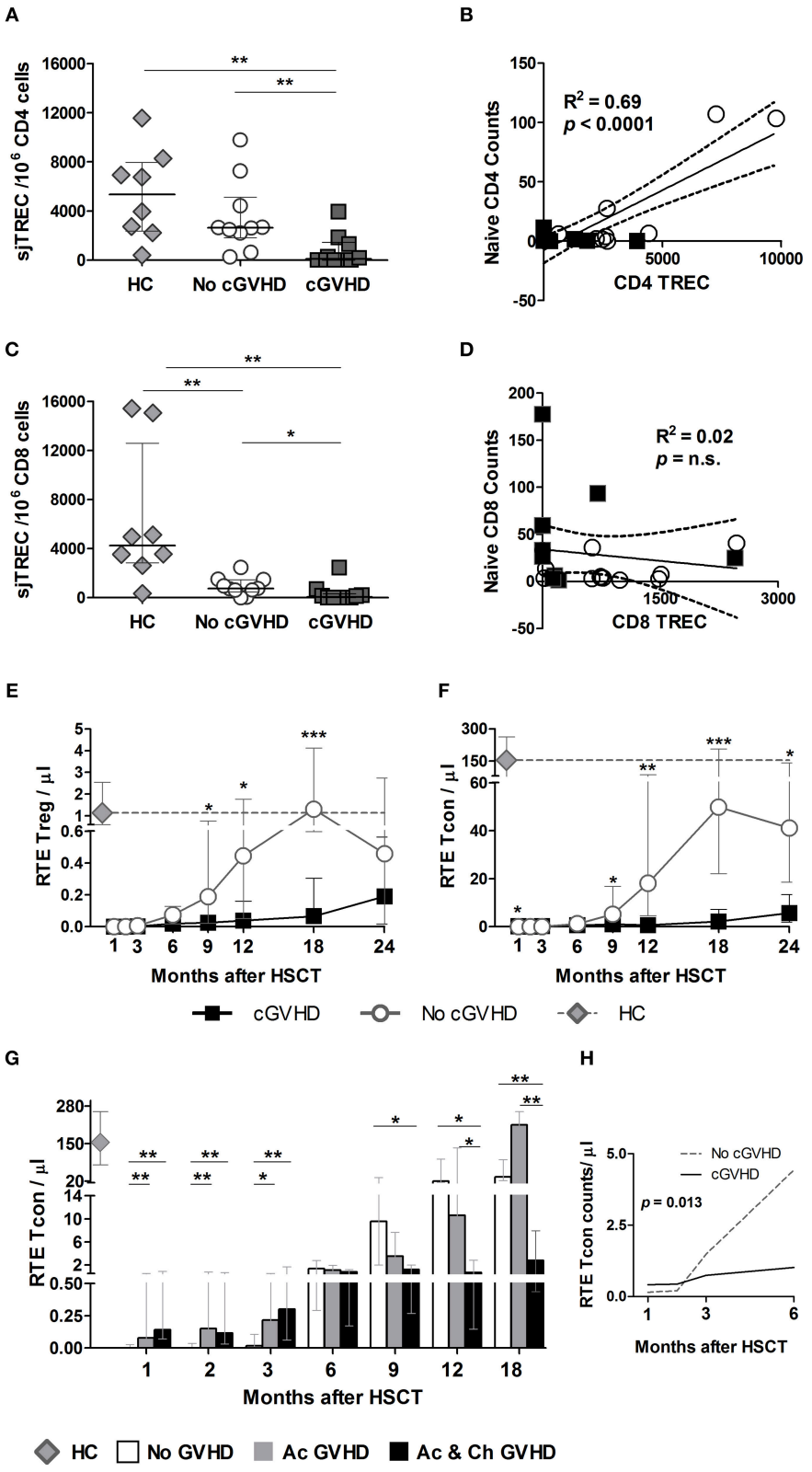

FIGURE 7 | sjTREC content and RTE analysis post-HSCT. sjTRECs were quantified in purified CD4 **(A)** and CD8 **(C)** T cells at months 9 and 12 post-HSCT. Median and IQR values for sjTREC content are shown per 10^6 CD8 or CD4 T cells in healthy controls (HC, gray diamonds), patients without cGVHD (No cGVHD, white

(Continued)

FIGURE 7 | circles), and patients with (cGVHD, dark gray squares). Linear regression analysis between Naive CD4 **(B)** or CD8 **(D)** absolute counts and sjTREC content where black squares represent cGVHD and while circles No cGVHD patients. Absolute counts of RTE Treg **(E)** and RTE Tcon **(F)** in patients with (black lines and squares) and without (gray lines and circles) cGVHD, as well as in HC (gray diamonds and gray dotted lines). **(G)** RTE Tcon counts in patients without GVHD (No GVHD, white bars), with acute GVHD only (Ac GVHD, gray bars), with acute and chronic GVHD (Ac & Ch GVHD, black bars), and in HC (gray diamond). **(H)** RTE Tcon counts on the first 6 months after HSCT in patients with (cGVHD, black line) and without (No cGVHD, dotted gray line) cGVHD, illustrating the results of the LME for these subsets. Median and IQR values are shown. Asterisks denote statistically significant differences between patient groups (*$p = 0.01$ to 0.05; **$p = 0.001$ to 0.01; ***$p = 0.0001$ to 0.001).

the development of T cell depletion strategies that, despite reducing GVHD incidence, are associated with delayed immune reconstitution and increased relapse (28). In order to further study the role played by naive and memory T cell subsets in cGVHD in a setting of partial *in vivo* T cell depletion, we studied a homogenous cohort of patients undergoing unrelated donor allo-HSCT up to 24 months after transplant. This patient cohort was transplanted at a single institution, received the same ATG-containing RIC and GVHD prophylaxis regimens. Hence, despite the negative effects of some immunosuppressive drugs such as CsA, which negatively impacts on Treg function (29), all patients were under the same regimen during the initial post-transplantation period. After 6–9 Months post-HSCT, CsA was discontinued in patients who did not present cGVHD and therefore findings at these later time points may relate to both cGVHD development and/or the immunosuppressive regimen. In this setting, we observed 45% cGVHD incidence (27.5% moderate and severe), likely associated to the fact that 13 out of the 18 patients received PBSC as the graft source and that the majority of these patients had some degree of HLA mismatch with their donors.

While most reports focus on the effects of ATG on cGVHD incidence and immune reconstitution, we investigated the association between naive and memory T cell homeostasis and cGVHD after an ATG-containing conditioning regimen, which is understudied. All our patients received thymoglobulin in the conditioning regimen, which is one of the available rabbit ATGs. Since all of these lymphocyte depleting products are different, it is possible that immune reconstitution in patients receiving another ATG could be somewhat different.

We performed a phenotypic study of T cell reconstitution evaluating Naive, CM, EM, and EMRA reconstitution at fixed time-points after HSCT. We further extend previous studies looking at T cell subsets in cGVHD by investigating the reconstitution of a recently described T cell subset with unique characteristics. SCM have been described as a subset of memory T cells with a Naive-like phenotype, that includes CD45RA, CD62L, and CCR7, that can be distinguished from Naive T cells by the expression of CD95 (30). SCM T cells originate from *in vivo* priming of Naive T cells and possess stem-cell-like properties, being able to generate other memory subsets (25, 30, 31), and are therefore thought to play a role in the maintenance of long term memory. In addition, increased SCM have been associated to autoimmune conditions (32, 33). In order to study the mechanisms involved in T cell reconstitution, we further evaluated proliferation through Ki-67 expression, susceptibility to apoptosis through Bcl-2 and CD95 levels, and evaluated thymic production of CD4 T cells through the identification of CD31-expressing RTE. At selected time-points, we performed TCR diversity and TREC content analysis. Despite the inherent variability of studies performed in human subjects with a distinct genetic makeup, we report interesting observations that shed some light into the biology of cGVHD in humans.

We observed altered Treg homeostasis in patients developing cGVHD. This is consistent with previous reports from our group and others, in the setting of multiple conditioning and GVHD prophylaxis regimens, showing impaired Treg reconstitution in cGVHD (34–37). Reduced Treg in cGVHD was not associated with altered expression of the apoptosis-related proteins Bcl-2 and CD95, but correlated with decreased proliferation, possibly associated with immunosuppressive therapies. Importantly, we demonstrate a clear association between cGVHD development and severely impaired Naive and SCM Treg reconstitution in this setting. Decreased Naive Treg may result from reduced thymic output following GVHD-induced thymic damage. Indeed, we found significantly decreased levels of the RTE-enriched $CD45RA^{+}CD62L^{+}CD31^{+}$ subset, a population that has been used as an indicator of thymic production, within Treg in cGVHD (26, 38). Decreased SCM Treg may result from decreased Naive Treg and/or differentiation into memory phenotypes.

Overall, our data on Treg subset reconstitution support the hypothesis that imbalances in T cell tolerance play an important role in the biology of cGVHD and reinforces the potential benefits of Treg-restoring therapies, particularly of Naive Treg as this subset is particularly depleted in cGVHD.

In line with the previously reported negative impact of ATG on CD4 T cell reconstitution (39–41), Tcon recovery was impaired in both patient groups. However, we did not find significant differences in total Tcon numbers when comparing patients with and without cGVHD. Nevertheless, we report significantly distinct Tcon subset composition when comparing both patient groups. Notably, Naive and SCM Tcon recovery were impaired in patients developing cGVHD. This was accompanied by a tendency for increased levels of the more differentiated subsets EM and EMRA in patients with cGVHD

T cell reconstitution is thought to occur through peripheral expansion and *de novo* thymic production (42, 43). In order to investigate the pathways leading to impaired Naive Tcon reconstitution, we measured *ex vivo* proliferation throughout the follow-up, using Ki-67 as a proliferation marker. We observed a tendency for reduced Ki-67 expression within total Tcon, as well as in Tcon subsets (data not shown), in patients developing cGVHD. Susceptibility to apoptosis, as assessed by the quantification of Bcl-2 and CD95 expression levels, was similar between patient groups, pointing to decreased

homeostatic proliferation as a contributing factor to impaired Naive Tcon reconstitution.

In order to have a measure for *de novo* thymic production, we quantified sjTREC in total CD4 T cells. TRECs are episomal DNA sequences formed during thymic T-cell development by TCR V-J gene rearrangements that correlate with thymopoiesis, particularly in the absence of extensive proliferation (44). sjTRECs were significantly reduced in CD4 T cells from cGVHD *versus* No cGVHD patients. Interestingly, CD4 sjTREC correlated with Naive CD4 numbers, suggesting that, in the absence of cGVHD, the Naive Treg and Tcon recovery is likely to result from *de novo* thymic production occurring after HSCT (45). In order to further detail the contribution of thymic output to CD4 T cell recovery post-transplant, we quantified RTE Tcon. We show that from month 9 onwards, RTE Tcon increased in the absence of cGVHD while patients who develop cGVHD show a severely impaired RTE reconstitution. It is therefore likely that a combination of decreased proliferation and impaired thymic output leads to impaired Naive Tcon and Treg reconstitution in cGVHD. In addition, CD4 TCRVB diversity was significantly reduced in patients, more so in cGVHD, further pointing to impaired thymic production in patients developing cGVHD.

When accessing the role played by acute GVHD in RTE Tcon recovery after HSCT, we found increased RTE Tcon at months 1–3, both in Acute GVHD and in Acute and Chronic GVHD patients, as compared to No GVHD. This suggests a potential deleterious role of RTE Tcon early after HSCT. It is unclear if these cells are of thymic origin or result from cytokine-driven proliferation of infused RTE (46). We speculate that RTE Tcon detected early after HSCT may contain self-reactive clones, while RTE Tcon present at later time-points in No cGVHD result from adequate thymic selection processes.

Overall, we observed impaired Naive and SCM Tcon reconstitution in cGVHD, associated with reduced peripheral expansion. In addition, decreased TREC content and RTE Tcon recovery further suggest that cGVHD results from alterations in *de novo* thymic production. Intriguingly, we found that cGVHD development was associated to increased RTE Tcon early after HSCT through yet undescribed mechanisms.

It is noteworthy that we report a distinct picture emerging for CD8 T cells. EM, EMRA and CM were the most abundant CD8 subsets in the post-transplantation period. Interestingly, cGVHD associated with increased Naive, SCM and EMRA as compared to patients who did not develop cGVHD.

Stem Cell Memory (SCM) have been reported to differentiate from Naive, to be increased in autoimmune disease (32, 33) and to induce lethal GVHD in mouse studies (47). Our findings of increased Naive and SCM CD8 in cGVHD vs. No cGVHD raise the possibility that Naive CD8 T cells may differentiate into SCM and be involved in GVHD development by yet undescribed mechanisms. Interestingly, when the effects of previous aGVHD were taken into consideration in our statistical analysis, we observed that during the first 6 months after transplant SCM Tcon and CD8 were significantly affected by aGVHD. The stratification of patient groups into No GVHD, Acute GVHD only and Acute & Chronic GVHD confirmed that during these initial stages after HCST patients developing GVHD show increased numbers of these cells. A sustained increase in this population in patients developing cGVHD was observed for CD8 but not Tcon, further suggesting a potential role for CD8 SCM is cGVHD development.

Other studies have shown that the thymus is a direct target of GVHD (7) resulting on the emergence of self-reactive clones and the autoimmunity associated to cGVHD (48, 49). We observed a high prevalence of skewed and oligoclonal TCR repertoires in CD8 from both patient groups. Interestingly, the significantly reduced CD8 sjTREC content in cGVHD vs. No cGVHD patients suggests reduced thymic output and/or a high level of homeostatic proliferation observed in these patients.

Our data raises the possibility that increased Naive and SCM CD8 present from early time-points after transplant in cGVHD patients, may originate from defective negative selection mechanisms resulting from thymic tissue damage, leading to the output of self-reactive CD8 clones that may further differentiate and mediate disease. Given the increased levels of homeostatic T cell proliferation in the post-transplantation period, we cannot exclude the possible contribution of cell proliferation to the observed increase in Naive and SCM CD8.

We therefore extend on the observations by Alho et al. (34) that reported increased Naive T cell subsets in patients developing cGVHD, by comparing patients with and without cGVHD at month 3 after HSCT (34). We now show that this occurs not only within Naive CD8 but also in the SCM CD8 T cell subset, by detailing increases in these cell populations in patients who develop cGVHD in a cohort of patients undergoing unrelated HSCT after an ATG-containing conditioning regimen. We further show that this occurs in CD8 T cells in a sustained manner from month 1 to 9 in both percentages and absolute counts. In addition, by stratifying our patient cohort with regards to previous history of acute GVHD, we observed that increased SCM in Tcon and CD8 occurs early after HSCT in patients developing aGVHD. Of note, we did not observe any statistically significant impact of adding aGVHD as a co-variate within the Treg subsets.

In summary, we show that after an ATG-containing RIC regimen, cGVHD development after allo-HSCT is associated with reduced Treg recovery, particularly of Naive and SCM subsets. We speculate that this is likely due to reduced thymic production within the CD4 T cell compartment, where significantly decreased TCRVB diversity is observed in patients developing cGVHD. We also show that cGVHD development is associated to increases in Naive, SCM and EMRA CD8 T cells. This becomes apparent early after HSCT and persists throughout our 24-month follow-up, suggesting a potential involvement of these cells in the development of cGVHD.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Ethics Committee of Lisbon Academic Medical Center with written informed consent from all subjects. All subject gave written informed consent in

accordance with the Declaration of Helsinki. The protocol was approved by Ethics Committee of Lisbon Academic Medical Center (ref. 459/13).

AUTHOR CONTRIBUTIONS

MS analyzed data and wrote the paper. RA designed experiments, performed experiments, analyzed data, and wrote paper. IF, SB, and AR performed experiments. AV performed cell sorting and flow cytometry support. PP and NC collected clinical data. DL performed the spectratyping and TREC analysis. RR performed statistical data analysis. AA and AS analyzed data. CM, FL, and RM recruited patients. JR and JL conceived and designed the study. JL recruited patients, designed experiments, and wrote the paper.

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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.2019.00334/full#supplementary-material

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Shaping of CD56^{bri} Natural Killer Cells in Patients With Steroid-Refractory/Resistant Acute Graft-vs.-Host Disease via Extracorporeal Photopheresis

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CD56^{bri} natural killer (NK) cells play an important role in the pathogenesis of graft-vs. -host disease (GVHD) and immune defense in the early period after allogeneic hematopoietic stem cell transplantation. Extracorporeal photopheresis (ECP) as an immunomodulating therapy has been widely used for GVHD treatment. However, the mechanism of action of ECP still remains to be elucidated, particularly the influence of ECP on NK cells. Thirty-four patients with steroid-refractory/resistant acute GVHD (aGVHD) $\geq$ °II and moderate to severe chronic GVHD (cGVHD) received ECP therapy. Patient samples obtained during intensive and long-term treatment were analyzed. Immunomonitoring with respect to cell phenotype and function was performed on rested peripheral blood mononuclear cells (PBMCs) using multiparametric flow cytometry. NK activity in terms of cytokine release was analyzed by intracellular cytokine staining after co-culture with K562 cells. Moreover, the proliferative capacity of NK cells, CD4$^+$, and CD8$^+$ T cells was determined by carboxyfluorescein succinimidyl ester (CFSE) staining. Clinically, 75% of aGVHD and 78% of cGVHD patients responded to ECP therapy. Moreover, our data show that aGVHD, cGVHD patients and healthy donors (HDs) present distinct NK patterns: aGVHD patients have a higher frequency of CD56^{bri} NK subsets with stronger NKG2D and CD62L expression, while CD56$^-$CD16$^+$ NK cells with higher expression of CD57 and CD11b stand out as a signature population for cGVHD. ECP therapy could significantly decrease CD56^{bri}CD16$^-$ NK cells with shifting the quality from a cytotoxic to a regulatory pattern and additionally mature CD56^{dim} NK cells via upregulation of CD57 in complete responding aGVHD patients. Moreover, ECP could keep the anti-viral and anti-leukemic effects intact via maintaining specialized

anti-viral/leukemic CD57^{+}NKG2C^{+}CD56^{dim} NK cells as well as remaining the quality and quantity of cytokine release by NK cells. The proliferative capacity of effector cells remained constant over ECP therapy. In conclusion, ECP represents an attractive option to treat GVHD without compromising anti-viral/leukemic effects. Shaping of CD56^{bri} NK cell compartment by downregulating the cytotoxic subset while upregulating the regulatory subset contributes to the mechanisms of ECP therapy in aGVHD.

Keywords: GvHD, ECP, immunomodulation, natural killer cells, anti-viral effect, anti-leukemia effect

INTRODUCTION

Extracorporeal photopheresis (ECP) is being widely used for the treatment of T cell-mediated diseases e.g., graft-vs.-host disease (GVHD) with established clinical benefits (1). ECP therapy can rebalance the destroyed immune system in the case of GVHD by (a) direct induction of alloreactive T cell apoptosis, (b) downregulation of proinflammatory cytokines, (c) selective modulation of trafficking patterns of alloreactive T cells, and (d) increase of different regulatory cells such as CD4^{+} and CD8^{+} regulatory T cells, regulatory B cells, and myeloid-derived suppressor cells (2–5). Nevertheless, the mechanism of action of ECP still needs to be further elucidated, particularly with regard to natural killer (NK) cells.

CD56^{bri} NK cells are important innate immune cells that are the first lymphocyte subset which reconstitutes after allogeneic hematopoietic stem cell transplantation (allo-HSCT) and as such provides a temporal bridge of protection from opportunistic infections and prevention of cancer relapse during the transient period of T-cell deficiency post-transplantation (6–8). Therefore, the early rapid reconstitution of CD56^{bri} NK cells is of crucial importance for the post-transplantation outcomes. This has been reported by several studies documenting CD56^{bri} NK cells in correlation to a better survival and less transplantation-related mortality (9–12). In addition, clinical data illustrated that a low frequency of CD56^{bri} NK cells is associated with the development of GVHD (12, 13). This theory was further confirmed by a recent study showing that ECP could reduce GVHD by upregulating CD56^{bri} NK cells (14). However, since CD56^{bri} NK cells are the most efficient cytokine producers (15), theoretically they can also contribute to the induction and exacerbation of GVHD through releasing of proinflammatory cytokines like interferon-γ (IFN-γ) and tumor necrosis factor (TNF-α) (16–18). The question arises therefore how and to which extent ECP induced CD56^{bri} NK cells might contribute to the control of GVHD and this is the aim of our current study.

SUBJECTS, MATERIALS, AND METHODS

Subjects

Thirty-four patients suffering from steroid-refractory/resistant acute GVHD (aGVHD) ≥ °II and moderate to severe chronic GVHD (cGVHD) from the University Hospitals Heidelberg and Greifswald in Germany as well as Chaim Sheba Medical Center in Israel were included. The study was approved by the Institutional Review Boards. Written informed consent was obtained from all patients.

ECP Treatment

ECP therapy was performed with a Therakos UVAR XTS® and a CELLEX® Photopheresis System involving *ex vivo* exposure of leukapheresed peripheral blood mononuclear cells (PBMCs) to ultraviolet-A light in the presence of 8-methoxypsoralen (8-MOP) and reinfusion of the treated cells to patients. aGVHD patients received intensive semiweekly treatment in the first 12 weeks, followed by biweekly treatment. cGVHD patients received either semiweekly treatment followed by a biweekly treatment or a biweekly treatment *upfront*. ECP therapy was administered until reaching the best response.

Clinical Evaluation

Clinical assessment of aGVHD and cGVHD was undertaken according to the current guidelines (19–21). Response to ECP treatment was defined as complete response (CR), partial response (PR), stable disease (SD), and no response (NR). CR was defined as the resolution of all reversible manifestations. PR was defined as the improvement of clinical symptoms of the involved organ concomitant with a reduction of steroid dose. SD was defined as the reduction of steroid dose with slight improvement of clinical symptoms. NR was defined as the absence of improvement of clinical symptoms.

Cell Preparation

Peripheral blood was drawn from the patients before the ECP treatment. Buffy coats from consenting healthy donors (HDs) were obtained from the Heidelberg blood bank after overnight storage. PBMCs were isolated by gradient centrifugation followed by washing twice with phosphate buffered saline (PBS) (Sigma). Afterwards, cells were stored in liquid nitrogen till immunomonitoring. A standard resting process was performed to restore the function and the antigenic expression of cells as previously described (22). Briefly, cells were resuspended at 2 × 10^6 cells/ml with complete medium [CM: RPMI medium 1640 (Gibco) containing 10% fetal bovine serum (FBS) (Sigma)] after thawing. Afterwards cells were rested at 37°C, 5% CO_2 for 18 hours in a horizontal position.

Cell Line

K562, a highly undifferentiated human erythroleukemic cell line, was maintained in CM at 37°C, 5% CO_2. Medium was changed

every 3 days to passage cells. Mycoplasma contamination was checked by polymerase chain reaction (PCR) before each experiment.

Stimulation of NK Cells

5×10^5 rested PBMCs were co-cultured with 5×10^4 K562 cells in the presence of CD107a antibody at 37°C, 5% CO_2 in a 96-U-bottom plate for 6 hours. After the first hour incubation, 1 μl of 100X monensin (Biolegend) and Brefeldin A (Biolegend) were added into each well. PBMCs alone group was served as negative control.

Multiparameter Flow Cytometry

The quality and quantity of expression of different markers were determined by multiparametric flow cytometry. Samples were stained by different combinations of antibodies against CD3, CD4, CD8, CD11b, CD14, CD16, CD19, CD27, CD56, CD57, CD62L, CD107a, CD159c (NKG2C), CD314 (NKG2D), IFN-γ, and TNF-α. Detailed information of antibody is shown in **Supplementary Table 1**. 7-Amino actinomycin D (7AAD) or Near-infrared (NEAR-IR) was used for live/dead cell discrimination. Fluorescence minus one, unstimulated, and autofluorescence controls were included in order to place the gate more accurately. To reduce the variation, samples from the same patient at different time points have been analyzed on the same day. The Fc receptors were blocked by blocking buffer A [50% fluorescence-activated cell sorting buffer (FACS) + 50% human serum] or blocking buffer B (50% perm buffer + 50% human serum) prior to surface marker staining or intracellular cytokine staining, respectively. All acquisitions were performed on a LSRII device (BD Biosciences) and the data were analyzed by FACS Diva software (BD Biosciences). The cellular division index was determined by Flowjo software (TreeStar).

Surface Marker Staining

After 10 min blocking at 4°C, 5×10^5 rested PBMCs were stained with different antibodies for 20 min at 4°C in the dark.

Intracellular Cytokine Staining

Briefly, 5×10^5 rested cells were stained with NEAR-IR for 30 min at 4°C in the dark. Thereafter, cells were stained with surface marker antibodies, followed by fixation and permeabilization according to the Miltenyi Foxp3 fix/perm buffer instruction. A 15 min blocking step was performed prior to the intracellular antibody staining [30 min, room temperature (RT)].

Assessment of Proliferation Function

Freshly thawed PBMCs were washed and resuspended with 1 ml PBS containing 5% FBS in 15 ml tubes. 1 μl of 5 mM carboxyfluorescein succinimidyl ester (CFSE) solution (Biolegend) was directly added into the cell suspension followed by 5 min incubation at RT in the dark. The reaction was stopped by adding 5 ml cold CM and cells were washed twice with 5 ml CM. After staining, 2×10^5 CFSE-labeled cells were seeded into each well and stimulated with either 100 ng/ml interleukin-15 (IL-15) (R&D systems) or 1 μg/ml staphylococcal enterotoxin B (SEB) (Sigma) for NK cells and T cells, respectively. Cell proliferation was analyzed after 3 and 7 days culture for NK cells and T cells, respectively.

Statistical Analysis

Analysis was undertaken using SPSS version 24 (IBM) for windows software. One-way ANOVA with Bonferroni *post-hoc* test was performed to assess the differences of the marker expression and the cytokine release pattern among HDs, patients with aGVHD and cGVHD within the five different NK subsets. Differences between two different time points and two different groups were determined by Wilcoxon signed-rank test and Mann-Whitney U test, respectively. A p-value < 0.05 was considered to be statistically significant.

RESULTS

Demographics and Clinical Response

Patient characteristics and clinical response to ECP therapy are summarized in **Tables 1**, **2**. Sixteen patients with steroid-refectory/resistant aGVHD (6 men and 10 women aged 23–68 years) and 18 patients with cGVHD (11 men and 7 women aged 32–70 years) were treated by ECP. The median time from GVHD onset to commencing ECP was 33.5 days for aGVHD (range: 7–373 days) and 363.5 days for cGVHD (range: 14–4240 days). The median number of ECP cycles was 11 (range: 5–34 cycles) and 37 (range: 4–90 cycles) for aGVHD and cGVHD patients, respectively. 75% (12/16) of the patients with aGVHD and 78% (14/18) of those with cGVHD responded to the ECP treatment. Only two patients with aGVHD had cytomegalovirus (CMV) reactivation after the first 2–3 ECP treatment while still on 2 mg/kg per body weight steroids. As control, 10 healthy donors (5 men and 5 women aged 20–66 years) have been analyzed in our study. Three of them were tested positive for CMV.

Distinct NK Cells Pattern

Based on CD56 and CD16 expression, we could define five different NK cell subsets, as shown in **Figure 1A**. Of note, HDs, patients with aGVHD and cGVHD displayed distinct patterns of these five different subsets. **Figure 1B** depicts representative dot plots among HDs, aGVHD, and cGVHD patients. Patients suffering from steroid-refractory/resistant aGVHD were characterized by a higher frequency of CD56^{bri} NK cells when compared with HDs and patients with cGVHD (**Figure 1C**). Moreover, this signature population of aGVHD, CD56^{bri}CD16$^-$ NK cells, can be significantly downregulated by ECP treatment in patients achieving complete response (**Figure 1D**) but not in patients with PR and NR (**Supplementary Figure 1**). In contrast, CD56^{dim}CD16$^+$ and CD56$^-$CD16$^+$ NK cell subsets were prominent in patients with cGVHD (**Figure 1C**). Especially, a significant increase of CD56$^-$CD16$^+$ NK cells was observed in the cGVHD cohort.

To further characterize these different NK cell subsets, the expression of cell surface markers and cytokine profile upon K562 stimulation were examined (**Figure 2**). In patients with aGVHD at baseline pre-ECP treatment, we observed a decreased

TABLE 1 | aGVHD patients' characteristics and clinical response to ECP.

# Pat.	Primary disease	Type of transplantation	Stem cell source	CMV status D/R	Prophylaxis for GVHD	aGVHD grade	Organ involved	CMV reactivation	ECP response
1	CLL	MUD	PBSC	–/–	ATG+MMF+TAC	°III	gut	–	ST
2	FL	MUD	PBSC	–/–	ATG+MMF+TAC	°III-IV	gut	–	CR
3	AML	MMUD	PBSC	–/–	ATG+MMF+TAC	°III	gut	–	PR
4	AML	Haplo	PBSC	–/+	MMF+TAC	°III	gut	–	ST
5	CLL	MUD	PBSC	+/+	ATG+MMF+TAC	°III	gut	–	NR
6	TPLL	MMUD	PBSC	–/–	ATG+MMF+TAC	°III	gut	–	NR
7	CML	MMUD	PBSC	–/–	ATG+CsA+MMF	°II	gut	–	CR
8	AML	MUD	PBSC	–/–	ATG+MMF+TAC	°III	gut	–	CR
9	AML	MRD	PBSC	+/+	CsA+MMF	°II	gut	–	PR
10	CLL	MUD	PBSC	–/–	ATG+MMF	°III	gut	–	PR
11	FL	MRD	PBSC	–/+	CsA+MMF	°IV	gut	+*	PR
12	MDS	MRD	PBSC	+/+	MMF+TAC	°III/°IV	gut, skin	+*	PR
13	AML	MUD	PBSC	–/–	ATG+CsA+MMF	°III	gut	–	PR
14	AML	MRD	PBSC	+/+	CsA+MMF	°III	gut	–	PR
15	CMML	MRD	PBSC	+/+	CsA+MMF	°III /°IV	gut	–	CR
16	CTCL	MUD	PBSC	+/+	ATG+TAC	°III	gut	–	PR

*aGVHD, acute GVHD; ECP, Extracorporeal photopheresis; # Pat., Patient number; CLL, Chronic Lymphocytic Leukemia; FL, Folicular Lymphoma; AML, Acute Myeloid Leukemia; TPLL, T cell Prolymphocytic Leukemia; CML, Chronic Myelogenous Leukemia; MDS, Myelodysplastic Syndromes; CMML, Chronic Myelomonocytic Leukemia; CTCL, Cutaneous T cell Lymphoma; MUD, Matched unrelated donor; MMUD, Mismatched unrelated donor; Haplo, Haploidentical Stem Cell Transplantation; MRD, matched related donor; PBSC, Peripheral blood stem cell; CMV, Cytomegalovirus; D, Donor; R, Recipient; –, negative; +, positive; ATG, Anti-thymocyte globulin; MMF, Mycophenolate mofetil; TAC, Tacrolimus; CsA, Cyclosporine A; ST, Short treatment; CR, Complete response; PR, Partial response; NR, No response; * means after the first 2–3 ECP treatment still under 2 mg/kg body weight steroids.*

expression of the maturation markers CD57 and CD11b on NK cell subsets (**Figure 2A**). By contrast, significantly higher expression of these maturation markers was detected on NK cell subpopulations in patients with cGVHD when compared to HDs and patients with aGVHD (**Figure 2A**). Furthermore, we observed a significant elevation of the NK activation marker NKG2D on NK cells in patients with aGVHD. In addition, the immature markers CD27 and CD62L as well as the CMV specific activating receptor NKG2C display a similar expression on these five different NK subsets among the HDs, aGVHD and cGVHD groups with exception of CD56^{bri}CD16^{+} NK cells that showed a high expression of CD62L in aGVHD patients.

Besides surface marker expression, the anti-tumor function of the NK subsets upon K562 stimulation was evaluated (**Figure 2B**). CD56^{dim}CD16^{-} subset showed the highest level of CD107a expression as well as the biggest amount of TNF-α and IFN-γ secretion compared to other subpopulations, suggesting their crucial role in the anti-tumor capacity of the NK cells. Interestingly, although the NK cells had less potency to secrete cytokines in aGVHD patients, a stronger CD107a expression on CD56^{bri}CD16^{-} NK cells could be induced by co-culturing with K562 cells (**Figure 2B**).

Maturation of NK Cells by ECP Therapy

After ECP treatment, the quality (**Figure 3A**) and quantity (**Figure 3B**) of the maturation marker CD57 were upregulated in both CD56^{dim}CD16^{-} and CD56^{dim}CD16^{+} subsets in patients with aGVHD achieving CR as compared to patients with PR, NR, and ST, which suggests that ECP can promote the maturation of CD56^{dim}CD16^{-} and CD56^{dim}CD16^{+} NK cells in patients with aGVHD with favorable outcome.

Shifting the Quality of NK Cells From Cytotoxicity to Regulation by ECP Treatment

Functional NK cell populations, cytotoxic, regulatory, and tolerant NK cells, were defined in our study based on the relative expression of CD27 and CD11b, as shown in a previous study (23). The components of regulatory NK cells (CD27^{+}CD11b$^{+/-}$) and tolerant NK cells (CD27^{-}CD11b^{-}) were significantly increased in patients with GVHD compared to HDs (**Figure 4A**). However, the distribution of these three functional NK subsets within these five different NK subcategories was similar among HDs, aGVHD, and cGVHD patients (**Figures 4B–D**) with exception of an increased tolerant NK cell population in the aGVHD group (**Figure 4D**).

To investigate whether the functional NK cell subsets are influenced by ECP treatment, a comprehensive analysis was performed. Our results show that a significant decrease of cytotoxic CD27^{-}CD11b^{+} NK cells was observed in aGVHD patients after ECP therapy (**Supplementary Figure 2A**), caused by the dramatic reduction of cytotoxic NK cells within the CD56^{bri} NK cell populations (**Supplementary Figure 2B**, **Figures 5A,B**). Furthermore, we observed a significant downregulation only in aGVHD responders while not in non-responders (**Figure 5B**). This confirms that this general reduction

TABLE 2 | cGVHD patients' characteristics and clinical response to ECP.

# Pat.	Primary disease	Type of transplantation	Stem cell source	CMV status D/R	Prophylaxis for GVHD	cGVHD grade	Organ involved	CMV reactivation	ECP response
17	LL	MRD	PBSC	+/–	CsA+MTX	moderate	skin	–	PR
18	DLBCL	MMUD	PBSC	–/–	ATG+CsA	severe	lung	–	SD
19	AML	MRD	BM	+/+	CsA	severe	lung	–	PR*
20	AILT	MRD	PBSC	+/–	MMF	moderate	skin	–	SD
21	TPLL	MUD	PBSC	+/+	ATG+TAC	moderate	skin	–	PR
22	AML	MRD	PBSC	+/-	MMF+SRL	severe	skin	–	ST
23	PTCL	MUD	PBSC	+/–	ATG+CsA+MMF	moderate	skin	–	CR
24	CLL	MRD	PBSC	+/+	MMF	moderate	skin	–	PR
25	TPLL	Haplo	BM	–/–	MMF+TAC	severe	skin	–	ST
26	OMF	MUD	PBSC	+/+	MMF+TAC	severe	skin	–	PR
27	CLL	MUD	PBSC	–/–	CsA+MMF	severe	skin	–	PR*
28	ALL	MRD	PBSC	+/+	MMF	severe	skin	–	SD
29	HL	MRD	PBSC	+/+	CsA+MMF	severe	skin	–	PR
30	BAL	MRD	PBSC	–/–	CsA	severe	extent	–	SD
31	AML	MRD	PBSC	+/+	CsA	moderate	skin	–	PR
32	AML	MUD	PBSC	–/–	ATG+CsA+MMF	severe	skin	–	SD
33	MDS	MRD	PBSC	+/+	PDN	severe	skin	–	PR
34	MCL	MRD	PBSC	–/–	CsA+MMF	severe	skin	–	PR

cGVHD, chronic GVHD; ECP, Extracorporeal photopheresis; # Pat., patient number; LL, Lymphoblastic Lymphoma; DLBCL, Diffuse Large B cell Lymphoma; AML, Acute Myeloid Leukemia; AILT, Angioimmunoblastic T cell Lymphoma; TPLL, T Prolymphocytes Leukemia; PTCL, Peripheral T cell Lymphoma; CLL, Chronic Lymphocytic Leukemia; OMF, Osteomyelofibrosis; ALL, Acute Lymphoblastic Leukemia; HL, Hodgkin's lymphoma; BAL, Biphenotypic Acute Leukemia; MDS, Myelodysplastic Syndromes; MCL, Mantle cell Lymphoma; MUD, mismatched unrelated donor; MMUD, matched unrelated donor; MRD, matched related donor; Haplo, Haploidentical Stem Cell Transplantation; PBSC, Peripheral blood stem cell; BM, Bone marrow; CMV, Cytomegalovirus; D, Donor; R, Recipient; –, negative; +, positive; CsA, Cyclosporine A; MTX, Methotrexate; ATG, Anti-thymocyte globulin; MMF, Mycophenolate mofetil; TAC, Tacrolimus; SRL, Sirolimus; PDN, Prednisolon; PR, Partial response; SD, Stable disease; PR, Partial response but progression afterward; ST, Short treatment; NR, No response; CR, Complete response.*

correlates with ECP response. In parallel, regulatory NK cells within CD56^{bri} NK cell subsets were significantly increased by ECP therapy (**Figure 5C**, **Supplementary Figure 2C**). However, there were no significant changes of tolerant NK cells (**Figure 5D**, **Supplementary Figure 2D**). Collectively, our data suggest that ECP therapy could shift the quality of CD56^{bri} NK cells from cytotoxic to regulatory NK cells.

Intact Anti-Viral/Tumor Capacity of NK Cells Under ECP Treatment

To determine the influence of ECP therapy on the anti-viral/tumor capacity of NK cells, a specialized anti-viral/tumor population, CD56^{dim}CD57^{+}NKG2C^{+} NK cells (24), as well as the quality and quantity of NK activity were monitored during ECP treatment. CD56^{dim}CD57^{+}NKG2C^{+} NK cells were identified in our study following the strategy as shown in **Figure 6A**. A stable frequency of CD56^{dim}CD57^{+}NKG2C^{+} NK cells during ECP therapy was observed in both aGVHD (**Figure 6B**) and cGVHD patients (**Figure 6C**).

The mean fluorescence intensity (MFI) reflecting the cytokine release on a per-cell basis has been the subject of this study, with further interest due to the quality of NK cell response. There was no significant alteration of the MFI of CD107a, TNF-α and IFN-γ during ECP therapy in our study (**Figure 7A**). The frequency of CD107a expression and the cytokine release by NK cells upon K562 stimulation *in vitro* were maintained as well (**Figure 7B**). Of note, even though the multifunctional NK cells which are associated with enhanced effector function showed different patterns among HDs, aGVHD and cGVHD patients (**Figure 8A**), the multifunctionality of NK cells was constant during ECP therapy (**Figure 8B**).

Since five different NK cell subsets were defined in our study, we compared the contribution of these five subsets to the anti-tumor function and further assessed whether it would be influenced by ECP therapy. We found a significant improvement of CD107a expression and IFN-γ release by CD56^{bri} NK cells in the aGVHD cohort (**Figure 9A**) as well as secretion of TNF-α by CD56^{bri} NK cells in cGVHD patients (**Figure 9B**). Apparently, our data suggest that ECP could maintain or even improve the functionality of NK cells with respect to the anti-viral/tumor capabilities via preserving the frequency of CD56^{dim}CD57^{+}NKG2C^{+} NK cells and keeping the quality and quantity of the cytokine profile of the NK cells.

Preserving Proliferative Function of NK and T Cells After ECP Treatment

Proliferative capacity as an important cell function was evaluated in our study. Freshly thawed PBMCs stained with CFSE were stimulated either by IL-15 or SEB to determine the proliferative capabilities of the NK cells and T cells, respectively. NK cells (**Figure 10A**), CD4^{+} T cells (**Figure 10B**), and CD8^{+} T cells (**Figure 10C**) from aGVHD patients had greater proliferative capacity than HDs and patients with cGVHD. In contrast, NK cells but not CD4^{+} and CD8^{+} T cells from cGVHD patients showed a lower proliferative potential than HDs (**Figure 10**).

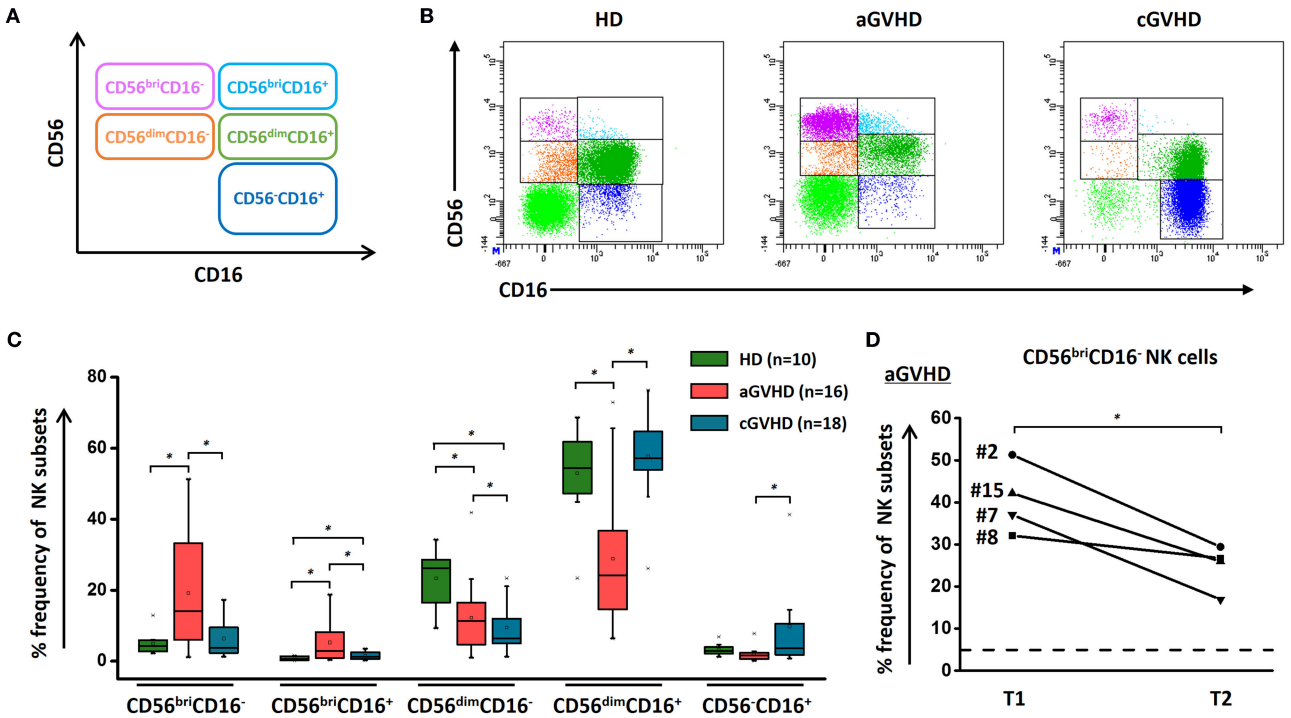

FIGURE 1 | NK subsets in GVHD and effect of ECP on CD56^{bri} NK subset. NK cells could be defined as five different subsets by relative expression of CD56 and CD16 **(A)**. The representative dot plots **(B)** and box plots **(C)** show that the component of NK cells is different among aGVHD patients ($n = 16$), cGVHD patients ($n = 18$), and HDs ($n = 10$). ECP could dramatically decrease the frequency of CD56^{bri}CD16^{-} NK cells in aGVHD patients with CR ($n = 4$) **(D)**. Dashed line represents the corresponding median value of frequencies observed in 10 healthy donors. $^{*}p < 0.05$.

Moreover, ECP therapy did not hamper neither NK cell (**Figure 10A**) nor T cell proliferative capacity (**Figures 10B, C**).

DISCUSSION

In corollary to our previous studies (5, 25), we further focused on NK cells to investigate their role in ECP therapy for GVHD patients. In the current study, we could show that (a) the heterogeneous NK cell population presents distinct patterns among HDs and patients with aGVHD and cGVHD. (b) A higher frequency of CD56^{bri} NK subset with stronger NKG2D and CD62L expression was found in patients with aGVHD when compared to those with cGVHD and HDs. In aGVHD patients achieving CR, ECP therapy could not only (c) decrease significantly CD56^{bri}CD16^{-} NK cells with shifting the property from cytotoxic to regulatory NK subsets but also (d) mature the CD56^{dim} NK cells through up-regulation of CD57. Most important, ECP could keep the intact anti-viral and anti-leukemic effects via (e) maintaining specialized anti-viral/leukemic CD57^{+}NKG2C^{+}CD56^{dim} NK cells as well as (f) preserving the quantity and quality in terms of the MFI of cytokines, polyfunctionality and proliferative capacity of NK cells under ECP treatment.

Immune reconstitution after allo-HSCT is a complex process. The unbalanced or the delayed and incomplete immune reconstitution could result in not only expansion of alloreactive T cells leading to GVHD but also in a higher incidence of opportunistic infections (5, 26). Thus, therapeutic strategies to accelerate immune reconstitution after allo-HSCT might be a reasonable way for both GVHD treatment and prevention of infections.

ECP therapy with promising clinical outcome constitutes an effective immunomodulatory therapy for GVHD. In our series, 75% of aGVHD and 78% of cGVHD patients obtained clinical response. Notably, our previous studies indicated that ECP therapy could support the immune recovery after allo-HSCT (5, 25). Therefore, ECP represents an attractive strategy to treat GVHD.

NK cells are known to be the first and predominant donor-derived lymphocytes to reconstitute after allo-HSCT (6, 12, 24, 27). The role of NK cells in the development and the prevention of GVHD is paradoxical. NK cells might enhance inflammation by secretion of TNF-α and IFN-γ and thus promote GVHD but on the other hand sustain homeostasis through targeted killing of activated alloreactive T cells and antigen presenting cells to control GVHD (28, 29). The heterogeneity within the NK subset might further contribute to these conflicting effects during GVHD. According to the expression of CD56 and CD16, we identified five different NK cell populations with different immunophenotype and function. Moreover, our results show that the composition of NK cells is explicitly divergent among HDs, aGVHD, and cGVHD patients, suggesting that

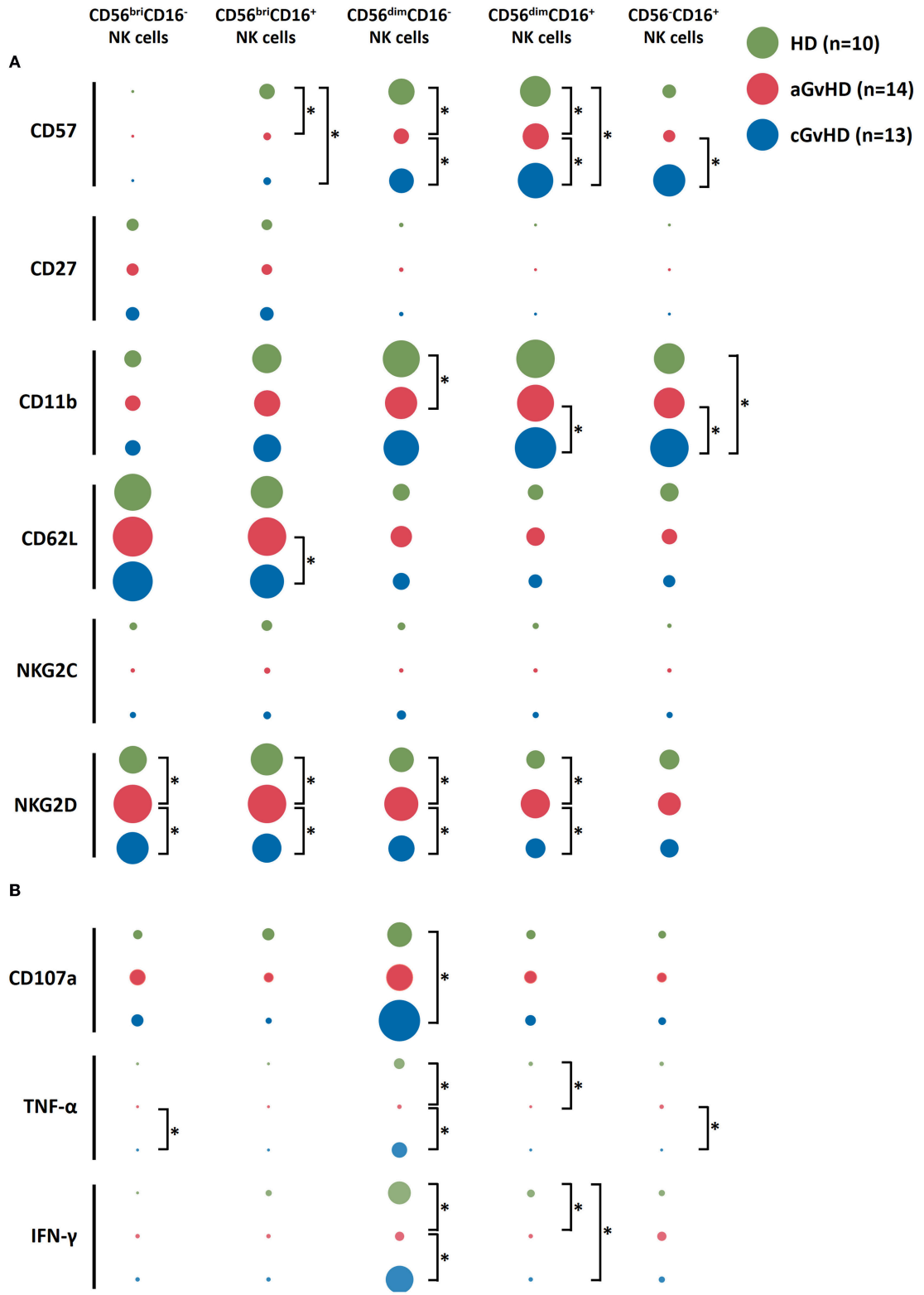

FIGURE 2 | Characterization of NK subsets. NK subsets display not a distinct immunophenotype based on the surface markers expression **(A)** but also a different functional profile upon K562 stimulation **(B)** among aGVHD ($n = 14$, excluding patient #5 and #8 due to the limited cell number of samples), cGVHD ($n = 13$, excluding patient #17, #18, #21, #22, and #25 due to the limited cell number of samples) and HD groups ($n = 10$). The figure was drawn by Excel software with "EasyCharts" package. The data were normalized automatically by the software. The diameter of the bubble presents the mean value of the percentage of the expression of each marker. *$p < 0.05$.

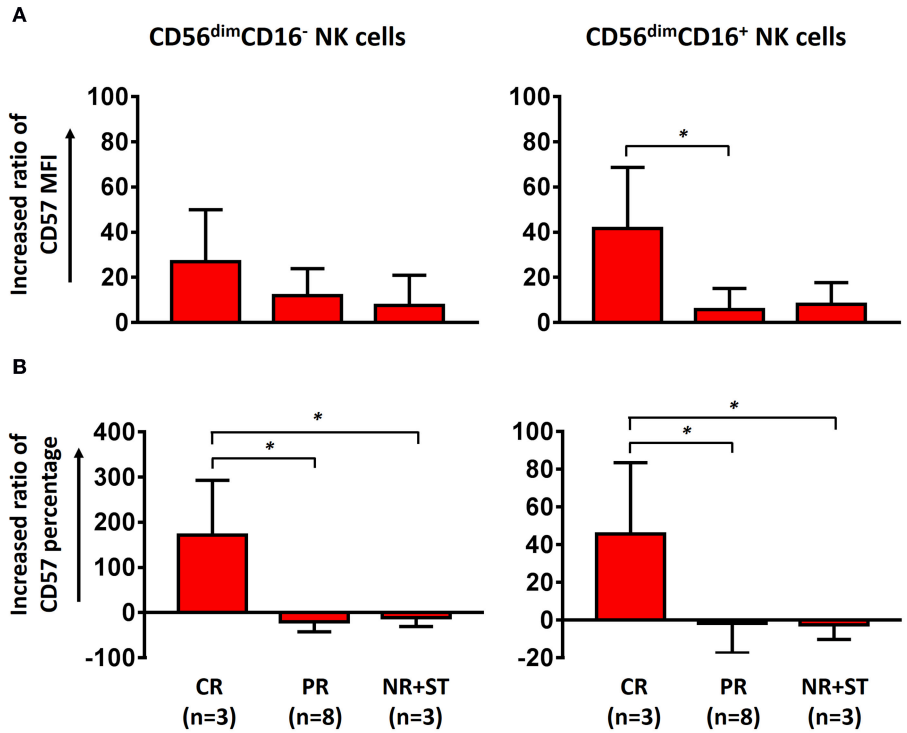

FIGURE 3 | Effect of ECP on maturation of CD56^{dim} NK cells. Both the MFI of CD57 on CD56^{dim} NK cells **(A)** and the frequency of CD57^{+}CD56^{dim} NK cells **(B)** were upregulated in aGVHD patients with CR ($n = 3$, excluding patient #8 due to the limited cell number of sample) but not in patients with PR ($n = 8$) and NR+ST ($n = 3$, excluding patient #5 due to the limited cell number of sample) after ECP treatment. The increasing ratio of the percentage was been calculated as followed: [(percentage of T2 – percentage of T1) × 100]/percentage of T1. The increasing ratio of the MFI was calculated as followed: [(MFI of T2 – MFI of T1) × 100]/MFI of T1. *$p < 0.05$.

different NK subsets are involved in the pathogenesis of aGVHD and cGVHD.

In patients suffering from steroid refractory/resistant aGVHD, we observed a dramatically increased in the CD56^{bri} NK subset compared to HDs and cGVHD patients, where CD56^{dim} NK cells constitute the majority of the NK cells. The origin of CD56^{bri} NK cells is still a hot debate. CD56^{bri} NK cells descending from common lymphoid progenitors or common myeloid progenitors are considered to be the immature precursors of CD56^{dim} NK cells in a linear-differentiation model (30, 31). In the early period post-HSCT, a high frequency of CD56^{bri} NK cells reflects a better immune reconstitution (9, 12, 32). This provides a biological explanation for a previous report indicating that ECP therapy could increase the CD56^{bri} NK cells in responding GVHD patients during the early ECP treatment phase (14). On the other hand, CD56^{dim} NK cells could upregulate CD56 after activation, suggesting a proportion of CD56^{bri} NK cells might be activated NK cells rather than immature precursors (31, 33). Both theories regarding the origin of CD56^{bri} NK cells are supported by our observation in aGVHD patients that CD56^{bri} NK cells express high levels of immature marker CD62L (27, 34) and also activation receptor NKG2D (35).

CD56^{bri} NK cells are believed to have a strong cytokine production capacity with a weakly cytolytic potential (12, 36, 37). Since the NKG2D ligands, MHC class I-related Chains A and B (MICA and MICB) as well as UL-16 binding proteins (ULBP1-4), are extensively induced in skin, gut, and liver during aGVHD, these ligands could recruit the activated cytokine-producing NKG2D^{+}CD56^{bri} NK cells into the target tissues to directly damage cells (38). In turn, the proinflammatory cytokines released by the injured tissue could cause increased secretion of TNF-α and IFN-γ *in situ* by activated CD56^{bri} NK cells creating an amplification loop that leads to further deterioration of GVHD by direct cell damage or indirect T cell-mediated tissue damage (18, 39, 40). This might explain our observation of a dramatic reduction of CD56^{bri} NK cells with decreasing NKG2D expression in aGVHD patients undergoing ECP therapy in association with a favorable clinical outcome.

This observation seems to be in conflict with a recent ECP study performed in GVHD patients reporting that an early increase of CD56^{bri} NK cells is a dominant effect and predicts response to ECP (14). However, the discrepancy between this study and our data might be explained by a longer immunomonitoring time span of patients under ECP therapy in our study. As described, CD56^{dim} NK cells display a mature phenotype and majorly contribute to immune defense and GVHD-reducing effect in contrast to CD56^{bri} subsets (24, 41, 42). Indeed, a higher expression of differentiation markers CD11b and CD57 on CD56^{dim} NK cells than on CD56^{bri} NK cells confirmed their mature phenotype in our study. Recently,

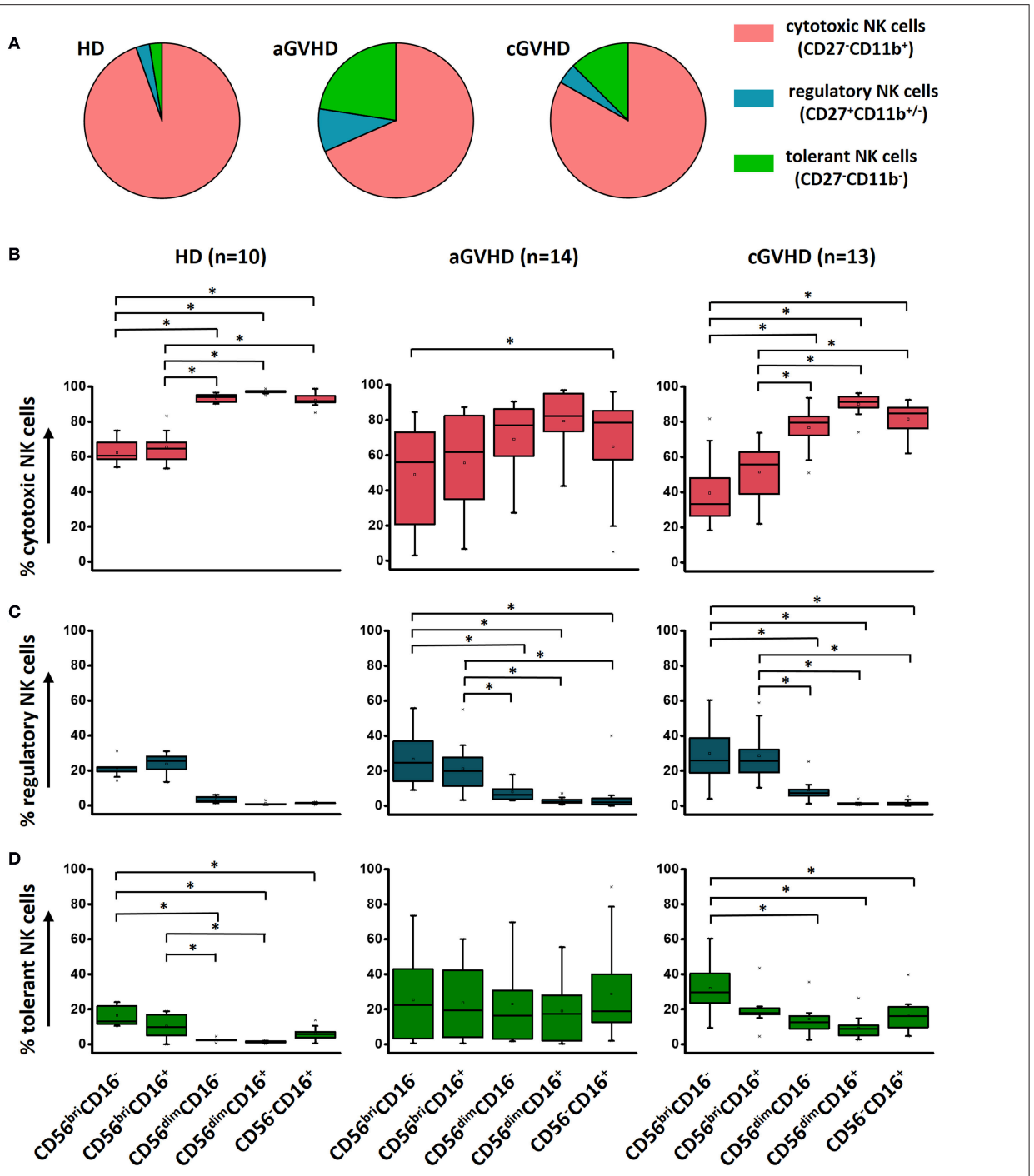

FIGURE 4 | Characterization of functional NK subsets. Based on the expression of CD11b and CD27, NK cells could be defined as three functional subsets: $CD11b^{+}CD27^{-}$ cytotoxic NK cells, $CD11b^{+/-}CD27^{+}$ regulatory NK cells and $CD11b^{-}CD27^{-}$ tolerant NK cells. Although the frequency of these three subsets is different among HDs ($n = 10$), aGVHD ($n = 14$, excluding patient #5 and #8 due to the limited cell number of samples) and cGVHD ($n = 13$, excluding patient #17, #18, #21, #22, and #25 due to the limited cell number of samples) groups **(A)**, the component of cytotoxic **(B)**, and regulatory **(C)** NK cells is similar in five subpopulations among three different groups except the tolerant NK cells **(D)**. *$p < 0.05$.

preclinical data showed that alloreactive T cells could impair the reconstitution and maturation of donor NK cells through competition for the critical survival/differentiation cytokine IL-15, switching NK cells toward early immature NK cells that are known to survive at low levels of IL-15 (32). Reasonably, we assume that priming of CD56^{bri} NK cells by ECP therapy in the early treatment phase is a prerequisite for sequential steps of NK cell differentiation. Apart from this, our previous study

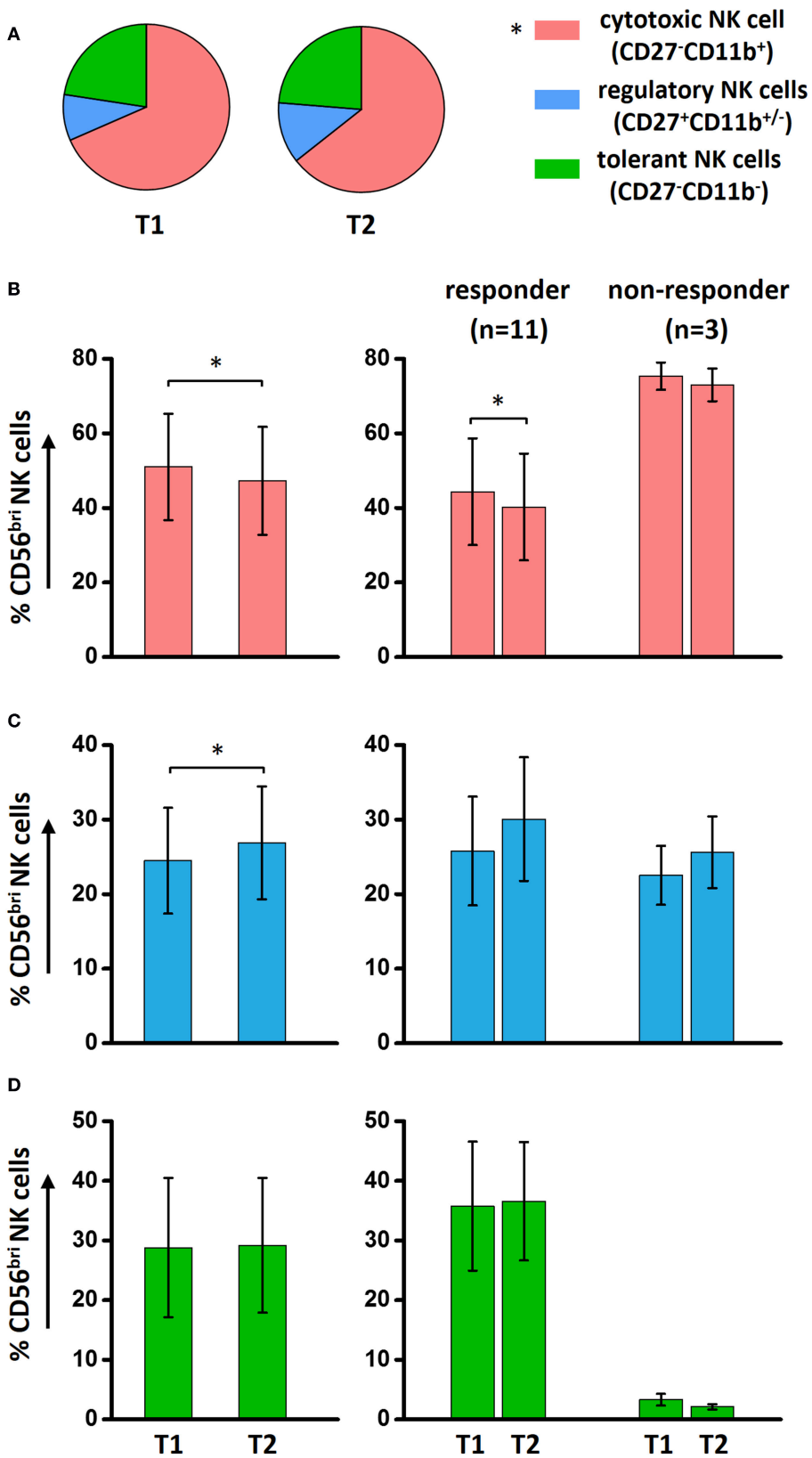

FIGURE 5 | Effect of ECP on functional NK subsets. ECP could reduce significantly the total cytotoxic NK cells while keeping the total regulatory and tolerant NK cells stable in aGVHD patients **(A)**. However, within the CD56^{bri} NK subsets, a dramatically decrease of cytotoxic NK cells **(B)** in conjunction with an increase of regulatory

(Continued)

FIGURE 5 | NK cells **(C)** has been observed in aGVHD patient with response under ECP therapy. **(D)** The effect of ECP therapy has not been observed on CD56^{bri} tolerant NK cells. The responder group includes the patients with CR ($n = 3$, excluding patient #8 due to the limited cell number of sample) and patients with PR ($n = 8$). The non-responder group includes the patients with NR ($n = 1$, excluding patient #5 due to the limited cell number of sample) and patients with ST ($n = 2$). $^{*}p < 0.05$.

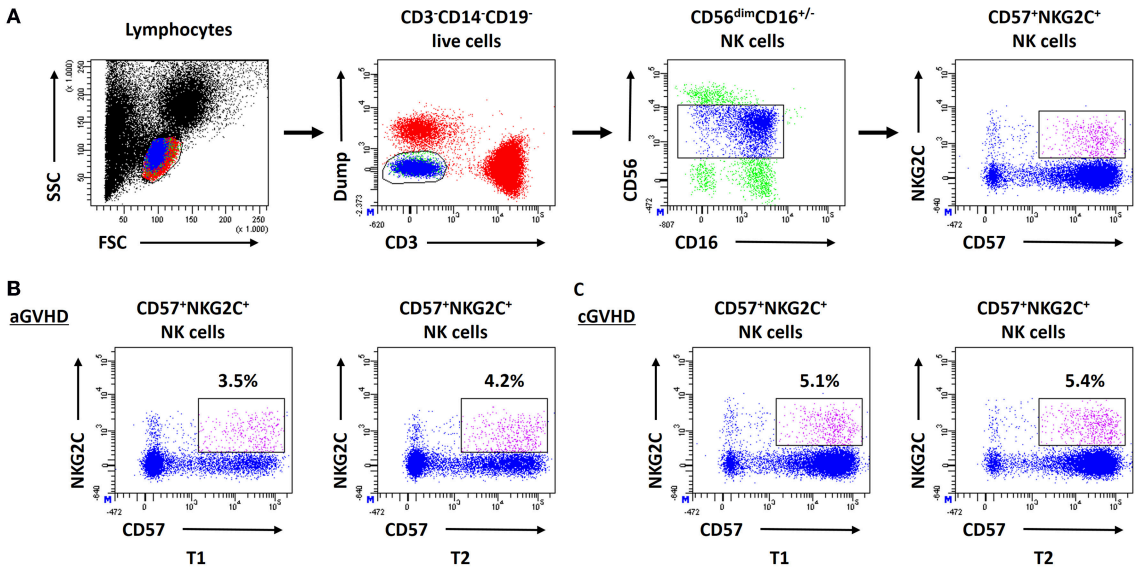

FIGURE 6 | Effect of ECP on CD56^{dim}CD57^{+}NKG2C^{+} NK cells. **(A)** shows the analysis strategy of CD56^{dim}CD57^{+}NKG2C^{+} NK cells. This specialized anti-viral/relapse subset could be preserved after long-term ECP treatment in both aGVHD **(B)** and cGVHD **(C)** patients.

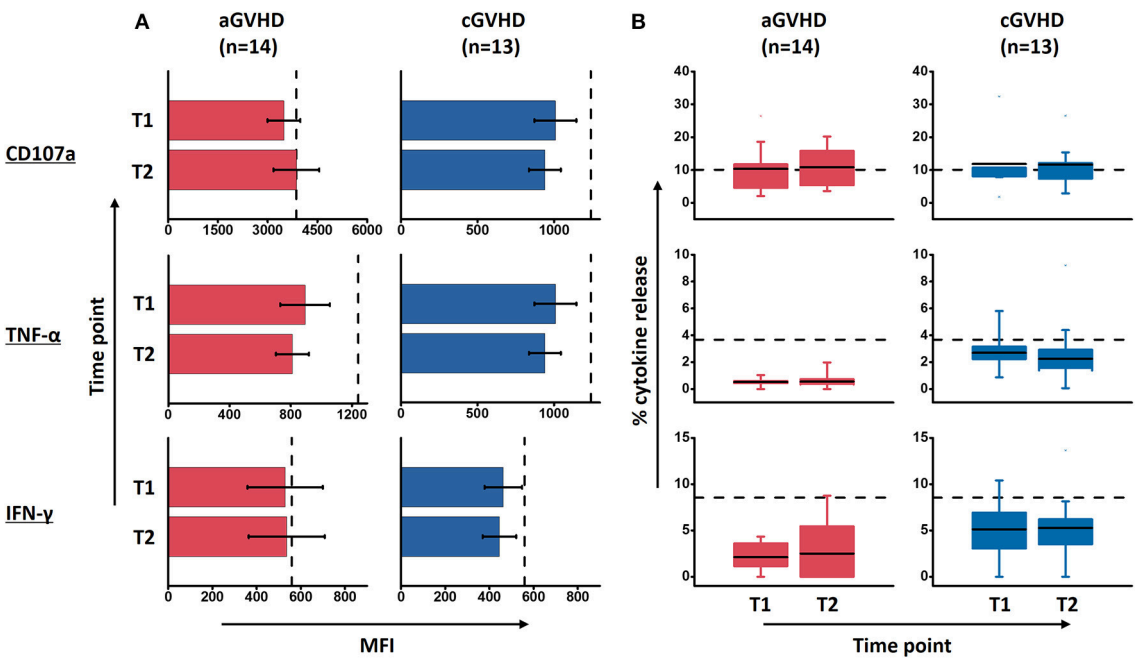

FIGURE 7 | Effect of ECP on the quality and quantity of NK cell function upon K562 stimulation. ECP therapy has no negative effect on the quality of NK cell function in terms of MFI of marker expression **(A)** as well as the quantity of CD107 expression and the cytokine release by NK cells **(B)**. Fourteen patients with aGVHD, excluding patient #5 and #8 due to the limited cell number of samples, and 13 patients with cGVHD, excluding patient #17, #18, #21, #22, and #25 due to the limited cell number of samples, were analyzed. The dashed lines represent the mean value of 10 healthy donor controls.

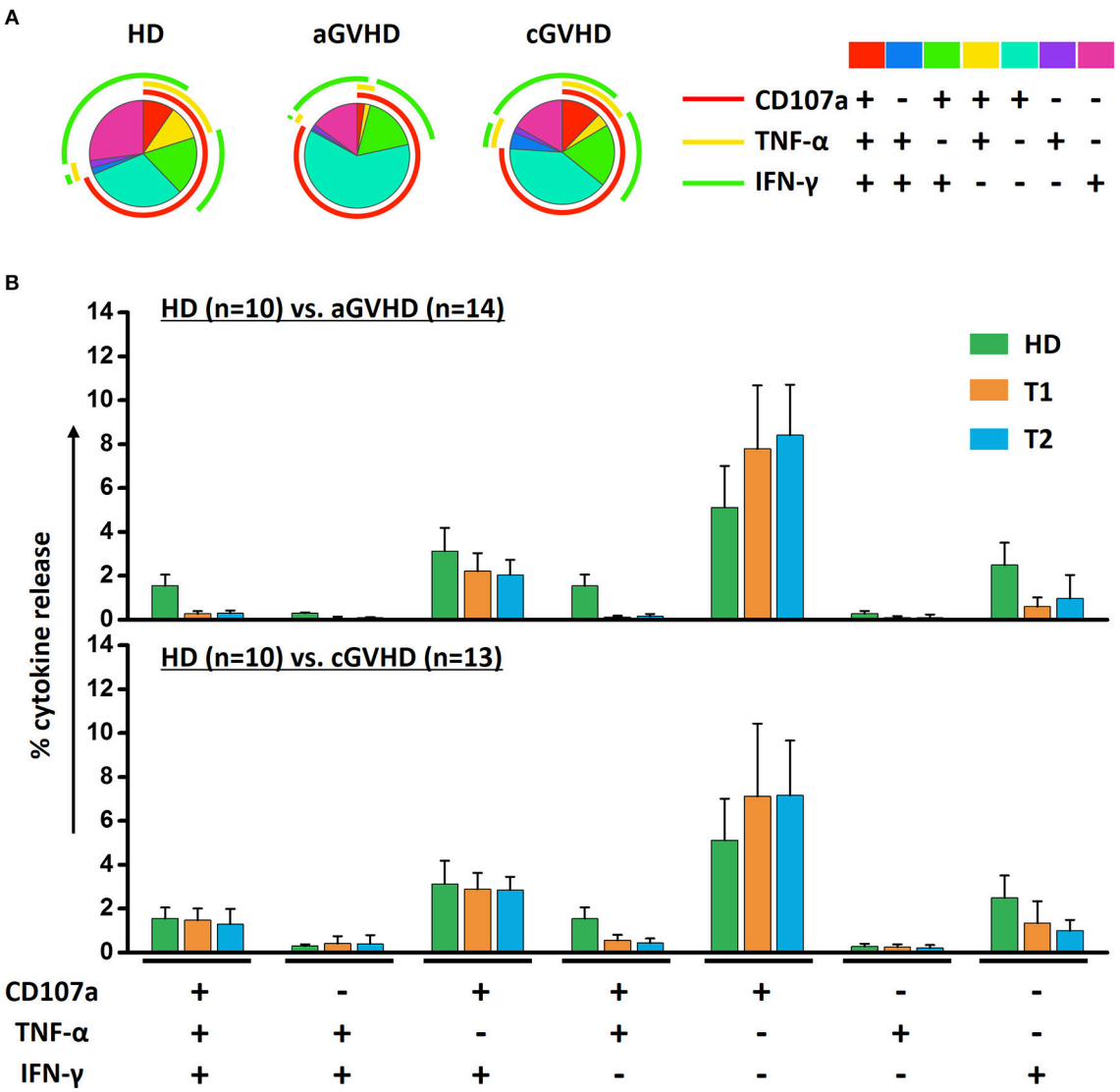

FIGURE 8 | Effects of ECP on the multifunctional NK cells. The polyfunctional NK cells were analyzed using Boolean gating strategy. The pattern of multifunctional NK cells was different among HDs ($n = 10$), aGVHD ($n = 14$, excluding patient #5 and #8 due to the limited cell number of samples) and cGVHD ($n = 13$, excluding patient #17, #18, #21, #22, and #25 due to the limited cell number of samples) patients **(A)**. After long-term ECP treatment, the polyfunctional NK cells could keep stable **(B)**.

indicated that ECP promotes the NK cell differentiation via losing immature receptor CD62L (5). In addition, the significant increase of the density of CD57 on CD56^{dim} NK cells and the frequency of CD57^{+}CD56^{dim} NK cells further support our theory of NK cell differentiation by ECP, since acquisition of CD57 on NK cells is an irreversible process for NK cell maturation (43–46).

Most interesting data emerge from the dissection of the components of NK cells based on the expression of CD11b and CD27. Significant increases of regulatory (CD27^{+}CD11b$^{+/-}$) and tolerant (CD27^{-}CD11b^{-}) NK cells were observed in GVHD patients, suggesting NK cells could have immunoregulatory properties under certain conditions. Those regulatory NK cells could control the inflammation via either induction of other regulatory cells such as regulatory T cells, tolerogenic dendritic cells and monocytes or via suppression of Th17 cells (23, 47). This pleiotropic nature of NK cells might be likely responsible for the variable and even conflicting roles in the development of GVHD. Of note, ECP could shift the NK cells from a cytotoxic to a regulatory/tolerant phenotype, especially, within the CD56^{bri} subset. This shaping effect might partly contribute to the induction of NK cells (48) and CD56^{bri} NK cells (14) by ECP therapy as well.

In the case of cGVHD, CD56^{-} NK cells stand out as a signature NK subset. Previous studies have shown that the existence of CD56^{-} NK cells is associated with chronic viral infection e.g., human immunodeficiency virus and hepatitis C, where NK cells display an impaired functionality with an exhausted phenotype (37, 49–53). In line with these studies, CD56^{-}CD16^{+} NK cells in cGVHD patients highly express

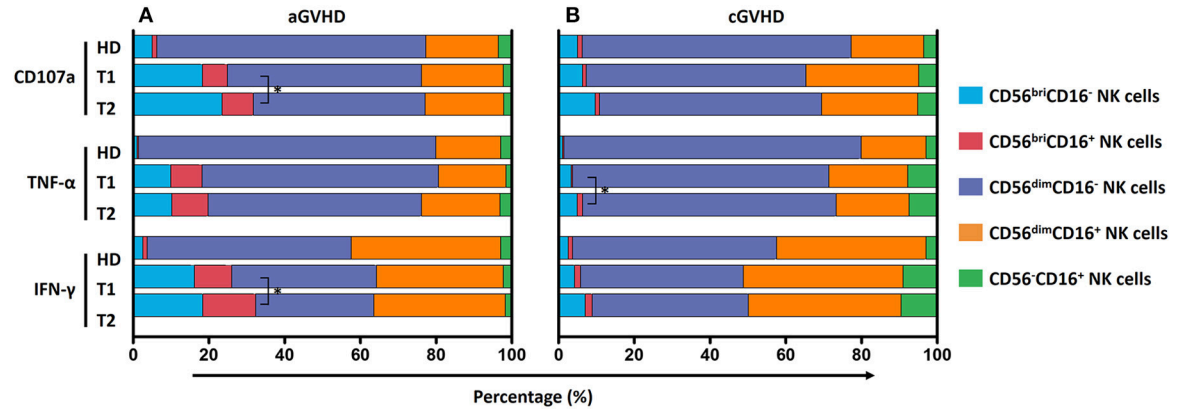

FIGURE 9 | Effect of ECP on the contribution of NK subsets to GVL effect. The contribution of five different NK subpopulations with the respect to CD107a expression, TNF-α and IFN-γ secretion was investigated in both aGVHD ($n = 14$, excluding patient #5 and #8 due to the limited cell number of samples) **(A)** and cGVHD patients ($n = 13$, excluding patient #17, #18, #21, #22, and #25 due to the limited cell number of samples) **(B)**. ECP therapy could improve CD107a expression and IFN-γ secretion by CD56^{bri}CD16$^{+/-}$ NK cells in aGVHD patients **(A)**, while improve TNF-α secretion by CD56^{bri}CD16$^{+/-}$ NK cells in cGVHD patients **(B)**. *$p < 0.05$.

terminally differentiated markers and display a low capacity of cytotoxicity and cytokine release upon K562 stimulation. However, the patients with higher frequency of CD56^{-} NK cells had no viral infection nor virus reactivation, suggesting that cGVHD with a persistent inflammation could drive mature NK cells toward the unfunctional CD56^{-} NK cells as well. However, we did not observe any effects on CD56^{-} NK subset by ECP therapy.

Although ECP therapy could induce immune tolerance and rebalance the immune system, there is no clinical reports showing that ECP is associated with an increased risk of infection and relapse of primary disease (5, 54, 55). In our previous study, we for the first time proved that ECP therapy preserves immunity against infections and the graft vs. leukemia (GVL) effect on the cellular level (5). Based on these findings, we further investigated whether ECP could influence the function of NK cells, since NK cells mediate important innate immunity that bridges the T-cell-deficient period after HSCT in order to control the viral infections and eliminate the residual malignant cells (32, 56, 57).

A specialized subset of NK cells with a CD56^{dim}CD57^{+}NKG2C^{+} phenotype that is highly associated with anti-viral and GVL effect was monitored in the current study. ECP had no negative influence on the frequency of this subset. Consequently, CD56^{dim}CD57^{+}NKG2C^{+} NK cells could still functionally mediate the GVL effect either through secreting TNF-α and IFN-γ or via NKG2C binding to HLA-E (24). Our data further confirmed that the production of TNF-α and IFN-γ by NK cells in response to K562 myeloid leukemia cells was not affected by ECP treatment. Similarly, lysosomal-associated membrane protein-1 (CD107a), a sensitive marker of NK activity (58), was stably expressed by NK cells upon K562 stimulation under ECP therapy.

The magnitude of immune response is a fundamental characteristic of NK cell-mediated immune defense. However, the quality of NK cell responses is more crucial for determining their functionality. With respect to this, the MFI reflecting the cytokine release on a per-cell basis and the polyfunctionality of NK cells associated with enhanced effector function were assessed in GVHD patients under ECP therapy (59). No significant changes were observed. Notably, ECP could even enhance the NK cell-mediated GVL effect via increase of cytokine release by CD56^{bri} NK cells. Furthermore, the proliferation of NK cells providing an expanded pool of effector cells against the pathogens was not hampered by ECP therapy as well. Summing up, our data suggest that ECP does not comprise the quantity and quality of NK activity for control of virus reactivation and anti-tumor immunity post-transplant.

Importantly, immunosuppressive medications should be considered for changes of different cell populations and markers as well. In our study, the major change in immunosuppressive therapy next to ECP treatment was the reduction of steroids. This reduction however was not associated with similar changes of cell subsets among patients with different clinical responses. Therefore, we assume that the reduction of steroids might not contribute to the changes of the NK subsets.

Incorporating the results from former studies, the underling mechanisms behind the effects of ECP on NK cells are summarized in **Figure 11**. ECP therapy could directly induce alloreactive T cell apoptosis which results in sparing of IL-15. Consequently, this promotes not only the recovery of immune reconstitution with an increase of CD56^{bri} NK cells but also a differentiation of NK cells from an immature phenotype CD56^{bri} to a mature state CD56^{dim} followed by further maturation of CD56^{dim} NK cells. Moreover, ECP could educate CD56^{bri} NK cells by shifting their quality from a cytotoxic to a regulatory function.

In conclusion, ECP therapy represents a safe and effective immunomodulatory treatment for GVHD patients through its effects on reconstitution, differentiation, maturation and education of NK cells.

ETHICS STATEMENT

The study was approved by the local Ethical Committees and all patients signed consent forms before treatment.

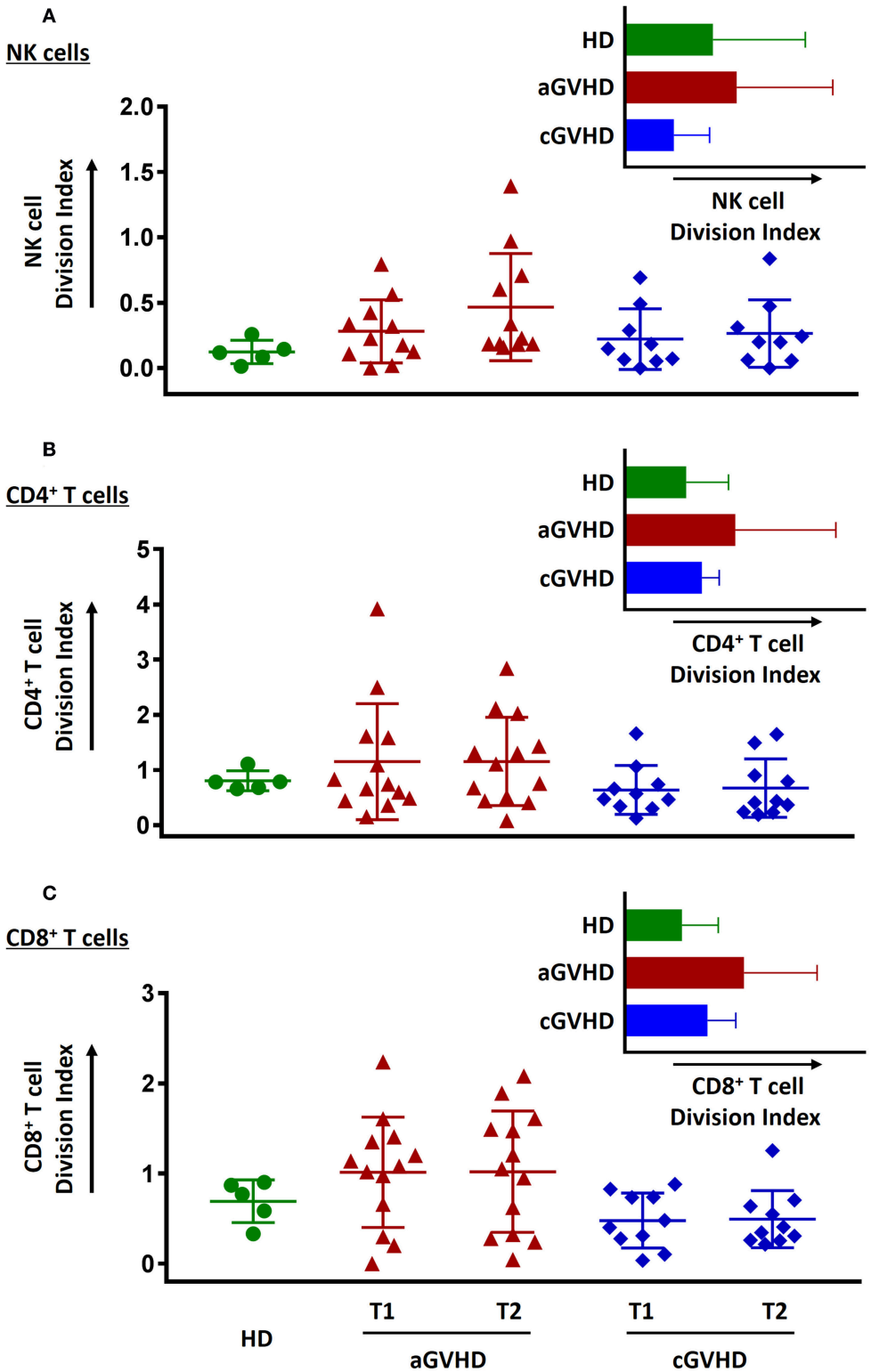

FIGURE 10 | Effect of ECP on cell proliferative function. The proliferative capacity of NK cells **(A)**, CD4$^+$ T cells **(B)**, and CD8$^+$ T cells **(C)** was evaluated using CFSE staining. None of them could be hampered by ECP therapy. All the proliferation assays were performed with 11 samples from aGVHD patients, excluding the samples from patient #5, #6, #8, #10, and #11 due to the limited cell number of samples, nine samples from cGVHD patients, excluding the samples from patient #17, #18, #19, #21, #22, #25, #26, #27, and #31 due to the limited cell number of samples, and five HDs.

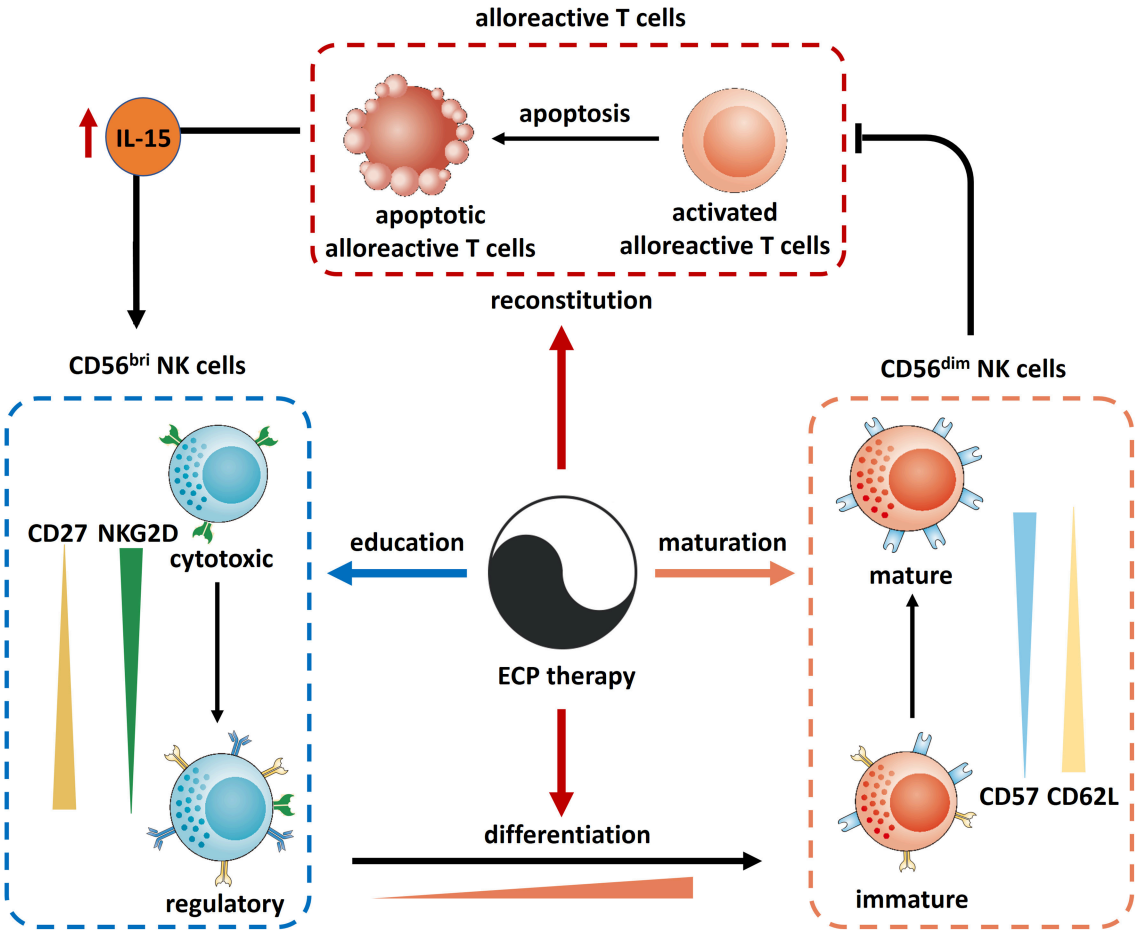

FIGURE 11 | Mechanism of action of ECP on NK cells in GVHD. Reconstitution: ECP could induce the apoptosis of alloreactive T cells resulting in an elevation of IL-15 which recoveries the immune reconstitution. Differentiation: IL-15 could also promote the differentiation of NK cells from CD56^{bir} subset to CD56^{dim} subset. In turn, CD56^{dim} NK cells could kill the alloreactive T cells. Maturation: CD56^{dim} NK cells could be matured through ECP therapy via upregulation of CD57 and downregulation of CD62L. Education: ECP could educate CD56^{bri} NK cells by shifting their quality from a cytotoxic to a regulatory function.

AUTHOR CONTRIBUTIONS

AS, MS, LW, and MN designed the research. MN, LW, and MY performed the experiments. AS, M-LS, TL, UH, SS, PW, WK, LS, MS, and RY treated the patients. KB collected and shipped the samples. MN and LW acquired and analyzed the data. MS, AS, MN, LW, AH-K, BC, PD, CM-T, RY, AN, BN, and WK discussed the organization of the manuscript. LW and MN wrote the manuscript. All authors critically reviewed the manuscript. MS, AS, PD, CM-T, and AN edited the manuscript. AS, MS, and LW supervised 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.2019.00547/full#supplementary-material

Supplementary Table 1 | Antibody list.

Supplementary Figure 1 | Effect of ECP on CD56briCD16- NK subsets in aGVHD patients without CR. The dramatic reduction of CD56briCD16- NK subset by ECP therapy could not be observed in patients with PR ($n = 8$), NR ($n = 1$), and ST ($n = 2$). Dashed line represents the corresponding median value of frequencies observed in 10 healthy donors.

Supplementary Figure 2 | Effect of ECP on functional NK subsets. **(A)** The effects of ECP on the functional NK cell subsets were evaluated in both aGVHD (left panel) and cGVHD patients (right panel). A significant downregulation of the frequency of cytotoxic NK cells in conjunction with a slight upregulation of regulatory and tolerant NK cells was observed in aGVHD patients. The changes of cytotoxic, regulatory and tolerant NK cells within five different NK subpopulations by ECP have be shown in **(B–D)**, respectively.

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ORIGINAL RESEARCH
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Human Double-Negative Regulatory T-Cells Induce a Metabolic and Functional Switch in Effector T-Cells by Suppressing mTOR Activity

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The recently discovered population of TCRαβ+ CD4–/CD8– (double-negative, DN) T-cells are highly potent suppressor cells in mice and humans. In preclinical transplantation models, adoptive transfer of DN T-cells specifically inhibits alloreactive T-cells and prevents transplant rejection or graft-vs.-host disease (GvHD). Interestingly, clinical studies in patients who underwent allogeneic stem cell transplantation reveal an inverse correlation between the frequency of circulating DN T-cells and the severity of GvHD, suggesting a therapeutic potential of human DN T-cells. However, their exact mode of action has not been elucidated yet. Investigating the impact of DN T-cells on conventional T-cells, we found that human DN T-cells selectively inhibit mTOR signaling in CD4 T-cells. Given that mTOR is a critical regulator of cellular metabolism, we further determined the impact of DN T-cells on the metabolic framework of T-cells. Intriguingly, DN T-cells diminished expression of glucose transporters and glucose uptake, whereas fatty acid uptake was not modified, indicating that DN T-cells prevent metabolic adaptation of CD4 T-cells upon activation (i.e., glycolytic switch) thereby contributing to their suppression. Further analyses demonstrated that CD4 T-cells also do not upregulate homing receptors associated with inflammatory processes. In contrast, expression of central memory-cell associated cell surface markers and transcription factors were increased by DN T-cells. Moreover, CD4 T-cells failed to produce inflammatory cytokines after co-culture with DN T-cells, whereas IL-2 secretion was enhanced. Taken together DN T-cells impair metabolic reprogramming of conventional CD4 T-cells by abrogating mTOR signaling, thereby modulating CD4 T-cell functionality. These results uncover a new mechanism of DN T-cell-mediated suppression, pointing out that DN T-cells could serve as cell-based therapy to limit alloreactive immune response.

Keywords: double-negative T-cells, immune tolerance, mTOR, T-cell metabolism, allogeneic hematopoietic stem cell transplantation, GvHD

INTRODUCTION

Allogenic hematopoietic stem cell transplantation (allo-HSCT) is often the only curative treatment option for patients with leukemia, lymphoma, and other malignancies of the hematopoietic system (1). Despite advances in allo-HSCT (2), life-threatening treatment-related complications can arise amongst others because donor T-cells recognize not only the recipient's malignant tumor cells (graft-vs.-tumor effect, GvT), but also target healthy tissue of transplanted recipients (graft-vs.-host disease, GvHD) (3). Standard therapy of GvHD with corticosteroids is insufficient as 50% of the patients are steroid-refractory and systemic immunosuppression carries the risk of cancer relapse and opportunistic infections (4). Alternative treatment strategies to specifically inhibit or modulate alloreactive T-cells could improve the outcome and survival rate of allo-HSCT (5). One promising approach to limit exaggerating T-cell responses could be the use of regulatory T-cells (Treg) as an adoptive cellular therapy. In first clinical trials, infusion of *ex vivo* expanded Tregs was reported to be safe, feasible, and capable of reducing GvHD after allo-HSCT (6, 7).

In fact, T-cell receptor (TCR) αβ+ CD4–/CD8– double-negative regulatory (DN) T-cells compose 1–5% of all T-cells in mice and humans and display immunoregulatory functions with therapeutic potential *in vitro* and *in vivo* (8–10). Notably, murine DN T-cells have been shown to suppress auto-, allo-, and xenogenic immune responses in a broad spectrum of murine disease models (11–15). Accordingly, adoptive transfer of DN T-cells prevented rejection of major histocompatibility complex (MHC–) mismatched organ transplants (10, 16) or the onset of diabetes (17). In particular, the transfer of murine DN T-cells after allo-HSCT resulted in induction of tolerance in allogenic T-cells, thereby avoiding GvHD while maintaining anti-leukemia effects (18). Moreover, clinical relevance for human DN T-cells was revealed since frequency of circulating DN T-cells in patients undergoing allo-HSCT is inversely correlated with the severity of acute GvHD (19). The observation that patients with frequencies of DN T-cells over 1% did not develop any severe acute GvHD favors these cells as a promising tool for cellular therapy. In addition, a recent report disclosed DN T-cell numbers to be lowered in patients at the point of chronic GvHD commencement (20). Of interest, human DN T-cells were also shown to delay the onset of xenogeneic GvHD in a humanized mouse model (21). Murine DN T cells have been reported to mediate immune suppression via Fas-FasL interactions, secretion of perforin/granzyme or indirectly via modification of dendritic cells (DCs) (11, 13, 14, 22). However, human DN T-cells do not eliminate responder cells, modulate DCs or deplete nutrients or T-cell growth factors. Although TCR activation, cell-cell-contact, and *de-novo* protein synthesis were essential for human DN T cell-mediated suppression (9), the manner in which DN T-cells shape reactive T-cells has not been defined.

In order to understand the impact of DN T-cells on alloreactive T-cells, we investigated the fate and function of DN T-cell-treated CD4 T-cells. We found that DN T-cells suppress proliferation, but also modify metabolism, characteristics, and effector functions of CD4 T-cells by selective blocking of the mTOR (mammalian target of rapamycin) signaling pathway. Taken together these results suggest that DN T-cells might bias CD4 T-cells toward a quiescent phenotype thereby inducing peripheral tolerance after allo-HSCT.

MATERIALS AND METHODS

Medium and Reagents

T-cells were cultured in RPMI 1640 medium supplemented with 10% human AB-serum (c.c.pro, Oberdorla, Germany). The following recombinant human cytokines were used: 100 U/ml IL-2 (Novartis, Basel, Switzerland), 500 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) (Sanofi, Paris, France), 5 ng/ml IL-4 and transforming growth factor beta (TGF-β) (PeproTech, Hamburg, Germany), 10 ng/ml IL-1β and tumor necrosis factor (TNF) (PromoKine, Heidelberg, Germany), 1,000 U/ml IL-6 (CellGenix, Freiburg, Germany), and 1 µg/ml prostaglandin E2 (PGE2) (Enzo Life Science, Lörrach, Germany).

Isolation and Culture of T-Cells

Peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation from leukapheresis products from healthy volunteers using Pancoll (PAN Biotech, Aidenbach, Germany). The study was approved by the Ethics committee of the University Erlangen-Nuremberg (protocol number 284_18 Bc). Informed consent was provided in accordance with the Declaration of Helsinki. Isolation of CD4 T-cells (human CD4+ T cell isolation kit) and DN T-cells (human double-negative T cell isolation kit) from PBMCs via magnetic separation was performed according to the manufacturer's instructions (Miltenyi Biotec, Bergisch-Gladbach, Germany). DCs were generated as previously described (23). In brief, monocytes were enriched by adherence to plastic surface of cell culture flasks for 2 h, then cultured with medium plus 10% fetal calf serum (FCS) supplemented with GM-CSF, IL-4, and TGFβ. On day 5, GM-CSF, IL-4, TNF, IL-6, IL-1β, and PGE2 were added to the culture for an additional 48 h, non-adherent cells were harvested and used for stimulation of DN T-cell cultures. DN T-cells (1×10^5/well) from donor A were co-cultured with allogeneic mature DCs (2.5×10^4/well) from donor B in 96-well plates in complete medium plus IL-2 (100 IU/ml). DN T-cells were re-stimulated weekly with allogeneic DCs for 2–5 weeks. Viability and purity of the T-cells was monitored by flow cytometry. Further purification via magnetic bead separation was performed if purity was <95%. DN T-cells were used for functional assays not earlier than 5 days after the last stimulation.

T-Cell Suppression Assays

Freshly isolated CD4 T-cells from donor A were labeled with a violet proliferation dye (VPD450 BD Biosciences, Heidelberg, Germany) and seeded in 96-well plates (5×10^4 CD4 T-cells per well). DN T-cells from donor A were used as suppressor cells in a responder to suppressor ratio of 1:1. Cells were activated with anti-CD3/CD28 coated Dynabeads (ThermoFisher, Waltham, USA) at a ratio of 25:1. After 1, 3, or 6 days of co-culture, cells were harvested, stained with monoclonal antibodies (mAbs) or dyes, and measured by flow cytometry. CD4 T-cells were

analyzed by gating on viable lymphocytes with forward scatter-area (FSC-A)/sideward scatter-area (SSC-A) and on singlets with forward scatter-height (FSC-H), followed by gating on CD4+ cells. The proliferation of CD4 responder T-cells was analyzed by the decrease in proliferation dye fluorescence. Unstimulated CD4 T-cells were used as a control. DN T-cells used in suppression assays for cytokine detection were additionally labeled with carboxyfluorescein succinimidyl ester (CFSE) (Sigma, Munich, Germany). For activation experiments, CD4 T-cells were labeled with VPD450 and incubated with the Akt/mTOR signaling activator SC79 (20 μg/ml, Calbiochem/Merck, Darmstadt, Germany), the mTOR signaling activator MHY-1485 (20 μM, Selleckchem, Munich, Germany) or medium only as a control. After 2 h, cells were washed twice and used as responder cells in a co-culture as described above. To analyze the impact of mTOR hyperactivation using MHY-1485 on DN T-cell induced alterations, cells were harvested at day 3 of co-culture and their phenotype and metabolism was assessed by flow cytometry. Proliferation of T-cells was determined by flow cytometry at day 6 of co-culture.

Transwell Assays

For transwell experiments, CD4 T-cells were activated with anti-CD3/CD28 coated beads in the bottom of a 24-well plate at a 25:1 ratio. In total 5×10^5 were seeded per well. DN T-cells were added to the bottom well or together with anti-CD3/CD28 coated beads to the top chamber (Corning, New York, USA). Top and bottom chamber were separated by a 0.4 μm permeable pore polycarbonate membrane that allows pass through of soluble factors, but not of cells. Controls were cultured in a 24-well plate as described above.

Flow Cytometry

Cells were stained with anti-human anti-CD4 (SK3), anti-CD27 (L128), anti-pS6 (pS240 N4-41), anti-p-p38 (p180/pY182), anti-CD95 (DX2), anti-CD98 (UM7F8), anti-IFN-γ (B27), anti-IL-2 (MQ1-17H12), anti-IL17-A (N49-653), anti-CD195 (2D7), anti-CCR5 (J252D4), anti-CD54 (HA58), anti-NF-κB p65 [(pS529) (K10-895.12.50)] (all from BD Biosciences, Heidelberg, Germany), anti-T-bet (eBio4510), anti-CD28 (10F3), anti-Eomes (WG1928, all from ThermoFisher, Waltham, USA), anti-CD197 (G043H7), anti-HIF-1α (546-16), anti-CD183 (G025H7), anti-GM-CSF (BV D2-21C11), anti-CD49d (9F10), and anti-Integrin β7 (FIB504) (all from Biolegend, San Diego, USA), anti-GLUT1 (EPR3915) and anti-GLUT3 (polyclonal, both from Abcam, Cambrige, United Kingdom) mAbs. Intracellular glucose transporter 1 (GLUT1), GLUT3, HIF-1α, interferon-gamma (IFN-γ), IL-2, GM-CSF, IL-17A were stained with the Cytofix/Cytoperm kit (BD Biosciences, Heidelberg, Germany), according to the manufacturer's protocol. Intracellular T-box transcription factor (T-bet) and eomesodermin (Eomes) were stained with Foxp3 Transcription Factor Staining Buffer Set (ThermoFisher, Waltham, USA), according to the manufacturer's protocol. Lymphocytes were determined by FSC-A/SSC-A, doublets were excluded by FSC-H and CD4+ cells were gated by the indicated mAbs. For detection of phosphorylated proteins on day 1 after co-culture, cells were stained with surface mAbs, washed, fixed with BD Cytofix/Cytoperm, washed again, and permeabilized with Perm Buffer III (BD Biosciences, Heidelberg, Germany) for 30 min. After washing with PBS (2% FCS), cells were incubated with the indicated mAbs for 40 min, washed, and fixed with CellFIX (BD Biosciences, Heidelberg, Germany). For assessment of glucose or fatty acid uptake cells were incubated with 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG) (200 μM, AAT Bioquest, Sunnyvale, California, USA) for 10 min or $\text{Bodipy}_{\text{C1}-12}$ (2 μM, life technologies, Carlsbad, California, USA) for 5 min at 37°C and washed twice with PBS (2% FCS). Flow cytometry data were acquired on a FACS Canto II (BD Biosciences, Heidelberg, Germany) and CD4 T-cells were analyzed for indicated mAbs with FlowJo software (TreeStar, Ashland, Oregon, USA).

Detection of Cytokines

To ascertain cytokines in CD4 T-cells, co-cultures were treated with phorbol-12-myristat-13-acetat (PMA) (2 μg/ml)/Ionomycin (1 μM, both Sigma-Aldrich, Munich, Germany) for 4 h on day 6 of co-culture. Intracellular staining was performed as indicated above. For measurement of secreted cytokines on day 6 of co-culture, DN T-cells and CD4 T-cells were separated using anti-CD4+ magnetic beads (Miltenyi Biotec, Bergisch-Gladbach, Germany). Purity was confirmed with flow cytometry (>95%). CD4 T-cells were stimulated with PMA/Ionomycin, supernatants were collected after 6 h and analyzed simultaneously for IL-2, IL-4, IL-6, IL-10, IL-17A, TNF, and IFN-γ secretion using the human Th1/Th2/Th17 cytokine cytometric bead array kit (BD Biosciences, Heidelberg, Germany).

T-Cell Migration Assay

To measure the migration of T-cells, CD4 T-cells were incubated unstimulated, with anti-CD3/CD28 beads or with anti-CD3/CD28 beads and DN T-cells as described above. On day 6 of co-culture CD4 T-cells and DN T-cells were separated using anti-CD4+ magnetic beads (Miltenyi Biotec, Bergisch-Gladbach, Germany). Purity was confirmed with flow cytometry (>95%). CD4 T-cells (10^5) were re-suspended in RPMI medium without human AB serum and deposited on the upper chamber of a transwell insert (5.0 μm pore size, Corning Inc., New York, USA). The bottom well contained RPMI medium only or with 100 ng/ml CXCL10, CCL3, or CXCL9 (Biolegend, San Diego, California, USA). Transwell plates were incubated for 2 h at 37°C. The content of the lower chamber was collected, stained with anti-human anti-CD4, and migrated cell numbers were quantified by usage of 123 counting Beads (Thermo Fisher, Waltham, USA).

ELISA

For measurement of Akt and p38 phosphorylation with Fast Activated Cell-based ELISA (FACE), CD4 T-cells were activated with anti-CD3/CD28 coated beads in the presence or absence of DN T-cells. After 24 h, CD4 T-cells were separated using anti-CD4+ magnetic beads (Miltenyi Biotec, Bergisch-Gladbach, Germany). Purity was confirmed with flow cytometry (>95%). CD4 T-cells (10^4) were plated on a Poly-D-Lysin coated (10

mg/ml) 96-well flat bottom plate and fixed with Formaldehyde. For detection of phosphorylation, we used the FACE p38 in-cell Western analysis for phospho-p38 (T180/Y182) and FACE AKT in-cell Western analysis for phospho-AKT (S473) (Active motif, Carlsbad, California, USA) according to the manufacturer's instructions.

Metabolic Flux Analyses

The CD4 T-cells' bioenergetics after DN T-cell co-cultured was assessed using an XFe96 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, Massachusetts, USA). CD4 T-cells were activated with anti-CD3/CD28 coated beads in the presence or absence of DN T-cells, unstimulated CD4 T-cells were used as control. On day 3 of co-culture CD4 T-cells and DN T-cells were separated using anti-CD4+ magnetic beads (Miltenyi Biotec, Bergisch-Gladbach, Germany). Purity and viability was confirmed with flow cytometry (>95%). To determine oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) CD4 T-cells were utilized for XF Mitochondrial Stress Test Kits and XF Glycolysis Stress Test Kits according to the manufacturer's recommendations (Seahorse Bioscience, North Billerica, USA) and as previously detailed (24, 25).

Statistical Analyses

Data were analyzed with Graphpad Prism software (GraphPad San Diego, USA). Results were compared using non-parametric (Mann-Whitney-U or Wilcoxon) tests. A $p < 0.05$ was considered significant.

RESULTS

DN T-Cells Modulate TCR Signaling in CD4 T-Cells

Human DN T-cells effectively inhibit CD4 T-cell proliferation but the consequences for suppressed CD4 T-cells remain elusive. Given that initial signal transduction after TCR ligation plays a pivotal role for the further fate of the cell, we first focused on whether human DN T-cells can influence CD4 T-cell signaling. We addressed this question by investigating phosphorylation of central signaling molecules in CD4 T-cells after stimulation with and without DN T-cells via flow cytometry. Activated CD4 T-cells exhibited high phosphorylation levels at the downstream molecule of the mTOR signaling pathway S6, while DN T-cell-treated CD4 T-cells revealed lower S6 phosphorylation (**Figure 1A** and **Supplemental Figure 1A**). However, DN T-cells did not influence the phosphorylation of the signaling molecule mitogen-activated protein (MAP) kinase p38, pointing to a selective modulation of signal transduction. To verify this finding, ELISA of total and phosphorylated Akt and p38 was performed. CD4 T-cells showed no differences of total-Akt expression in presence or absence of DN T-cells, while phospho-Akt was reduced in CD4 T-cells after co-culture with DN T-cells (**Supplemental Figure 1B**). In contrast, phosphorylation of p38 was not affected by DN T-cells. To further elucidate signaling alterations caused by DN T-cells, we analyzed if downstream targets of mTOR and p38 were also affected by DN T-cells. Notably, DN T-cells diminished the upregulation of mTOR-regulated transcription factor HIF-1α in CD4 T-cells, whereas activation of transcription factor NF-κB was not impaired (**Figure 1B** and **Supplemental Figure 1C**). Given that the cell surface molecule CD98 is upregulated due to mTOR activity (26) while CD54 is induced by p38 signaling (27), we analyzed the expression of these proteins as surrogate markers. CD98 but not CD54 expression was abrogated in activated CD4 T-cells after co-culture with DN T-cells (**Figure 1C** and **Supplemental Figure 1D**), underpinning that DN T-cells selectively inhibit mTOR signaling. To test the physiological relevance of mTOR signaling for DN T-cell-mediated suppression, we hyperactivated this pathway in CD4 T-cells using the small molecule SC79 as described in *Materials and Methods*. SC79 did not modify the proliferation of unstimulated and anti-CD3/CD28-coated beads activated CD4 T-cells. In contrast, pretreatment of CD4 T-cells with the mTOR-activator rendered the CD4 T-cells unsusceptible to DN T-cell-mediated suppression (**Figure 1D**). Taken together, these findings indicate that DN T-cells mediate their suppressive activity by decreasing mTOR activity rather than blocking entire signaling processes in CD4 T-cells.

DN T-Cells Inhibit Glycolytic Reprogramming of CD4 T-Cells

Given that DN T-cells selectively inhibit the glycolytic key regulators mTOR and HIF-1α, we next considered whether DN T-cells affect CD4 T-cell metabolism. We therefore analyzed the regulation of glucose transporter 1 (GLUT1) and 3 (GLUT3) in the absence or presence of DN T-cells. Activation of CD4 T-cells with anti-CD3/CD28 coated beads induced high GLUT1 and GLUT3 expression that peaked after three days (**Figure 2A** and **Supplemental Figure 2A**). This upregulation of glucose transporters was impaired in the presence of DN T-cells, suggesting a reduced capacity for glycolysis. As a result of declined activation of CD4 T-cells after 6 days, differences between activated and DN T-cell-suppressed CD4 T-cells had vanished at that point. To verify the impact of mTOR signaling on DN T-cell-caused metabolic alterations, we activated this pathway using the specific activator MHY-1485 as described in *Materials and Methods*. MHY-1485 did not modify expression of GLUT1 in unstimulated and activated CD4 T-cells, while downregulation of GLUT1 expression was not present in mTOR hyperactivated CD4 T-cells in co-culture with DN T-cells (**Supplemental Figure 2B**). To further confirm the effect of DN T-cells on CD4 T-cell metabolism, we directly measured glucose and fatty acid uptake using the fluorescent analogs 2-NBDG and $\text{Bodipy}_{\text{C1}-12}$. As expected, activation of CD4 T-cells with anti-CD3/CD28 coated beads resulted in high 2-NBDG consumption that was strongly impaired by DN T-cells (**Figure 2B** and **Supplemental Figure 2C**). In contrast, DN T-cells did not affect enhanced fatty acid uptake of CD4 T-cells (**Figure 2C**), demonstrating a selective inhibition of glucose utilization. In addition, real-time analyses of extracellular acidification rate (ECAR, indicative for aerobic glycolysis) as well as of oxygen consumption rate (OCR, indicative for mitochondrial respiration) of CD4 T-cells declared a reduced

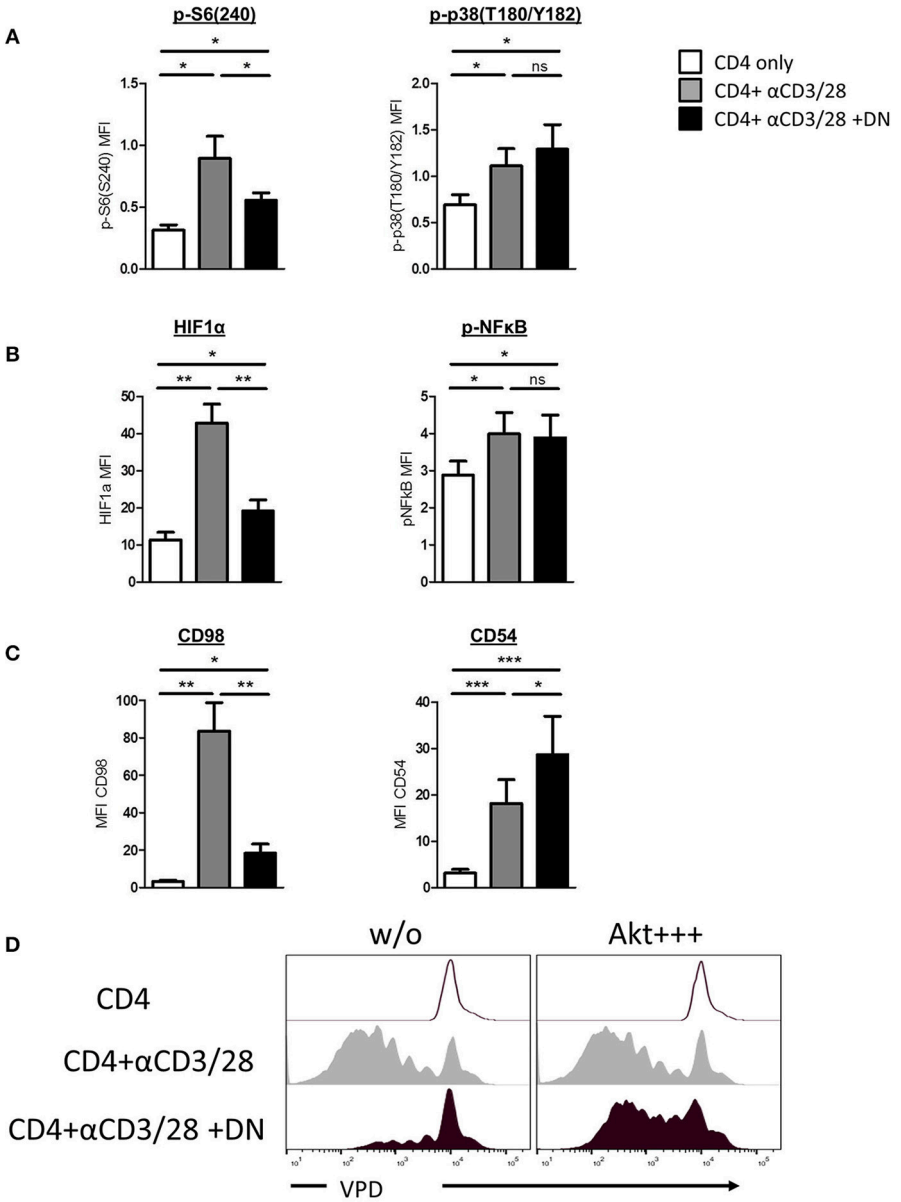

FIGURE 1 | DN T-cells inhibit mTOR activation but not MAPK p38 signaling in CD4 T-cells. Freshly isolated CD4 T-cells were incubated with anti-CD3/CD28 coated beads in absence (gray) or presence (black) of DN T-cells. Unstimulated CD4 T-cells were used as negative control (white). **(A)** Phosphorylation of ribosomal protein S6(S240) (left) and MAPK p38(T180/Y182) (right) in CD4 T-cells after 24 h culture was quantified by flow cytometry. Graphs show MFI +/-SEM of at least six independent experiments. **(B)** Expression of HIF-1α and NFκB(p65) was analyzed in CD4 T-cells after 24 h co-culture, graph represent MFI +/–SEM of 7 experiments. **(C)** Expression of CD98 and CD54 was measured after 3 days, MFI +/SEM of at least five experiments is shown. Ns, not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.01$. **(D)** Freshly isolated VPD-labeled CD4 T-cells were incubated with SC79 (Akt+ + +) for 2 h at 37°C and washed intensively. Treated and untreated VPD-labeled CD4 T-cells were activated with anti-CD3/CD28 coated beads in presence or absence of DNT-cells for 6 days. Cells were analyzed by flow cytometry, histograms were gated for CD4 T-cells.

glycolytic rate upon incubation with DN T-cells (**Figure 2D**). When analyzing the OCR/ECAR ratio, an indicator for the balance between aerobe glycolysis and oxidative phosphorylation (OXPHOS), we noticed that the activation-related skewing of CD4 T-cells toward ECAR was abrogated by DN T-cells suggesting DN T-cells inhibited upregulation of glycolysis in activated CD4 T-cells (**Figure 2D**).

DN T-Cells Affect the Phenotype of CD4 T-Cells

To further explore the consequences of an altered metabolism in CD4 T-cells, we performed flow cytometry staining of intracellular and surface markers on day 6 of co-culture. First, we analyzed whether DN T-cells manipulate T-bet and Eomes expression in CD4 T-cells. Interestingly, DN T-cells diminished

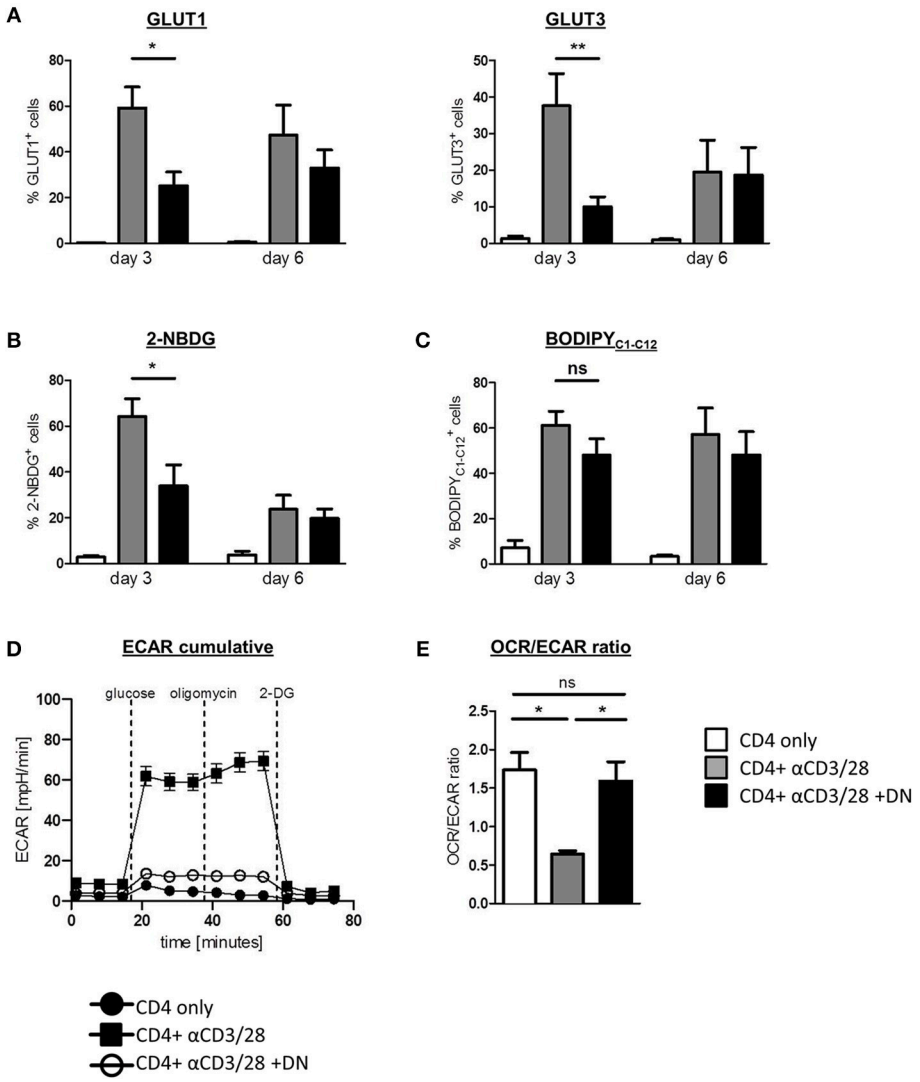

FIGURE 2 | DN T-cells impair metabolic reprogramming of CD4 T-cells. Freshly isolated CD4 T-cells were incubated with anti-CD3/CD28 coated beads in absence (gray) or presence (black) of DN T-cells, unstimulated CD4 T-cells served as negative control (white). Cells were analyzed by flow cytometry after 3 and 6 days. **(A)** Expression of GLUT1 and GLUT3 in CD4 T-cells was determined by flow cytometry after 3 and 6 days. **(B)** Uptake of the glucose analog 2-NBDG and **(C)** the fatty acid $Bodipy_{C1-C12}$ in CD4 T-cells was measured as described in *Materials and Methods*. Data of at least seven independent experiments +/- SEM are shown. **(D)** On day 3 of co-culture CD4 T-cells were re-isolated by magnetic sorting and ECAR was measured in CD4 T-cells, using an XFe96 flux analyzer. **(E)** The OCR/ECAR ratio indicative for the energetic balance between OXPHOS and aerobic glycolysis was calculated for unstimulated CD4 T-cells and for activated CD4 T-cells in presence or absence of DN T-cells ($n = 4$). ns, not significant, $^{*}p < 0.05$, $^{**}p < 0.01$.

the induction of T-bet in activated CD4 T-cells, whereas Eomes expression was further enhanced after co-culture (**Figure 3A** and **Supplemental Figure 3A**). Furthermore, DN T-cells did not strengthen the expression of the transcription factor FoxP3 in CD4 T-cells (**Supplemental Figure 3D**). Since transcription factors orchestrate the expression of distinct T-cell markers, DN T-cells suppressed upregulation of the co-stimulatory cell surface molecule CD28 and the death receptor Fas on CD4 T-cells (**Figure 3B** and **Supplemental Figure 3B**). In contrast, expression of the co-stimulatory receptor CD27 was not reduced but further enhanced in presence of DN T-cells. To test the physiological relevance of mTOR signaling for DN T-cell-mediated characteristic changes, we overactivated mTOR in CD4 T-cells via MHY-1485. Notably, MHY-1485 treated activated CD4 T-cells were resistant to DN T-cell-induced downregulation of CD98 and CD28, whereas controls were not altered by mTOR hyperactivation (**Supplemental Figure 3C**). To further assess the differentiation of CD4 T-cells we stained for CCR7 and CD45RO (**Supplemental Figure 3E**). Unstimulated CD4 T-cells express CCR7 but are negative for CD45RO, while activated CD4 T-cells upregulate CD45RO and loose CCR7 on their surface. Of interest, DN T-cell co-cultured CD4 T-cells express CD45RO as well as

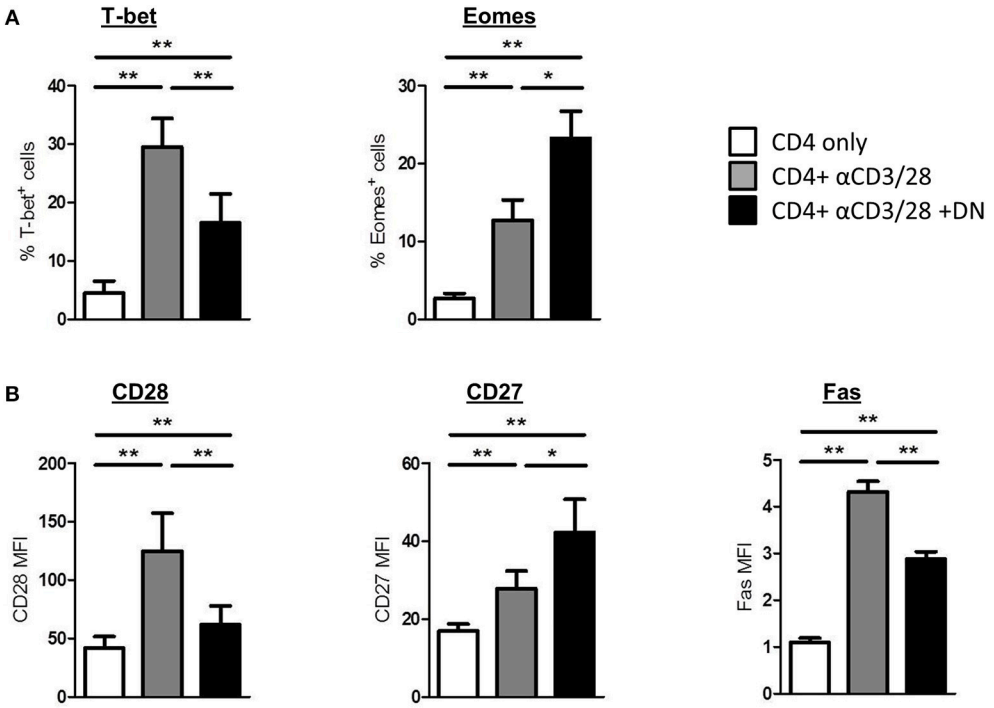

FIGURE 3 | DN T-cells modulate expression profiles of CD4 T-cells. Freshly isolated CD4 T-cells were cultured with anti-CD3/CD28 coated beads in absence (gray) or presence (black) of DN T-cells, unstimulated CD4 T-cells were used as negative control (white). Cells were harvested on day 6 of co-culture and analyzed by flow cytometry. **(A)** CD4 T-cells were analyzed for the expression of transcription factors T-bet and Eomes. **(B)** Expression of CD28, CD27, and Fas on CD4 T-cells is shown. Data represent results of at least seven independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$.

CCR7. Overall, these results indicate that DN T-cells modulate the phenotype of suppressed CD4 T-cells.

DN T-Cells Change Migratory Capacity of CD4 T-Cells

To investigate whether DN T-cells can restrict homing of alloreactive T-cells, we analyzed the expression of pro-inflammatory chemokine receptors CXCR3 and CCR5 that are associated with GvHD induction and severity (28, 29). Both CXCR3 and CCR5 and also the integrin α4β7, which is essential for homing of cells to the gut, were upregulated on CD4 T-cells after activation (**Figure 4A** and **Supplemental Figure 4A**). Of importance, DN T-cells diminished expression of GvHD-associated chemokine receptors, whereas CCR7 and CXCR5 were upregulated (**Figure 4B** and **Supplemental Figure 4B**), suggesting an augmented potential of CD4 T-cells to migrate to lymphoid organs. Moreover, mTOR hyperactivation with MHY-1485 reversed DN T-cell-related suppression of CXCR3 in CD4 T-cells (**Supplemental Figure 4C**), indicating that blocking expression of pro-inflammatory homing receptors was controlled by mTOR inhibition. To test the functional impact of differently expressed chemokine receptors after DN T-cell co-culture on the migratory capacity of CD4 T-cells, we performed an *in vitro* migration assay. CXCL9 and CXCL10 are ligands of CXCR3, while CCL3 binds to the CCR5 receptor. After activation CD4 T-cells migrated toward CXCL9, CXCL10, and CCL3 gradient (**Figure 4C**). However, migration of CD4 T-cells that were co-cultured with DN T-cells was declined pointing toward a limited ability of these cells to home to GvHD-target organs. These data extend our previous finding that DN T-cells modify CD4 T-cell expression profiles, thereby shaping their migratory patterns.

DN T-Cells Modulate Effector Functions in CD4 T-Cells

We next sought to investigate whether CD4 T-cells offer significant functional changes after co-culture with DN T-cells. Therefore, we assessed cytokine production of CD4 T-cells by flow cytometry. Activated CD4 T-cells produced substantial amounts of IFN-γ (**Figures 5A,B**). Strikingly, CD4 T-cells activated in presence of DN T-cells did not show enhanced IFN-γ production but more IL-2 expression. Moreover, we observed that the GvHD-associated effector cytokines IL-17A and GM-CSF were also reduced in CD4 T-cells after DN T-cell co-culture (**Figure 5B**). To confirm these findings, we re-separated CD4 T-cells from DN T-cells and performed cytometric bead arrays. Activation of CD4 T-cells with anti-CD3/CD28 coated beads resulted in an enhanced secretion of cytokines IFN-γ and IL-17A, suggesting a differentiation into CD4 effector T-cells (**Figure 5C**). In contrast, CD4 T-cells cultured with DN T-cells did not upregulate secretion of effector cytokines after re-stimulation, while IL-2 production was increased compared to

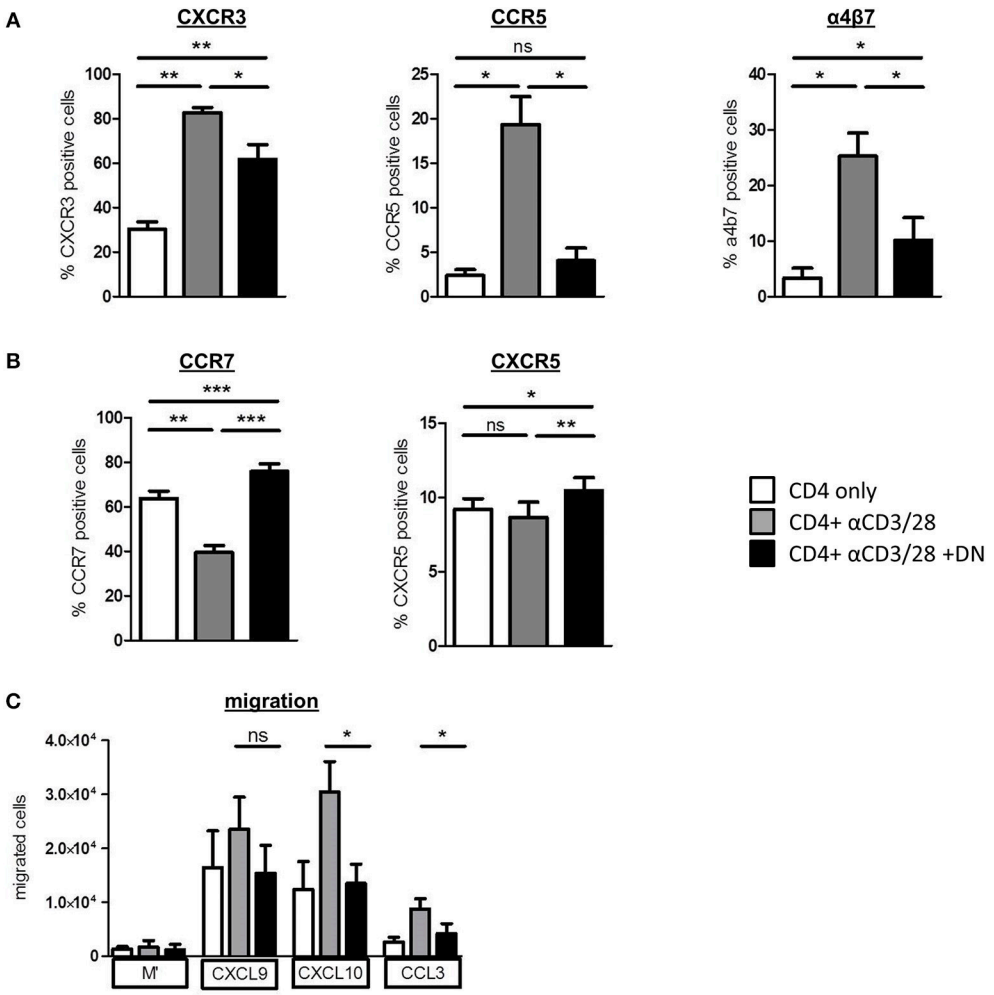

FIGURE 4 | DN T-cells influence migratory capacity of CD4 T-cells. CD4 T-cells were incubated with anti-CD3/CD28 coated beads absence (gray) or presence (black) of DN T-cells, unstimulated CD4 T-cells were used as negative control (white). After 6 days, cells were analyzed by flow cytometry. Data represent expression of **(A)** CXCR3, CCR5, integrin a4b7, and **(B)** CCR7 and CXCR5 on CD4 T-cells. Mean percentages +/– SEM of seven independent experiments is shown. **(C)** On day 6 of co-culture CD4 T-cells were re-isolated by magnetic sorting and added to the upper chamber of an *in vitro* Transwell migration-assay with the indicated chemokines in the lower chamber. Chemokine dependent CD4 T cell migration was determined after 2 h with 123-counting beads by flow cytometry. ns, not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

unstimulated or activated CD4 T-cells. In summary, our findings provide evidence that DN T-cells inhibit proliferation but also reprogram effector functions of CD4 T-cells.

Modulation of CD4 T-Cells Is Cell-Cell-Contact Dependent

Next we addressed the question whether modulation of CD4 T-cell metabolism and function is induced by cell-cell-contact between DN T-cells and responder CD4 T-cells or results from competition for nutrients or stimulation. First, supernatants obtained from suppression assays were not able to exert any DN T-cell-included alterations when added to freshly with anti-CD3/CD28 coated beads activated CD4 T-cells (data not shown). Moreover, we co-cultured CD4 and DN T-cells in a transwell plate to prevent cell-cell contact but maintain diffusion of nutrients and cytokines. As illustrated in **Figures 6A**, and **B** DN T-cells were limited to suppress CD98 expression and glucose uptake in CD4 T-cells when direct cell contact was blocked. Furthermore, CD28 reduction and inhibition of IFN-γ production (**Figures 6C,D** and data not shown) were reduced in CD4 T-cells after co-cultivation with DN T-cells in the transwell system. These data disclose that cell-cell-contact is indispensable for the alteration of CD4 T-cells by DN T-cells. In summary, these data indicate that human DN T-cells suppress proliferation but also modulate migratory and effector functions of CD4 T-cells via controlling mTOR signaling and metabolic reprogramming (**Figure 7**).

DISCUSSION

In this study, we examine the impact of human DN T-cells on CD4 T-cell fate and function and reveal that DN T-cells not

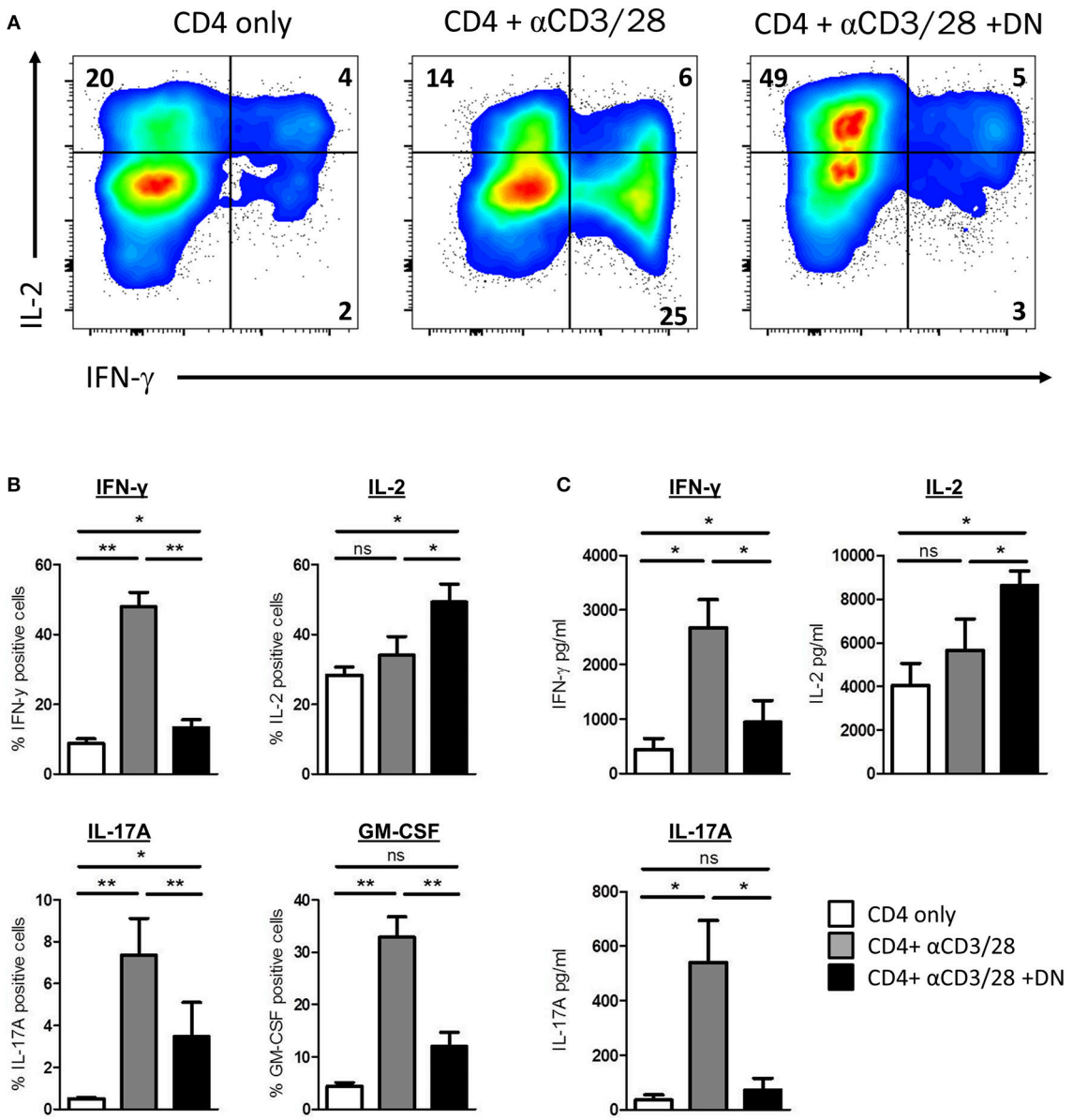

FIGURE 5 | DN T-cells altered cytokine profile of CD4 T-cells. Freshly isolated CD4 T-cells were incubated with anti-CD3/CD28 coated beads in presence or absence of DNT-cells. **(A,B)** On day 6 of co-culture cells were re-stimulated with PMA/Ionomycin in the presence of monensin. Expression of cytokines was determined by intracellular flow cytometry staining. **(A)** Representative dot plots were gated for viable CD4 T-cells. **(B)** Graph represent mean percentages+/– SEM of IFN-γ+, IL-2+, GM-CSF, and IL-17A+ CD4 T-cells of at least seven independent experiments. **(C)** On day 6 of co-culture CD4 T-cells were re-isolated by magnetic sorting and stimulated with PMA/Ionomycin for 6 h. Secretion of effector cytokines in the supernatant was analyzed for of IFN-γ, IL-2, and IL-17A by cytometric bead array. Graphs show concentration +/– SEM of indicated cytokines, data represent results of at least seven independent experiments. ns, not significant, $^{*}p < 0.05$, $^{**}p < 0.01$.

only efficiently suppress proliferation of CD4 T-cells (9, 30), but also modulate their metabolic programming and functionality. These findings unveil new mechanisms how DN T-cells can affect induction or maintenance of peripheral tolerance. Notably, DN T-cells have emerged as a promising therapeutic option for a number of entities including GvHD after allo-HSCT. In murine models, DN T-cells were demonstrated to restrict the development of GvHD while mediating beneficial anti-leukemic effects (18). Moreover, the transfer of human DN T-cells in a humanized mouse model was shown to delay onset of xenogeneic GVHD (21). Clinical studies in patients after allo-HSCT revealed an inverse correlation between DN T-cell levels and severity of both acute and chronic GvHD (19, 20), suggesting a therapeutic potential of human DN T-cells.

Using DN T-cells to modulate functionality of T-cells could offer an additional advantage over standard therapy

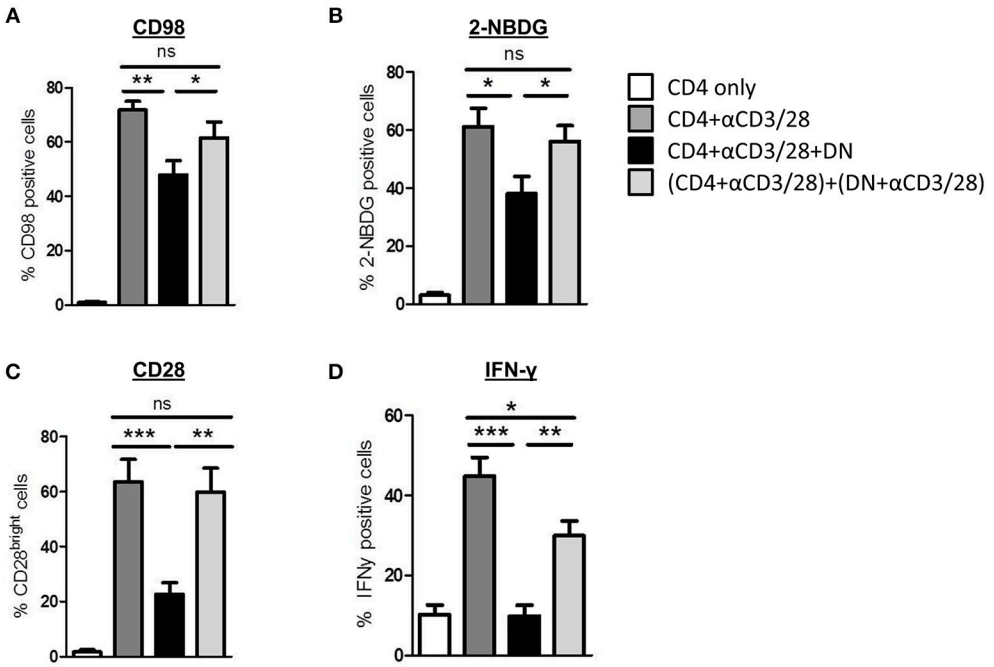

FIGURE 6 | DN T cell-mediated modifications of CD4 T cell phenotype, metabolism, and cytokine production are cell-cell-contact dependent. CD4 T-cells were incubated with anti-CD3/CD28 coated beads, DN T-cells were added directly to the culture or to the top chamber of a transwell system as described in *Materials and Methods*. **(A,B)** Expression of CD98 **(A)** and uptake of 2-NBDG **(B)** was assessed after 3 days, data show mean percentages +/– SEM of at least seven independent experiments. **(C)** Expression of CD28 was assessed after 6 days, data show mean percentages of CD28 bright-cells +/– SEM of seven independent experiments. **(D)** On day 6 of co-culture cells were stimulated with PMA/Ionomycin in the presence of monensin for 4 h. Expression of IFN-γ was determined by intracellular flow cytometry staining. Graphs illustrate mean percentages +/– SEM of IFN-γ on CD4 T-cells, data of seven independent experiments is shown. ns, not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

with immunosuppressive drugs in GvHD treatment. The metabolic master regulator mTOR integrates nutrient and energy sensing pathways and controls proliferation, differentiation and metabolism of T-cells. Dysregulation of mTOR signaling has been reported in various autoimmune diseases and transplantation settings and can be targeted by immunosuppressive drugs like sirolimus and everolimus (31, 32). Recent reports have clearly demonstrated that mTOR signaling induce metabolic reprogramming of alloantigen activated T-cells after allo-HSCT. Moreover, the authors have identified glycolysis as the predominant metabolic process used by alloreactive T-cells to promote GvHD (33). Our results showed that DN T-cells can especially inhibit the mTOR pathway in CD4 T-cells but spare other T-cell signaling pathways, as p38 and NFκB were unaffected. Consistent with this observation, we found that DN T-cells downregulate glucose metabolism and uptake in CD4 T-cells, whereas uptake of fatty acids was not affected. In the literature various molecular mechanisms have been described to regulate mTOR signaling and metabolic reprogramming of T-cells as for example the inhibitory molecules CTLA-4 and PD-1 engage distinct phosphatases to terminate mTOR phosphorylation (34, 35). Interestingly, the inhibitory receptor PD-1 has also been reported to block glycolysis but favors fatty acid oxidation in T-cells (36). Further analysis has to be done to determine the underlying mechanism of DN T-cells-mediated metabolic alterations. Anyhow, these findings are of particular interest as selective targeting of metabolic pathways in T-cells offers new opportunities to specifically suppress alloreactive T-cells (37).

Several studies have shown that cell metabolism determines T-cell fate and function. Notably, mTOR signaling and metabolic reprogramming are involved in the differentiation of memory and effector T-cells by regulation of the transcription factors T-bet and Eomes (38). We found that CD4 T-cells revealed elevated levels of Eomes but reduction of T-bet expression after co-culture with DN T-cells. Moreover, these cells displayed an altered phenotype with enhanced expression of CD27, CCR7, and CXCR5, which are described to be discriminatory for long-living central-memory T-cells (39–41). Of interest, Rapamycin-treated T-cells were reported to be dependent on oxidative phosphorylation and more prone to become long-living memory T-cells, suggesting that specific mTOR inhibition induce these phenotypic alterations (42).

Of particular importance was the observation that suppressed CD4 T-cells have different effector functions, namely decreased IFNγ, IL-17A, and GM-CSF levels but amplified IL-2 production. Altered secretion of effector cytokines by CD4 T-cell due to DN T-cells could have important implications for the onset of GvHD in a clinical setting after allo-HSCT. The main Th1 effector cytokine IFN-γ plays an essential role in the induction of GvHD

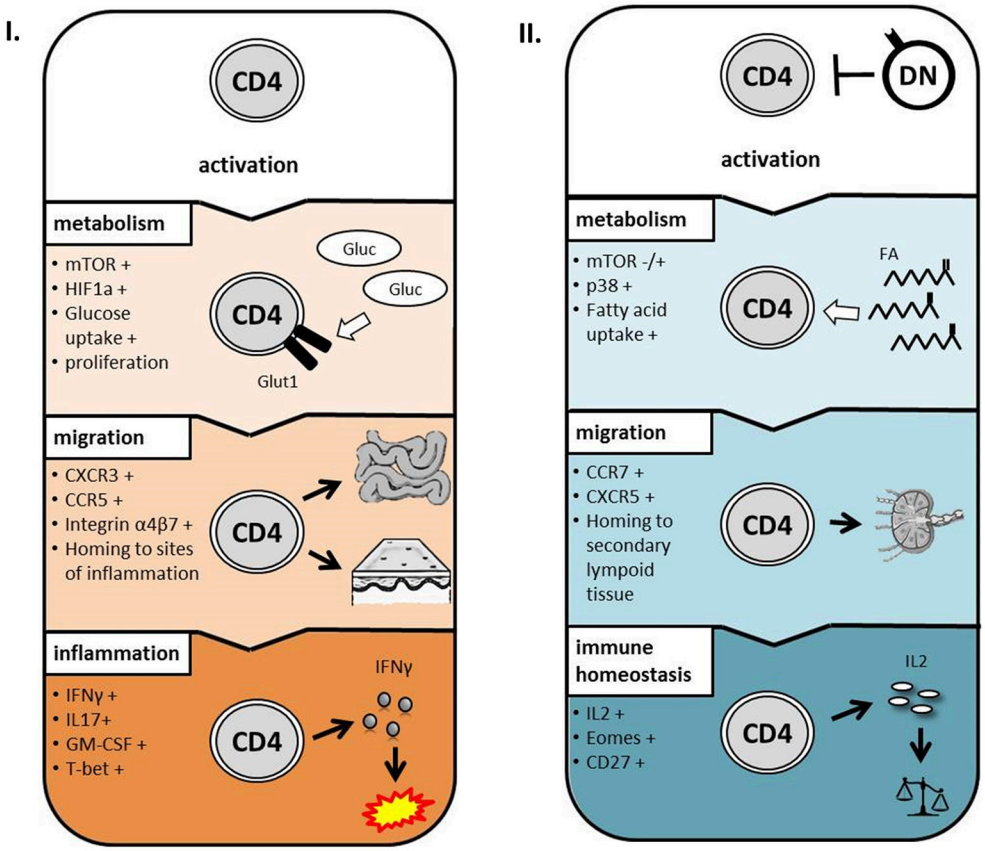

FIGURE 7 | Proposed model for the impact of DN T-cells on CD4 T-cells. After activation, CD4 T-cells upregulate mTOR signaling, glucose metabolism, and chemokine receptors to inflammatory sites resulting in inflammatory conditions **(I.)**. In presence of DN T-cells, metabolism, and migratory capacity of CD4 T-cells are altered during activation resulting in immune homeostasis **(II.)**.

as grafts of IFN-γ-gene knockout donors could not cause GvHD in recipient mice (43) and, in turn, GvHD could not be induced in IFN-γ-signaling deficient mice (44). These defects in cytokine production are in support of the idea that DN T-cells impair glucose metabolism as aerobic glycolysis is fundamental for IFN-γ translation (45). In addition, IL-17A and GM-CSF secretion of CD4 T-cells were diminished by DN T-cells. Both IL-17A serum levels and Th17 infiltrating cells are associated with GvHD after allo-HSCT (46, 47). IL-17A expression is associated with glucose metabolism as the transcription factor HIF-1α controls the activation of the IL-17A promoter and Th17 differentiation (48). Moreover, a recent study has demonstrated GM-CSF producing T-cells to be sufficient to promote GvHD (49). In contrast, our data revealed enhanced IL-2 production by CD4 T-cells after co-culture with DN T-cells. IL-2 is required for T-cell activation, differentiation, and survival but can also be favorable as IL-2 selectively can restore the immunosuppressive function of FoxP3 Tregs without activation of T-cells or abrogation of anti-leukemia effects (50). In addition to cytokines, chemokines also play a crucial role in T-cell effector function. Indeed, the pro-inflammatory chemokine receptors CXCR3 and CCR5 are involved in the induction of GvHD by orchestrating the migration and infiltration of effector T-cells to their target tissue (28, 29). Moreover, the integrin α4β7 is exclusively responsible for alloreactive T-cells homing to the gut (51). Of note, an antagonist of CCR5 (Maraviroc) or therapeutic antibodies against α4β7 (Vedolizumab, Natalizumab) are currently examined in clinical trials and raise hopes for a novel therapy to treat GvHD (52, 53). Our findings suggest that DN T-cells also have potential to shift the expression of chemokine receptors on CD4 T-cells and thus induce homing to secondary lymphoid organs rather than sites of inflammation. Since coordinated migration of cells by chemokine receptors is required for the appropriate execution of T-cell effector function, DN T-cells open up another possibility to interfere with T-cell function after allo-HSCT.

In summary, T-cells do not maintain their naïve phenotype after DN T-cell co-culture, but display characteristics akin to long-living central-memory T-cells. Our observations shed light on the molecular process of DN T-cell-mediated suppression, since the T-cells are not rendered senescent or anergic. This is in contrast with recent studies that have shown FoxP3+ Tregs to induce senescence in T-cells and FoxP3+ Treg-treated T-cells do not exhibit a similar modulation of surface molecules or cytokine production (54). Furthermore, cellular-based therapy

after HSCT with FoxP3+ Tregs has been already tested. Initial clinical studies obtained first incidence that adoptive transfer of *ex vivo* expanded FoP3+ Tregs can prevent GvHD (6, 7). DN T-cells are another promising regulatory T-cell subset, which not only abolishes but modulates target cell function. In addition, DN T-cells also might be able to support FoxP3+ Tregs after adoptive transfer by enhancing IL-2 production of T-cells.

In conclusion, our results reveal new and various targets of DN T-cells to selectively modulate signaling and metabolic programming of T-cells resulting in functional altered effector cells. These findings could pave the way to use DN T-cells for cellular therapy as an alternative treatment strategy to prevent and diminish GvHD after allo-HSCT.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Ethics committee of the University Erlangen-Nuremberg with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Ethics committee of the University Erlangen-Nuremberg (protocol number 284-18 Bc).

AUTHOR CONTRIBUTIONS

TH, AM, and SV designed the research. TH, MA, MP, CS, and SV performed experiments. TH, MA, KH, DM, HB, AM, and SV analyzed and interpreted data. TH, AM, and SV wrote the manuscript. HB, DM, and AM provided advice and revised the manuscript. All of the authors edited the manuscript.

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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.2019.00883/full#supplementary-material

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

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Dynamics of Expression of Programmed Cell Death Protein-1 (PD-1) on T Cells After Allogeneic Hematopoietic Stem Cell Transplantation

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Immune exhaustion contributes to treatment failure after allogeneic hematopoietic stem cell transplantation (HSCT) for hematological malignancies. Immune checkpoint blockade, including programmed cell death protein-1 (PD-1) blockade, is a promising strategy to improve the antitumor effect of allogeneic HSCT with high rates of response reported in patients treated for disease relapse. However, severe and sometimes fatal Graft- vs.-Host-Disease (GvHD) has been reported as a complication. Little is known about the dynamics of PD-1 expression on immune effector cells after allogeneic HSCT. In the present study, we analyzed PD-1 expression on T cell subpopulations isolated from 105 allogeneic HSCT recipients. Our analysis revealed a significant increase in proportions of PD-1-expressing CD4 and CD8 T cells early after allogeneic HSCT followed by a progressive normalization of PD-1 expression at CD8 but not CD4 T cell surface. Analysis of co-expression of two other exhaustion markers, 2B4 and CD160, revealed a preferential expansion of PD-1-single positive cells. Moreover, the analysis of granzyme B and perforin expression in PD-1+ and PD-1- CD8 T cells from HSCT recipients did not reveal any impairment in cytotoxic molecules production by PD-1-expressing CD8 T cells. Analyzing the association between clinical factors and the expression of PD-1 on T cells, we identified the use of *in vivo* and/or *ex vivo* T-cell depletion as the factor most strongly associated with elevated PD-1 levels on T cells. Our results extend our knowledge of the regulation of PD-1 expression at T cell surface after allogeneic HSCT, a crucial information for the optimization of post-transplantation PD-1 blocking therapies.

Keywords: PD-1, checkpoint inhibitors, HSCT, transplantation, exhaustion

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) is a well-established therapeutic modality for a broad variety of hematological malignancies, unfortunately still associated with a significant risk of cancer relapse. Mechanisms of disease relapse after allogeneic HSCT include resistance to chemotherapy but also escape of tumor cells from the control of the alloreactive

immune responses (1). Immune exhaustion of donor-derived immune cells contributes to treatment failure and immune checkpoint blockade is a promising strategy to improve the antitumor effect of the transplantation procedure (2, 3). Several studies reported the association of programmed cell death protein-1 (PD-1) up-regulation at T-cell surface with disease relapse (4–7) and high rates of antitumor responses have been reported in patients treated with programmed cell death protein-1 (PD-1)/programmed death-ligand 1 (PD-L1) blockade administered for disease relapse after allogeneic HSCT (8–10). However, the PD-1/PD-L1 axis plays an important role in maintenance of immune tolerance after allogeneic HSCT as revealed by preclinical studies (11–14) and supported by the high incidence of severe, and in some cases fatal, Graft-vs.-Host-Disease (GvHD) in patients receiving PD-1 blockade for post-transplant relapse (8–10). Little is known about the dynamics of expression of PD-1 at immune effectors' cell surface during immune-reconstitution after transplantation, a crucial information for the optimization of antibody-based PD-1/PD-L1 blockade therapies in the context of allogeneic HSCT. In the present study, we measured PD-1 expression on T cells subpopulations isolated from 105 patients analyzed at different time points after allogeneic HSCT. Our analysis revealed an early and long lasting increase in proportions of PD-1+ CD4 T cells after allogeneic HSCT while we observed only a transient increase in PD-1+ CD8 T cells. Moreover, we identified the use of *in vivo* and/or *ex vivo* T-cell depletion as the clinical factor most strongly associated with elevated PD-1 levels on T cells.

PATIENTS AND METHODS

Study Design and Patients' Characteristics

We prospectively analyzed 148 samples isolated from 105 patients who underwent allogeneic HSCT at our center and were seen at follow-up visits in our outpatient clinic between November 2015 and November 2016. Samples from 24 age-matched, sex-matched healthy blood donors were used as controls. Written informed consent was provided by all individuals enrolled in the study and the study was approved by the ethics committee of the Geneva University Hospitals. Clinical data were retrospectively extracted from patient's medical records. Patients' characteristics are summarized in **Table 1**. Forty-nine patients (47%) received grafts from an HLA-identical sibling (SIB) and 39 patients (37%) from an HLA-matched unrelated donor (MUD), whereas 3 (3%) patients received grafts from an HLA-mismatched unrelated donor (MMUD). Fourteen patients (13%) received grafts from haploidentical donors. Myeloablative conditioning (MAC) mostly consisted of cyclophosphamide (120 mg/kg) in combination with busulfan (12.8 mg/kg) or total body irradiation (10–12 Gy). Reduced-intensity conditioning (RIC) mainly consisted of fludarabine (120 mg/m^2) associated with low dose busulfan (6.4 mg/kg) or melphalan (140 mg/m^2). Most patients (82 patients, 78%) received some form of *in vivo* and/or *ex vivo* T-cell depletion (TCD). *In vivo* TCD by anti-thymocyte globulin (ATG) (Thymoglobulin® 7.5 mg/kg or ATG-Fresenius® 25 mg/kg) was part of conditioning for all patients treated with RIC and for patients receiving grafts from an unrelated donor after a MAC. *Ex vivo* partial TCD was obtained through grafts incubation with alemtuzumab (Campath [Genzyme Corporation, Cambridge, MA]) *in vitro* washed before infusion, administered at day 0,

TABLE 1 | Clinical characteristics of HSCT recipients.

	Patients n = 105	
AGE	**YEARS (RANGE)**	
Age at transplant	50 (18–70)	
SEX	**n**	**%**
Male	65	62
Female	40	38
PRIMARY DISEASE	**n**	**%**
AML	36	34
ALL	11	10
Lymphoma/CLL	16	15
MDS	15	14
MPS/MDPS	11	10
Multiple Myeloma	4	4
CML	7	7
Aplastic Anemia	4	4
Other	1	1
CELL SOURCE	**n**	**%**
PBSC	88	84
BM	17	16
DONOR TYPE	**n**	**%**
SIB	49	47
MUD	39	37
MMUD	3	3
Haploidentical	14	13
CONDITIONING REGIMEN	**n**	**%**
MAC	58	55
RIC	47	45
T-CELL DEPLETION (TCD)	**n**	**%**
No TCD	23	22
ATG	29	28
Ex vivo	14	13
ATG + Ex vivo	25	24
Post-Tx Cy	14	13
IMMUNOSUPPRESSION	**n**	**%**
CSA	4	4
CSA, MMF	41	39
CSA, MTX	38	36
Tacrolimus, MTX	8	8
Tacrolimus, MMF, Cy	14	13
CMV SEROSTATUS	**n**	**%**
Recipient+	64	61
Donor+	60	57

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; ATG, Anti-thymocyte globulin; BM, bone marrow; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; CSA, cyclosporine; MAC, myeloablative conditioning; MDS, myelodysplastic syndrome; MMF, mycophenolate mofetil; MMUD, mis-matched unrelated donor; MPS, myeloproliferative syndrome; MTX, methotrexate; MUD, matched unrelated donor; PBSC, peripheral blood stem cells; RIC, reduced-intensity conditioning; Post-Tx Cy, post-transplant Cyclophosphamide; SIB, matched related sibling; TCD, T-cell depletion.

followed on day +1 by an add-back of donor T cells (usually 100×10^6/kg donor T cells) (15). Twenty-nine patients (28%) received ATG alone, 14 patients (13%) *ex vivo* TCD alone and 25 patients (24%) both ATG and *ex vivo* TCD. Fourteen patients (13%) receiving grafts from haploidentical donors were treated with post-transplantation cyclophosphamide as *in vivo* TCD as previously described (16). Graft- vs.-host disease (GvHD) prophylaxis mainly consisted of cyclosporine (for 3 months duration in the absence of GvHD in the case of partial T cell depletion and for 6 months for T cell–replete graft recipients) in combination with either methotrexate, in case of MAC regimen, or mycophenolate mofetil for patients transplanted after RIC. *Ex vivo* TCD graft recipients also received methylprednisolone on days −2 and −1. Donor lymphocyte infusions (DLI) at incremental doses (starting with 5×10^5 CD3/kg for unrelated- and 1×10^6/kg for related donors) were given at 3 months to all patients who had received *ex vivo* TCD grafts with RIC in absence of GvHD. Acute or chronic GvHD was treated with corticosteroids alone or in combination with mycophenolate mofetil and/or cyclosporine.

Flow Cytometry Analysis

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh anticoagulated blood by Ficoll density gradient centrifugation. PBMCs were then stained and analyzed using a ten-color flow cytometry panel. The following conjugated antibodies were used for surface staining: anti-CCR7 (FITC), anti-CD57 (PE-CF594), anti-CD45RA (allophycocyanin-H7), and anti-CD3 (V500) were from BD Biosciences (San Jose, CA); anti-PD-1 (PE), anti-2B4 (PerCp-Cy5.5), anti-CD160 (APC), anti-CD27 (Alexa Fluor 700), anti-CD4 (PECy7), anti-CD8 (BrillantViolet421) were from Biolegend. Intracellular staining for cytotoxic molecules was performed using anti–granzyme B (Alexa Fluor 700, clone GB11 [BD Biosciences]) and anti-Perforin (FITC, clone B-D48 [Diaclone]) on fixed and permeabilized cells following manufacturer's instructions (eBioscience). Samples were acquired on Gallios 3 cell analyzer (BD Biosciences), and data files were analyzed using FlowJo software (Tree Star).

Statistical Analysis

Statistical analysis was performed using Prism version 7 (GraphPad Inc), R version 3.5.1 (Comprehensive R Archive Network (CRAN) project (http://cran.us.r-project.org) with R studio Version 1.1.453 and EZR version 1.37 (Saitama Medical Center, Jichi Medical University, Saitama, Japan) (17). *P*-values <0.05 were considered statistically significant.

RESULTS

PD-1 Expression Is Differentially Regulated at CD4 and CD8 T Cell Surface After Allogeneic HSCT

We first analyzed the expression of PD-1 at the surface of CD4 and CD8 T cells from healthy control subjects (HC) and from patients after HSCT. HSCT recipients displayed significantly higher proportions of PD-1 expressing CD4 [median (range), 61% (15–97%)] and CD8 [38% (6–100%)] T cells compared with HC [CD4 19% (7–31%), $p < 0.0001$; CD8 32% (8–56%), $p = 0.0124$] (**Figures 1A,B**). Given the severe immune homeostasis alteration present immediately after HSCT because of the severe lymphopenia and the pro-inflammatory environment secondary to the conditioning regimens, we next investigated whether the observed increase in PD-1 expression at T cell surface was only a transient or rather a sustained, long-lasting T cell abnormality after HSCT. We found a significant negative correlation between the time elapsed since transplantation and the proportion of PD-1 expressing CD4 ($r = -0.3755$, $p < 0.0001$; **Figure 1A**) and CD8 ($r = -0.3176$, $p < 0.0001$; **Figure 1B**) T cells. Interestingly, we observed a significantly higher proportion of PD-1+ CD4 T cells isolated from HSCT recipients compared with HC at all-time points studied including patients studied more than 5 years post-transplantation (**Figure 1A**). Conversely, CD8 T cells isolated from patients at 1 and 3 months post-transplantation exhibited increased levels of PD-1 expression compared to healthy controls while we failed to detect any significant difference between HSCT and HC at later time points (**Figure 1B**). Immune reconstitution after HSCT is associated with an altered distribution of T cell subsets, with an over-representation of effector/memory cells and a reduction in naïve cells (**Figures 2A,B**). As PD-1 is constitutively expressed at higher proportions on effector/memory cells compared to naïve cells, we analyzed PD-1 expression in T cell subpopulations to determine whether the observed increase in PD-1 positive T cells in HSCT recipients is a mere consequence of the increased proportions of effector/memory subsets or an actual up-regulation of PD-1 at T cell subsets surface. When T cell subsets heterogeneity was taken into account, we observed higher proportions of PD-1 expressing cells in all effector/memory CD4 T cell subsets from HSCT recipients, including CD45RA- CCR7+ CD27+ central memory (CM), CD45RA- CCR7- CD27+ transitional memory (TM) and CD45RA- CCR7- CD27- effector memory (EM) (**Figure 2C**). Interestingly, such increase was sustained during time, PD-1 expression being significantly higher in HSCT recipients even after more than 24 months since transplantation. Conversely, among CD8 T cell subsets we only observed a significant PD-1 up-regulation within CD8 CM T cells from HSCT recipients during the first 6 months after transplantation, while no difference was observed in TM, EM and effector memory re-expressing CD45RA (TEMRA) at any of the time points studied (**Figure 2D**). Collectively these results point to different dynamics of PD-1 expression in CD4 and CD8 T cells after HSCT, CD4 T cells up-regulating PD-1 expression on all effector/memory cells for more than 2 years after transplantation, CD8 T cells only transiently expressing higher proportions of PD-1 as a consequence of the effector/memory skewing after transplantation as well as of a transient PD-1 overexpression on CM cells.

PD-1 Expression at T Cell Surface After HSCT Is Only Partially Associated With Co-Expression of Other Exhaustion Markers

PD-1 represents the most accepted marker of T cell exhaustion identified so far and the only one being a direct target of

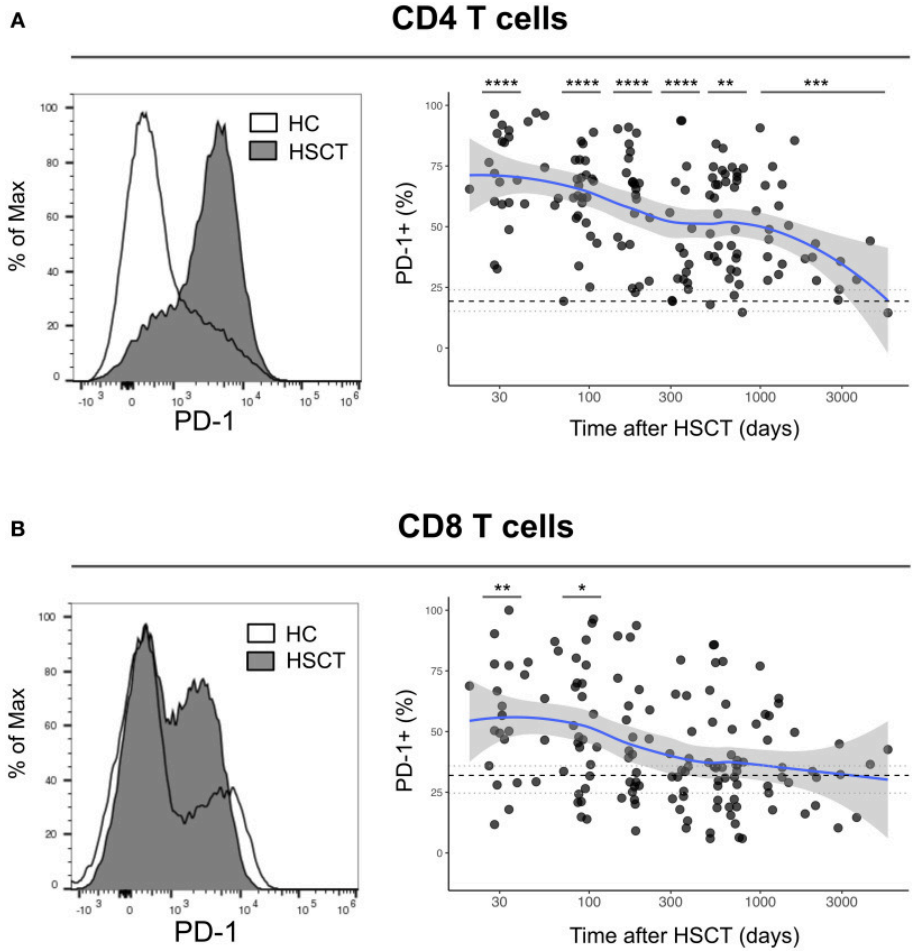

FIGURE 1 | Dynamics of PD-1 expression on CD4 and CD8 T cells after allogeneic HSCT. **(A,B)** Histograms representing PD-1 expression on CD4 **(A)** or CD8 **(B)** T cells from HC (open line) or a HSCT recipient at day 30 post-HSCT (gray filled histogram). Graphs represent proportions of PD-1 expressing CD4 **(A)** or CD8 **(B)** T cells from HSCT recipients and their relationship with time since HSCT. Dark gray dots represent each sample, blue lines represent medians and the gray area represents the confidence interval. Median (black dashed line), 25th and 75th percentiles (gray dotted lines) of PD-1 expressing T cells from HC are represented. The *p*-values of expression in HSCT recipient groups at 1, 3, 6, 12, and >24 months compared with HC are indicated (Mann-Whitney *U*-test; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

approved immunotherapeutic strategies. However, PD-1 is also up-regulated during T cell activation and co-expression with other surface markers, including 2B4 and CD160, seems to better identify truly exhausted cells (18–20). We, therefore, assessed the expression of 2B4 and CD160 on T cells after HSCT (**Figure 3A**, left panels). No difference in the expression of 2B4 was detected on CD4 or CD8 T cells from HSCT recipients compared with HC (**Figure 3A**, middle panels). Conversely, we observed slight but significant up-regulation of CD160 on T cells from HSCT recipients compared to HC, on CD4 at all-time point studied and on CD8 T cells recovered at 1 month as well as more than 24 months post-transplantation (**Figure 3A**, right panels). We next analyzed the co-expression of the exhaustion markers PD-1, 2B4, and CD160 in T cells from HSCT recipients. As shown in **Figures 3B,C**, we observed a strong increase in the proportion of single positive PD-1 CD4 T cells with only limited 2B4 and CD160 co-expression (**Figures 3B,C**, upper panels). Interestingly, the expansion of this PD-1-single positive CD4 T cells was greatest at early time points but still persisted more than 24 months after transplantation. Conversely, the increased PD-1 expression in CD8 T cells was secondary to the expansion of both PD-1 single positive cells and PD-1 cells co-expressing 2B4 and/or CD160 (**Figures 3B,C**, lower panels). Importantly, this analysis confirmed the transient nature of the increase in PD-1 expressing CD8 T cells after transplantation (**Figures 3B,C**). Collectively, these results confirm the minimal and transient increase in PD-1 expression on CD8 T cell subsets and show a sustained expansion of single positive PD-1-expressing CD4 T cells without any accompanying up-regulation of the other T cell exhaustion markers 2B4 and CD160.

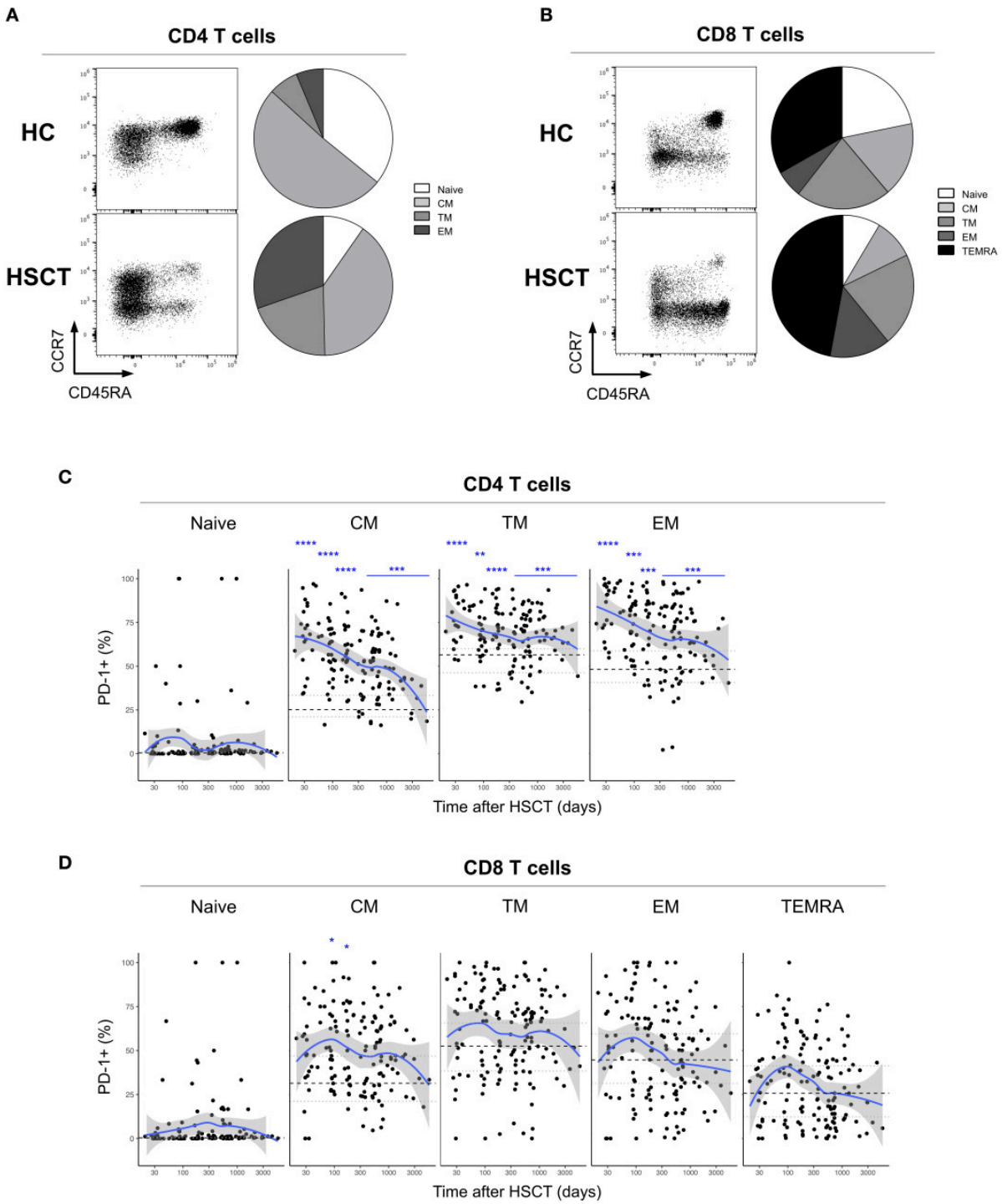

FIGURE 2 | PD-1 expression on CD4 and CD8 T cell subsets after allogeneic HSCT. **(A,B)** Representative FACS dot plots and pie charts representing the proportions of T cell subsets identified based on CD45RA, CCR7, and CD27 expression (Naive: CD45RA+ CCR7+ CD27+; central memory (CM): CD45RA- CCR7+ CD27+; transitional memory (TM): CD45RA- CCR7- CD27+; effector memory (EM) CD45RA- CCR7- CD27-; effector memory re-expressing CD45RA (TEMRA): CD45RA+ CCR7- CD27-). Different colors correspond to subsets as indicated. **(C,D)** proportions of PD-1 expressing CD4 **(C)** or CD8 **(D)** T cell subsets from HSCT recipients (black filled circles) and their relationship with time since HSCT. Blue lines represent medians and the gray area represents the confidence interval. Median (black dashed line), 25th and 75th percentiles (gray dotted lines) of PD-1 expressing T cells from HC are represented. The *p*-values of expression in HSCT recipient groups at 1, 3, 6, 12, and >12 months compared with HC are indicated (Mann-Whitney *U*-test; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

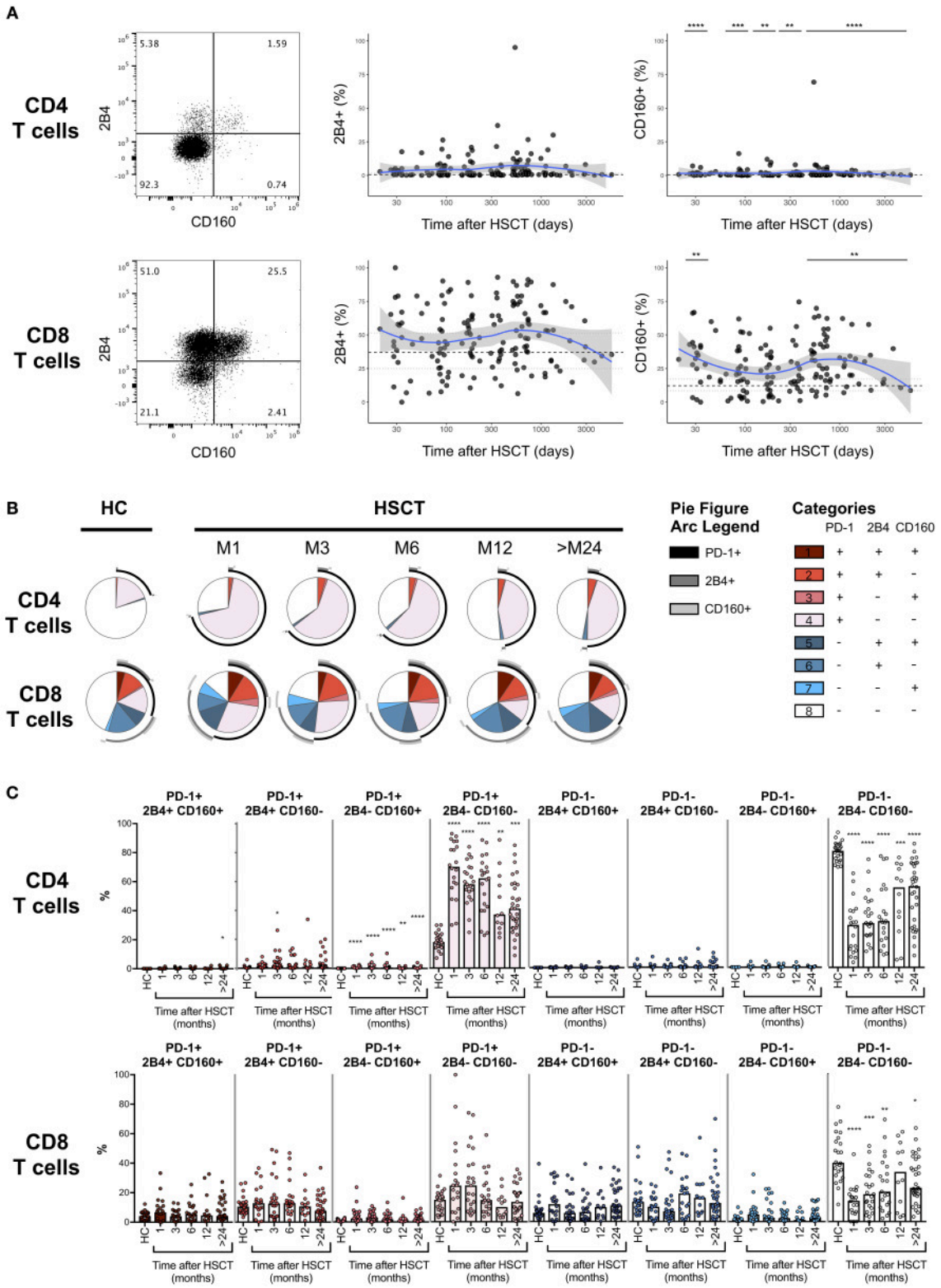

FIGURE 3 | Co-expression of PD-1, 2B4, and CD160 on CD4 and CD8 T cells after allogeneic HSCT. **(A)** Representative dot plots (left panels) and proportions of 2B4 and CD160 expressing CD4 or CD8 T cell subsets from HSCT recipients (black filled circles) and their relationship with time since HSCT. Blue lines represent medians and the gray area represents the confidence interval. Median (black dashed line), 25th and 75th percentiles (gray dotted lines) of 2B4 and CD160 expressing T cells

(Continued)

FIGURE 3 | from HC are represented. The *p*-values of expression in HSCT recipient groups at 1, 3, 6, 12, and >24 months compared with HC are indicated (Mann-Whitney *U*-test; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$). **(B)** Pie charts representing co-expression of 2B4 and CD160 by PD-1 negative (blue gradient colors) and PD-1 positive (red gradient color) CD4 (upper pies) or CD8 (lower pies) T cells. Arcs represent single molecules expression accordingly to the indicated colors. **(C)** Proportions of PD-1, 2B4, and CD160 co-expressing CD4 or CD8 T cell subsets in HC and HSCT recipients. The *p*-values of expression in HSCT recipient groups at indicated time compared with HC are shown (Kruskal-Wallis test; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$).

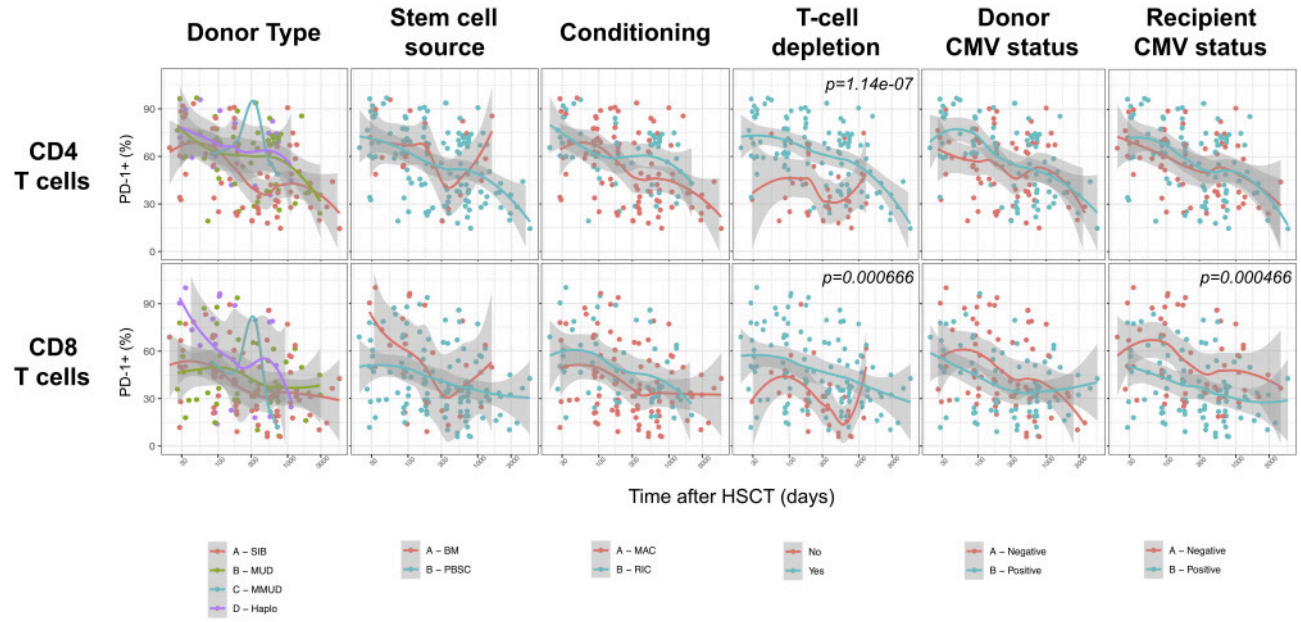

FIGURE 4 | Impact of clinical factors on PD-1 expression at CD4 and CD8 T cells surface after allogeneic HSCT. Comparison of PD-1 expression on CD4 and CD8 T cells from HSCT recipients depending on donor type, stem cell source, conditioning, T-cell depletion and donor/recipient CMV status. The *p*-values resulting from the multivariable linear regression analysis are indicated when significant.

PD-1 Expression at CD8 T Cell Surface Is Not Associated With Impaired Cytotoxic Molecules Production After Allogeneic HSCT

To assess whether PD-1 expression at CD8 T cell surface was potentially associated with functional impairment, we analyzed the expression of the cytotoxic molecules granzyme B and perforin in CD8 T cells isolated from an independent cohort of 10 healthy controls and 10 patients within 12 months since HSCT. As shown in **Supplemental Figure 1A**, no difference was observed between PD-1+ and PD-1- CD8 T cells in terms of perforin and granzyme B expression. Interestingly, both PD-1+ and PD-1- CD8 T cells from HSCT recipients expressed higher levels of perforin and granzyme B compared with their counterparts from HC (**Supplemental Figure 1A**) potentially reflecting the different distribution in CD8 T cell subsets in HSCT recipients. We therefore assessed perforin and granzyme B expression within CD8 effector and memory T cell subsets, namely CM, EM and TEMRA. We did not perform this analysis within the naïve CD8 T cell compartment as, in agreement with data reported in **Figure 2D**, this cell subset was virtually deprived of PD-1+ cells. As represented in **Supplemental Figures 1B–D**, we did not observe any reduction in perforin or granzyme B content in PD-1+ CM, EM and TEMRA CD8 T cells compared to their PD-1- counterparts. Interestingly, PD-1+ CM CD8 T cells displayed significantly higher levels of granzyme B compared to PD-1- CM CD8 T cells both in healthy controls and HSCT recipients (**Supplemental Figure 1B**). Collectively, these data do not support the existence of any impairment in cytotoxic molecules production in PD-1+ CD8 T cells recovered from both healthy controls and HSCT recipients.

T-Cell Depletion Induces Further PD-1 Up-Regulation on T Cells After Allogeneic HSCT

We next assessed the impact of clinical factors on PD-1 expression levels on T cells performing a multivariable linear regression analysis taking into account time elapsed since transplantation, stem cell source, donor type, conditioning regimen, recipient/donor CMV serostatus and use of T-cell depletion. This analysis confirmed the inverse relationship between the time elapsed since HSCT and PD-1 expression on both CD4 ($p < 0.0001$) and CD8 ($p = 0.007$) T cells. We found no association of donor/recipient matching, stem cell source, type of conditioning regimen and donor CMV

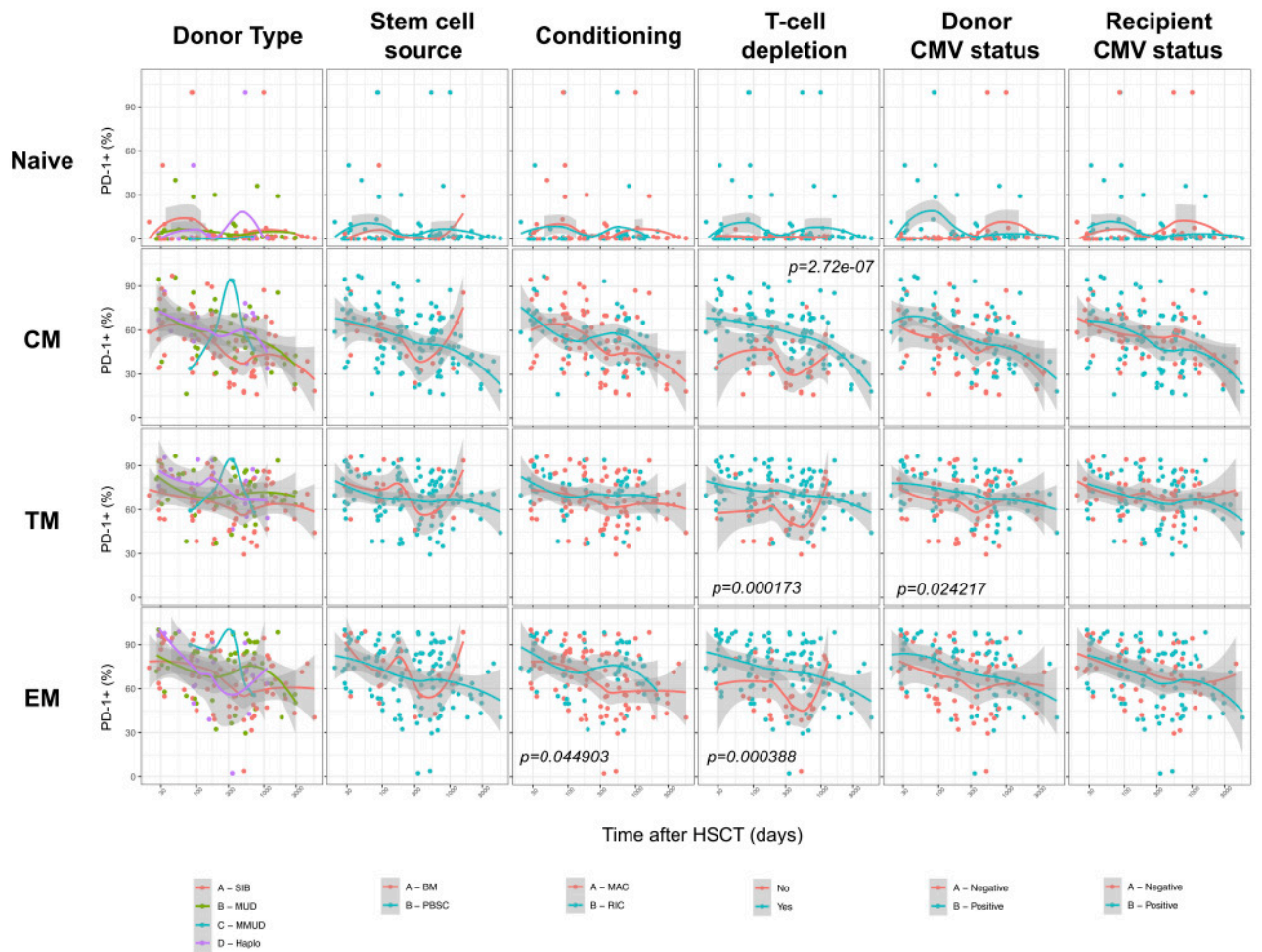

FIGURE 5 | Impact of clinical factors on PD-1 expression at CD4 T cell subsets surface after allogeneic HSCT. Comparison of PD-1 expression on CD4 T cell subsets from HSCT recipients depending on donor type, stem cell source, conditioning, T-cell depletion and donor/recipient CMV status. The *p*-values resulting from the multivariable linear regression analysis are indicated when significant.

serostatus with PD-1 expression on CD4 and CD8 T cells (**Figure 4**). Positive recipient CMV serostatus was associated with a statistically significant reduction of PD-1 expression on CD8 (estimate ± Standard Error, -14 ± 4; $p = 0.0005$) but not CD4 T cells (**Figure 4**). Interestingly, use of T-cell depletion was strongly associated with increased PD-1 expression on both CD4 (23 ± 4; $p < 0.00001$) and CD8 (16 ± 4.5; $p = 0.0007$) (**Figure 4**). Given the altered cell subsets distribution after HSCT (**Figures 2A,B**), we performed similar analysis on CD4 (**Figure 5**) and CD8 (**Figure 6**) T cell subsets. Such analysis confirmed the significant impact of T-cell depletion on effector and memory CD4 T cell compartments (**Figure 5**) while failed to confirm this association in CD8 T cell subsets (**Figure 6**) suggesting that in this latter case T-cell depletion is affecting T cell subsets distribution more than PD-1 expression itself. Conversely, this analysis confirmed the association between a negative CMV serostatus in the recipient and higher levels of PD-1 in CD8 TM, EM and TEMRA cells (**Figure 6**). To further investigate the association between T-cell depletion and PD-1 expression at T cell surface, we analyzed the impact of different TCD methods. Use of *in vivo* TCD by ATG administration alone was associated with increased expression of PD-1 at CD4 (**Figure 7A**) but not CD8 T cell surface (**Figure 7B**). Conversely, *ex vivo* TCD, alone or in combination with *in vivo* ATG administration, was associated with strong PD-1 up-regulation on both CD4 and CD8 T cells. Finally, use of *in vivo* TCD by administration of post-transplantation cyclophosphamide in the context of HSCT from haploidentical donors was significantly associated with increased PD-1 expression on CD4 and CD8 T cells (**Figures 7A,B**). We performed a similar analysis to evaluate the potential contribution of immune

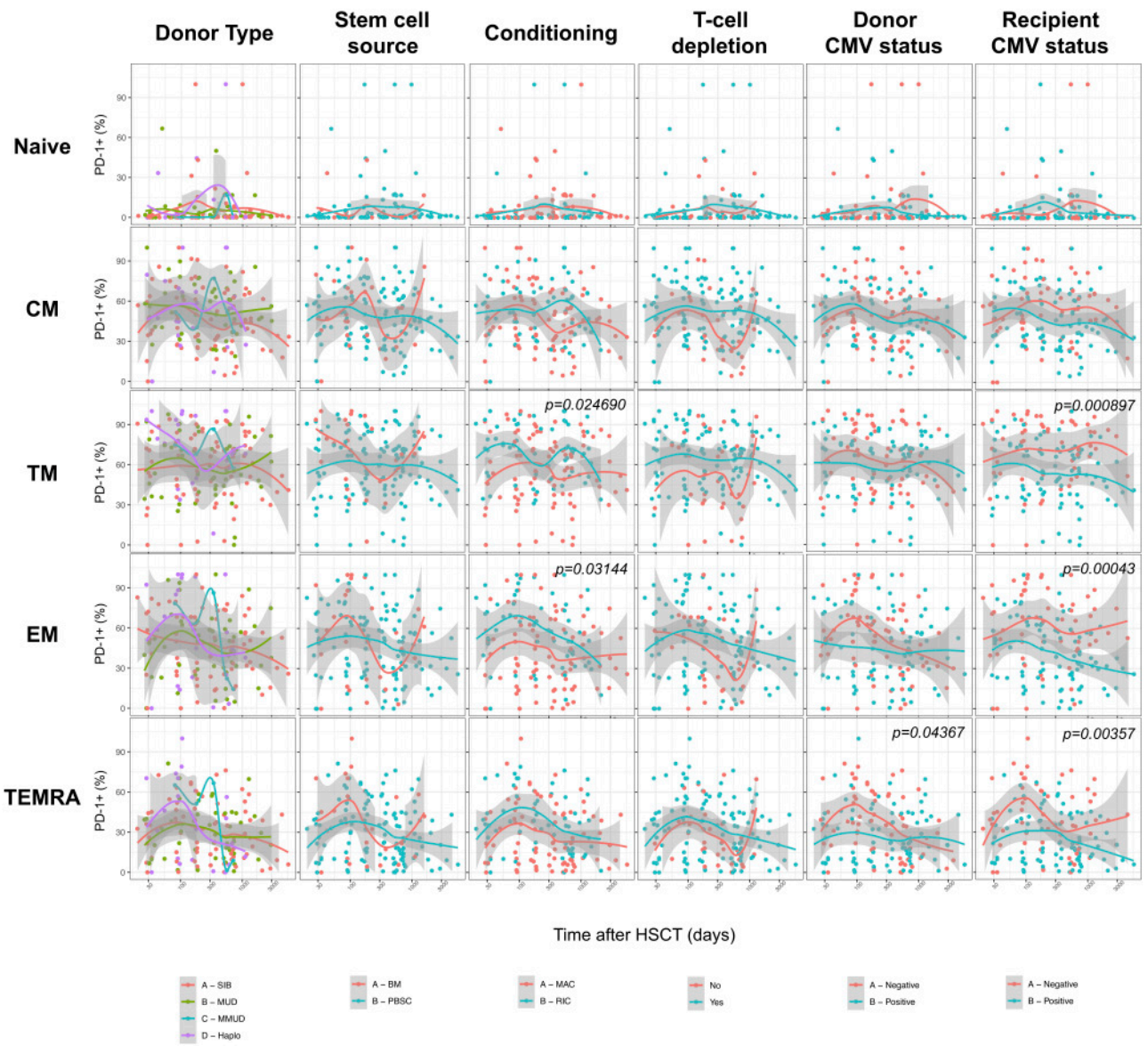

FIGURE 6 | Impact of clinical factors on PD-1 expression at CD8 T cell subsets surface after allogeneic HSCT. Comparison of PD-1 expression on CD8 T cell subsets from HSCT recipients depending on donor type, stem cell source, conditioning, T-cell depletion and donor/recipient CMV status. The *p*-values resulting from the multivariable linear regression analysis are indicated when significant.

suppression administered for GvHD prophylaxis on PD-1 expression (**Supplemental Figure 2**). This analysis revealed a weak, although significant positive association between the use of methotrexate-containing prophylaxis regimens and PD-1 expression in CD4 and CD8 T cells (**Supplemental Figure 2**). Collectively, these results demonstrate a strong association between the use of TCD and PD-1 upregulation on T cells after HSCT, suggesting a role for post-transplant lymphopenia in the regulation of PD-1 expression at T cell surface.

Limited Influence of GvHD on PD-1 Surface Expression on T Cells After Allogeneic HSCT

We finally analyzed the relationship between PD-1 expression at T cell surface and post-transplant complications. We failed to detect any significant difference in PD-1 expression on CD4 and CD8 T cells from patients with active acute GvHD (**Figure 8**). Conversely, chronic GvHD was associated with a slight but significant reduction in PD-1 expression at CD8

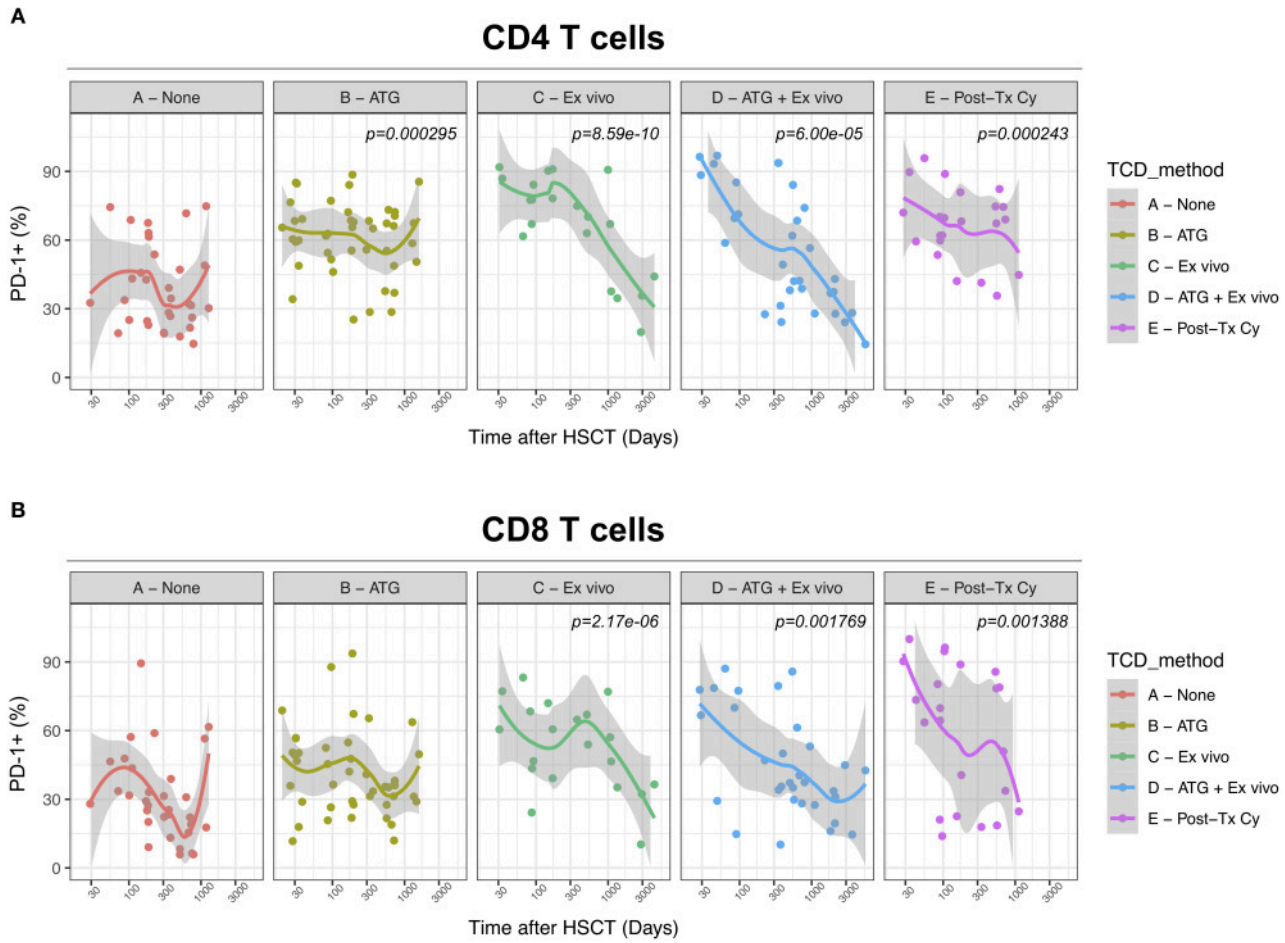

FIGURE 7 | Impact of different methods of T-cell depletion on PD-1 expression on CD4 and CD8 T cells after allogeneic HSCT. Comparison of PD-1 expression on CD4 **(A)** and CD8 **(B)** T cells from HSCT recipients receiving the indicated type of T-cell depletion. The *p*-values resulting from the multivariable linear regression analysis are indicated when significant.

but not CD4 T cell surface (**Figure 8**). The limited number of patients displaying an active disease relapse at the time of sampling in our cohort ($n = 5$) precluded any assessment of the relationship between this complication and PD-1 expression. Collectively, these results point to only a minor impact of GvHD on PD-1 expression levels on T cells after allogeneic HSCT.

DISCUSSION

Our prospective study revealed a significant increase in the proportions of PD-1-expressing CD4 and CD8 T cells at early phases after allogeneic HSCT followed by a progressive normalization of PD-1 expression at CD8 but not CD4 T cell surface. This expression pattern suggests that the targets of PD-1 blocking treatments might differ depending on time since transplantation at which this treatment is administered. According to our results, PD-1 blockade during the early time after transplantation would affect both CD4 and CD8 T cells. Conversely, administration at later time points, when PD-1 expression at CD8 T cell surface already returns to normal levels, would probably result mainly in anti-PD-1 binding on CD4 T cells. It is therefore tempting to speculate that the effects of PD-1/PDL-1 blockade might differ depending on the time of administration since HSCT with early administration eliciting both helper and cytotoxic T-cell responses while later administration essentially activating the CD4 helper compartment. Similarly, we can speculate that the risk of GvHD exacerbation following PD-1 blockade might differ depending on the time of administration, later administration being potentially associated with higher risk of GvHD development as a result of CD4 rather than CD8 stimulation. Detailed analysis of PD-1 expression at T cell surface during clinical trials investigating safety and efficacy of PD-1 blockade after allogeneic HSCT will address this question.

In our study, we analyzed the relationship between clinical factors at time of HSCT and PD-1 expression. Multivariable

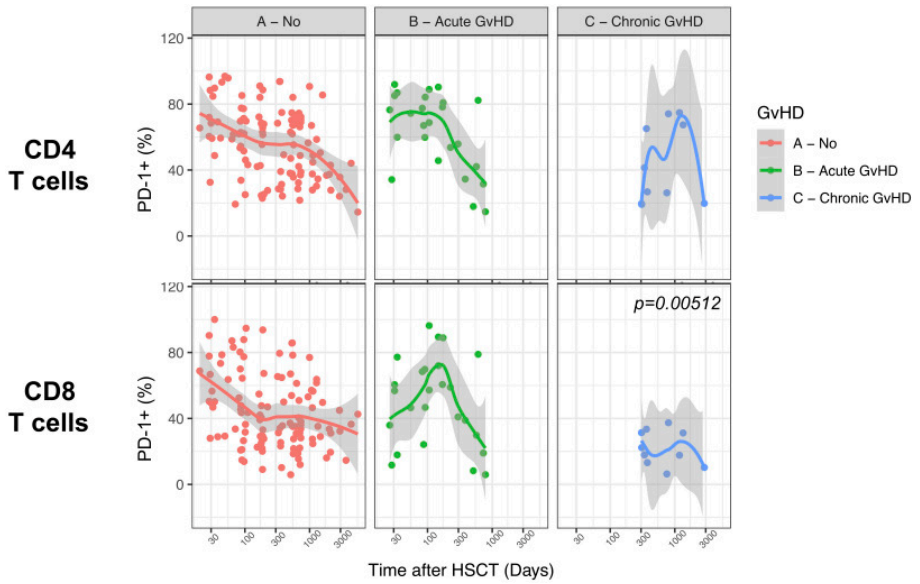

FIGURE 8 | Impact of acute and chronic GvHD on PD-1 expression on CD4 and CD8 T cells after allogeneic HSCT. Comparison of PD-1 expression on CD4 and CD8 T cells from HSCT recipients with no GvHD, acute GvHD or chronic GvHD. The *p*-values resulting from the multivariable linear regression analysis are indicated when significant.

linear regression analysis revealed the strongest association between T-cell depletion (TCD) and PD-1 expression at T cell surface, pointing to post-transplant lymphopenia as a major driver of PD-1 upregulation after HSCT. Our results are in agreement with a previous study by Beider et al. reporting higher levels of PD-1 expression on CD4+ CD25+ T cells isolated from HSCT recipients receiving ATG as part of the conditioning regimen compared with patients receiving no TCD (21). Interestingly the observed up-regulation of PD-1 in our cohort was consistent in all methods of TCD including *ex vivo* and *in vivo* TCD, employing either ATG or post-transplant cyclophosphamide. We can hypothesize that PD-1 up-regulation could contribute to the higher risk of relapse reported after TCD. In this case, HSCT recipients receiving TCD grafts could represent a patient group that might particularly benefit from PD-1 blocking strategies. These are hypotheses that need further investigation. Importantly, our multivariable analysis failed to show any association between donor/recipient HLA-matching with PD-1 expression, suggesting that alloreactivity is not necessary for PD-1 upregulation after HSCT. This result, together with the aforementioned impact of lymphopenia on PD-1 expression, is consistent with previous preclinical (22) and clinical (23) studies revealing PD-1 upregulation even after autologous HSCT.

PD-1 is up-regulated at T cell surface during activation (24) and in some cases has been shown to reflect immune activation rather than cell exhaustion in contexts of acute viral infection (25), cancer (26) and inflammatory disorders (27). Whether PD-1 expression at T cell surface after allogeneic HSCT reflects a true exhausted status or rather identifies activated cells remains unclear. The analysis of co-expression of other exhaustion markers, namely CD160 and 2B4, failed to detect any preferential expansion of cells co-expressing two or more exhaustion markers, supporting the hypothesis that the single-positive PD-1 compartment is mainly constituted of activated rather than exhausted T cells. A complementary analysis performed on a subsets of patients failed to show any differences between PD-1+ and PD-1- CD8 T cells in terms of effector cytotoxic molecules expression further supporting the hypothesis that, after allogeneic HSCT, PD-1 expression might mainly reflect a general cellular activation status. Further studies, characterizing PD-1 expressing cells from HSCT recipients at the transcriptomic (27) and epigenomic (28) level, will help to solve this question.

Our study has several limitations. First, the proportion of patients displaying an active disease relapse at time of sampling in our cohort was too small to draw any solid conclusions about the association between PD-1 expression at T-cell surface and disease relapse after HSCT that has been reported by several independent groups (4–6). Second, our analysis is limited to lymphocytes isolated from peripheral blood of HSCT recipients. The dynamics of PD-1 expression reported here might therefore not reflect the pattern of expression at tissue sites. In particular, bone marrow infiltrating T cells might display different PD-1 expression, more specific to this anatomic compartment. Several reports demonstrated in mice (12, 29) and humans (7, 30) that CD8 T cells infiltrating the bone marrow after allogeneic HSCT express significantly higher levels of PD-1 at their surface compared to peripheral blood cells, a difference that might derive

from the interaction with the micro-environment and/or with tumor cells in case of disease persistence or relapse. In a very elegant study comparing bone marrow-infiltrating T cells from HSCT recipients displaying relapse of acute myeloid leukemia after transplantation and from patients maintaining complete remission, Noviello et al. (7) recently reported higher proportions of T cells expressing inhibitory receptors, including PD-1, in relapsing patients than in patients maintaining complete remission. Moreover, by studying PD-1 co-expression with co-stimulatory molecules and T-box transcription factors, the authors further show that PD-1+Eomes+T-bet– phenotype in bone marrow-infiltrating CD8 T Memory Stem cells allows prediction of disease relapse (7).

In summary, our results indicate that the dynamics of PD-1 expression on T cells after allogeneic HSCT are differentially regulated in CD4 and CD8 T cells. These results suggest potentially different cellular targets, and consequently effects, depending on the time since transplantation at which PD-1/PD-L1 blockade may be used. Moreover, we identify the use of T-cell depletion as a major contributor to the induction of PD-1 upregulation on T cells after allogeneic HSCT. These results may have important implications for the optimization of PD-1/PD-L1 blocking therapies and stress the importance of performing detailed immune-monitoring studies during future clinical trials evaluating PD-1/PD-L1 blockade after allogeneic HSCT.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Commission Cantonale d'Ethique de la Recherche Scientifique de Genève with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Commission Cantonale d'Ethique de la Recherche Scientifique de Genève (Protocol #12–138).

AUTHOR CONTRIBUTIONS

FS conceived and coordinated the study, analyzed the data, prepared the figures and wrote the manuscript. AP and CB performed the experiments and critically revised the manuscript. SM-L, CD, AK, and YT collected the clinical data and critically revised the manuscript. ER and YC conceived the study, provided overall guidance, and edited the manuscript.

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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.2019.01034/full#supplementary-material

Supplemental Figure 1 | Perforin and Granzyme B expression in PD-1+ and PD-1- CD8 T cells. Proportions of perforin (left panels) and granzyme B (right panels) expressing cells among PD-1+ and PD-1- CD8 T cells **(A)** and CD8 T cell subsets **(B–D)** from HC (white filled dots) or HSCT recipients (black filled dots). The *p*-values are indicated when significant (Wilcoxon signed-rank test).

Supplemental Figure 2 | Impact of different immunosuppressive drugs for GvHD prophylaxis on PD-1 expression on CD4 and CD8 T cells after allogeneic HSCT. Comparison of PD-1 expression on CD4 (**Upper Panel**) and CD8 (**Lower Panel**) T cells from HSCT recipients receiving the indicated GvHD prophylaxis. The *p*-values resulting from the multivariable linear regression analysis are indicated when significant.

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REVIEW
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The Epitope-Specific Anti-human CD4 Antibody MAX.16H5 and Its Role in Immune Tolerance

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T cell modulation in the clinical background of autoimmune diseases or allogeneic cell and organ transplantations with concurrent preservation of their natural immunological functions (e.g., pathogen defense) is the major obstacle in immunology. An anti-human CD4 antibody (MAX.16H5) was applied intravenously in clinical trials for the treatment of autoimmune diseases (e.g., rheumatoid arthritis) and acute late-onset rejection after transplantation of a renal allograft. The response rates were remarkable and no critical allergic problems or side effects were obtained. During the treatment of autoimmune diseases with the murine MAX.16H5 IgG_1 antibody its effector mechanisms with effects on lymphocytes, cytokines, laboratory and clinical parameters, adverse effects as well as pharmacodynamics and kinetics were studied in detail. However, as the possibility of developing immune reactions against the murine IgG_1 Fc-part remains, the murine antibody was chimerized, inheriting CD4-directed variable domains of the MAX.16H5 IgG_1 connected to a human IgG_4 backbone. Both antibodies were studied *in vitro* and in specific humanized mouse transplantation models *in vivo* with a new scope. By *ex vivo* incubation of an allogeneic immune cell transplant with MAX.16H5 a new therapy strategy has emerged for the first time enabling both the preservation of the graft-vs.-leukemia (GVL) effect and the permanent suppression of the acute graft-vs.-host disease (aGVHD) without conventional immunosuppression. In this review, we especially focus on experimental data and clinical trials obtained from the treatment of autoimmune diseases with the murine MAX.16H5 IgG_1 antibody. Insights gained from these trials have paved the way to better understand the effects with the chimerized MAX.16H5 IgG_4 as novel therapeutic approach in the context of GVHD prevention.

Keywords: T cell modulation, anti-human CD4 antibody, MAX.16H5, autoimmune disease, graft-vs.-host disease, graft-vs.-leukemia effect

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INTRODUCTION

Besides the T cell receptor (TCR) and the CD3 antigen, other molecules are expressed on T cells but are also present on other hematopoietic cells (1). Monoclonal antibodies targeting such antigens (other than the TCR or CD3) can therefore bind to several antigen-expressing cell types. The CD4 molecule is expressed on T cells, monocytes and macrophages and contains four immunoglobulin-like domains (D1–D4) (2). It acts as a co-receptor during antigen presentation and associates with the TCR upon major histocompatibility complex (MHC) binding (1, 2). In the past, several

therapeutic strategies using CD4-directed antibodies were investigated for the treatment of several autoimmune diseases [reviewed in Wofsy (3) and Burmester et al. (4)]. In this context, the human $CD4^+$ T-cell clone 2C11 was generated (5) for immunization of BALB/c mice to produce the monoclonal anti-human CD4 antibody MAX.16H5 (initial name 30F16H5) (6, 7). Therefore, splenocytes of the immunized mice were fused with X63-Ag8.653 mouse myeloma cells to generate hybridoma cells (6–8). To examine their binding properties, antibody-containing hybridoma supernatants were incubated with $CD4^+$ T cells prepared from peripheral blood (PB) which were subsequently analyzed using enzyme-linked immunosorbent assays (ELISA) and cytofluorometric analyses (6). For the development as a therapeutic antibody, MAX.16H5 IgG_1 was selected because of its high affinity to CD4 (9). In this study, researchers compared 225 different CD4-directed antibodies regarding their CD4 binding properties and kinetics showing that MAX.16H5 IgG_1 shared some fine specificities with gp120 with regard to the recognition of different mutated CD4 versions (9). At the same time, experiments were performed to obtain information about the binding properties of gp120 and MAX.16H5 IgG_1 to the CD4 molecule by using peptides (10). The peptide $T_bYIC_bE_bVEDQK_{Ac}EE$ was reported to inhibit CD4 binding of both gp120 and MAX.16H5 IgG_1 (10). During the early years, the antibody was tested in several different assays thereby obtaining solid information not only about antigen-binding properties, but also about antibody-mediated effector mechanisms. Throughout the clinical development of the murine MAX16.H5 IgG_1, pharmacodynamic and pharmacokinetic data were collected. Upon administration in patients, the mode-of-action of the antibody, the induced CD4 and immunomodulation were studied intensively. Since MAX.16H5 IgG_1 was applied systemically, clinical data implementing cytokine profiles, acute-phase-reactant evaluation and side effects were obtained and documented. This review summarizes the clinical development of the therapeutic use of MAX.16H5 IgG_1 for the treatment of autoimmune diseases toward a promising treatment option for hematopoietic stem cell transplantation (HSCT)-related GVHD.

Abbreviations: AA, amino acid; ADCC, antibody dependent cellular cytotoxicity; aGVHD, acute graft-vs.-host disease; AML, acute myeloid leukemia; BW, body weight; CDC, complement dependent cytolysis; CEA, carcinoembryonic antigen; CRP, C-reactive protein; ELISA, enzyme-linked immunosorbent assay; ESR, erythrocyte sedimentation rate; $F(ab)_2$, fragment antigen binding region; Fc, fragment crystallizable region; FcR, Fc receptor; FLT3, Fms like tyrosine kinase 3; FoxP3, forkhead box protein P3; GVHD, graft-vs.-host disease; GVL effect, graft-vs.-leukemia effect; HAMA, human anti-mouse antibody; HIV, human immunodeficiency virus; HLA, human leukocyte antigen; HSC, hematopoietic stem cell; HSCT, hematopoietic stem cell transplantation; IBD, inflammatory bowel disease; Ig, immunoglobulin; IL, interleukin; ITD, internal tandem duplication; ITAM, immunoreceptor tyrosine-based activating motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; i.v., intravenous; JCA, juvenile chronic arthritis; Lck, lymphocyte-specific protein tyrosine kinase; mAb, monoclonal antibody; LPS, lipopolysaccharide; MHC, major histocompatibility complex; NSG, NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ; PB, peripheral blood; PBMC, peripheral blood mononuclear cell; PD-1, programmed cell death protein 1; PD-L1, programmed cell death 1 ligand 1; PHA, phytohemagglutinin; p.i., post injection; RA, rheumatoid arthritis; RF, rheumatoid factor; sCD14, soluble CD14; SLE, systemic lupus erythematosus; TCR, T-cell receptor; T_h cell, T-helper cell; T_{reg}, regulatory T cell; TT, tetanus toxoid.

MAX.16H5 IgG_1 IN THE TREATMENT OF AUTOIMMUNE DISEASES

In total, 47 patients have been treated with the murine wild type antibody MAX.16H5 IgG_1 (7, 11–24). The individuals suffered from varying diseases or conditions: rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), inflammatory bowel disease (IBD), or acute late-onset rejection after transplantation of a renal allograft (**Table 1**). Thereby, RA was the most studied disease. Besides the studies focusing on the applicability of MAX.16H5 IgG_1 as a promising therapeutic antibody format, several studies were published about the use of ^{99m}Tc-labeled MAX.16H5 IgG_1 in RA patients to report the localization/accumulation, the pharmacokinetics, and the elimination process of the antibody (7, 11, 23, 24). A broad variety of parameters was obtained during these clinical trials to evaluate the safety and efficacy of the systemic therapeutic administration of MAX.16H5 IgG_1. **Table 1** provides an overview of the studies performed in humans.

Since it is known that murine antibodies can cause immunological reactions in humans, the administration of the murine MAX.16H5 IgG_1 was particularly examined. Immunological reactions against the Fc-part of the murine antibody were expected, and in three different studies that administered MAX.16H5 IgG_1 in the background of RA the production of human anti-mouse antibodies (HAMAs) was investigated (13, 16, 21). Based on the obtained datasets authors concluded that HAMA production followed by MAX.16H5 IgG_1 administration was rather low and that HAMA activities are directed against specific determinants of the antibody, including anti-idiotypic reactivity (16). Furthermore, it was shown that MAX.16H5 IgG_1 $F(ab)_2$ directed HAMA (IgG) levels did not exceed levels higher than 0.7 mg/l after the first and 1.7 mg/l after the second course of therapy (13). Compared to HAMA activities exceeding 100 mg/l measured in other studies using monoclonal murine antibodies against cancer antigens (25, 26) in immunocompetent patients, the HAMA amounts directed against MAX.16H5 IgG_1 were rather low, but detectable. Overall, even in studies using the murine IgG_1 isotype of MAX.16H5, only low HAMA amounts were detected which allowed for further treatment cycles without loss of efficacy (16).

MAX.16H5 IgG_1 Mediated Effector Mechanisms

Antibodies can mediate effector mechanisms by both binding the antigen via the Fab domain and binding Fc receptors (FcRs) expressed on effector cells through the Fc part. Mouse IgG_1 is known to bind two different murine Fc receptors, mFcγRIIb and mFcγRIII (27). The mFcγRIIb is an immunoreceptor tyrosine-based inhibitory motif (ITIM)-carrying receptor, which is highly expressed on murine B cells, granulocytes, macrophages, monocytes and dendritic cells (27). In contrast, mFcγRIII is absent on B cells but highly expressed on monocytes, macrophages, dendritic cells, and granulocytes

TABLE 1 | Results of human studies using MAX.16H5 IgG_1.

Underlying disease	*N*	MAX.16H5 IgG_1 treatment	RR	Adverse effects and HAMA development	References
Active, severe RA	6	5/6 patients: 200–300 μg iv (370-550 MBq) ^{99m}Tc-mAb 1/6 patients: ≥10 MBq of lymphocytes treated *in vitro* with ^{99m}Tc-mAb	n.r.	No adverse effects observed	(7)
Active, severe RA	10	0.3 mg/kg BW [20 mg/day (14)] iv on 7 consecutive days; repeated treatment cycle after 8 weeks (4/10 patients)[a]	9/10[b]	2/10 patients: chills with fever, possibly due to lymphokine release syndrome (13) 2/10 patients: urticaria, 1/2 with severe allergic reaction possibly triggered by keeping a rodent as a pet, patient withdrawn from study (13) Chills, tremor, elevated body temperature, and nausea (15) Systemic side effects correlated with elevated levels of TNF-α, IL-2, and IFN-γ (15) 5/10 patients: HAMA production after 1st treatment cycle → of these 3/4 showed HAMA production after 2nd treatment cycle (16)	(13–16) (19) (17) (18)
Chronic active steroid-resistant or steroid-dependent IBD	3	0.3 mg/kg BW iv on 7 consecutive days[c]	3/3[d]	No adverse effects observed	(20)
Severe acute rejection after renal allograft	11[e]	5/11 patients: 0.6 mg/kg BW iv on 3 consecutive days[e]	3/5[e]	No adverse effects reported	(22)
Intractable severe SLE	1	0.3 mg/kg BW iv on 7 consecutive days[f]	1/1[g]	No adverse effects observed	(12)
Active, severe RA	4	3/4 patients: ≤250 μg ^{99m}Tc-mAb iv[h] 1/4 patients: ≥ 10 MBq of lymphocytes treated *in vitro* with ^{99m}Tc-mAb[h]	n.r.	No adverse effects observed	(11)
Active, severe RA or healthy control	8	200–300 μg (370–550 MBq) ^{99m}Tc-mAb and/or 1 mg (370 MBq) iv polyclonal HIG	n.r.	No adverse effects reported	(23)
Active, severe RA	1	2 mg (810 MBq) ^{99m}Tc-anti-CEA IgG_1 iv, 9 days later 250 μg (910 MBq) ^{99m}Tc-MAX.16H5 IgG_1 iv	n.r.	No adverse effects reported	(24)
Active, severe systemic onset JCAF	2	2 courses of 0.3 mg/kg BW iv on 7 consecutive days (time interval: 8 weeks)[i]	3/3[j]	No side effects after first treatment course 1/2 JCA patients: urticarial rash after first infusion of the second course	(21)
"adult type" RA	1	single course of 0.3 mg/kg BW iv on 7 consecutive days		1/2 JCA patients: fever up to 39.5°C with chills after the first antibody infusion of the second course Further infusions of the second treatment course well tolerated HAMA development detected in both JCA patients after two treatment courses No alterations in organ function, no infections observed either during treatment or during a 6months follow up in JCA patients No side effects reported for third patient with "adult type" RA	

The study references are mentioned, as well as the study population N, the underlying disease of the patients, the treatment conditions with MAX.16H5 IgG_1, the response rate RR, and—if so—observed adverse effects and HAMA development.

BW, body weight; CEA, carcinoembryonic antigen; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; HAMA, human anti-mouse antibody; HIG, human immunoglobulin; IBD, inflammatory bowel disease; inj., injection; iv, intravenous; JCA, juvenile chronic arthritis; mAb, monoclonal antibody; n.r., not reported; NSAID, non-steroidal anti-inflammatory drug; RA, rheumatoid arthritis; RR, response rate; SLE, systemic lupus erythematosus.

[a] *NSAID and steroid schedules were not changed during the observation period. "Treatment with slow acting anti-rheumatic agents had been discontinued in all but one patient at least 8 weeks before treatment" (18). Low-dose cyclophosphamide (50 mg/day) was maintained in one patient. Analysis of HAMA production in a 2nd treatment cycle after 6–8 weeks (16).*

[b] *"Responders were defined by a reduction of the Ritchie articular index of more than 30% of the initial levels 4 and 8 weeks after treatment or by a decrease of ESR and CRP values of more than 50%" (18). In 3 patients, clinical improvements were only achieved after the second treatment course. One patient withdrew from the study due to an apparently allergic reaction and was excluded from the discussion.*

[c] *Aside from the antibody treatment, 1.5 g (2 patients) or 3 g (1 patient) mesalazine were given together with 10 mg prednisolone throughout the observation period.*

[d] *One patient's clinical parameters improved for 3 weeks after the treatment; after 4 weeks he had a mild relapse. The second patient underwent a transient improvement but relapsed after 1 month. The last patient had a complete clinical, endoscopic, and biochemical remission for more than 5 months.*

[e] *Six other patients with severe acute rejection 1.5–8 years post transplantation received 3 × 1 g methylprednisolone alone without antibody therapy./ Responders to anti-CD4 therapy were characterized by creatinine levels below 50% of maximum increase 4 weeks after rejection treatment.*

[f] *Prednisolone therapy (50 mg/day) was continued throughout the observation period.*

[g] *Clinical and laboratory improvements lasted for 4 weeks after the antibody therapy. At this time point, methylprednisolone was given as bolus therapy for 5 days (750 mg daily) resulting in complete remission proven by the (for the first time) negative anti-DNA-antibody titer.*

[h] *"Four weeks before scintigraphy, conventional anti-inflammatory therapy was stopped whereas ongoing steroid treatment was continued with <10 mg/d" (11).*

[i] *One patient was concomitantly treated with 3 × 25 mg diclofenac, 15 mg prenisone (reduced to 10 mg during the second treatment course), and 2 × 100 mg cyclosporine (solely during the first treatment course) daily, and the other patient with 2 × 250 mg naproxen, 10 mg prednisone (reduced to 7.5 mg during the second treatment course), and 17.5 mg methotrexate daily.*

[j] *A 50% reduction of the Ritchie index, 65% reduction of the number of swollen joints, and disappearance of morning stiffness as well as a clear improvement of the CRP levels was defined as treatment success. "There were immediate beneficial clinical effects of treatment in one patient, while in the other marked beneficial effects were achieved only by repeated treatment. These effects could not be attributed to longstanding treatment with immunosuppressants" (21). Moreover, concomitant medication could be reduced in both JCA patients after the first treatment course.*

thereby mediating activating signals via an immunoreceptor tyrosine-based activation motif (ITAM) [reviewed in Bruhns (27)]. Mice produce three different IgG subclasses which do not only differ in their FcR binding specificity but also bear diverse capacity to activate the complement system (27–30). Based on serum bactericidal activity measurements the following hierarchy of murine IgG induced complement activity was proposed: IgG3 > IgG2 > IgG1 (30). It has to be noted that in these assays human serum was used as a source of complement (30).

To this date, no MAX.16H5 IgG_1 mediated complement-dependent cytotoxicity (CDC) (using rabbit serum as a source of complement) or antibody-dependent cell-mediated cytotoxicity (ADCC) using granulocytes or peripheral blood monocytes as effector cells was obtained in *in vitro* assays (13, 31).

Effects on Lymphocytes

In general, the MAX.16H5 IgG_1 treatment results in a decrease of $CD4^+$ cells in RA patients and therefore to an overall reduction of $CD3^+$ cells (13, 14). Neither $CD8^+$ nor B-cell values were changed in RA patients (13, 14). Immune cells from RA patients treated with MAX.16H5 IgG_1 showed reduced proliferation to various stimulatory agents 1 h post injection (*p.i.*) (13). However, in four out of nine patients, increased mitogen responses were induced after 8 days, which was indicative for unaltered clinical effects in these patients (13). Blood samples from IBD patients treated with MAX.16H5 IgG_1 showed a reduced lymphocyte proliferation after stimulation with mitogens and recall antigens (20), too.

Immortalized and interleukin (IL)-2-dependent $CD4^+$ T cells revealed reduced mitotic activity (not increased apoptosis) after incubation with MAX.16H5 IgG_1 or its $F(ab')_2$ (32). The same effect was observed with the Fab of MAX.16H5 and gp120 of HIV which could be prevented by high concentrations of IL-2 (32). The authors showed that this effect was connected to a decreased amount of lymphocyte-specific protein tyrosine kinase (Lck) bound to the intracellular domain of CD4 (32).

For further investigation of intracellular signaling pathways, the calcium release after TCR stimulation was examined in peripheral blood mononuclear cells (PBMCs) of RA patients or healthy donors treated with MAX.16H5 IgG_1 (33, 34). In one study, samples from healthy donors showed reduced intracellular calcium levels after MAX.16H5 IgG_1 incubation and TCR stimulation *in vitro*, but only if MAX.16H5 IgG_1 was still bound to the CD4 molecule (33). In a second study, the intracellular calcium concentration did not increase after solely incubation with MAX.16H5 IgG_1 (34). Only after cross-linking of CD3 and CD4 by anti-mouse goat serum an increased intracellular calcium signaling was obtained if MAX.16H5 incubation was performed for a maximum of 5 min (34). For longer incubation periods, the calcium signal decreased again indicating that full T cell activation by CD3 occurs rapidly within a short time (34). These data indicate a strong dependence of preincubation time. Furthermore, the increased calcium release only after cross-linking of CD3 and CD4 leads to the following speculation: the T cell activation was impaired due to transient but asynchronous activity of different kinases in T cells and intercellular cross-talk between T cells and monocytes was required (34).

At the time the studies were conducted, regulatory T cells (T_{regs}) were not yet identified as important targets to follow in clinical GVHD research: By 2000, T_{regs} were identified as suppressors of autoimmunity *in vitro* and in mouse models [reviewed in Shevach (35) and Sakaguchi (36)]. However, the flow cytometric identification of T_{regs} remained difficult until they were specified as positive for CD4, CD25, and forkhead box protein P3 (FoxP3) in 2003 (37–39). Therefore, the clinical data on MAX.16H5 IgG_1 lack information on the T_{reg} population.

Effects on B-Cell Crosstalk With T-Helper Cells and Immunoglobulin Secretion

PBMCs of healthy individuals were assessed for effects of MAX.16H5 IgG_1 incubation on B-cell differentiation and resulting IgG and IgM production (40). It was found that incubation with MAX.16H5 IgG_1 inhibited B-cell differentiation and following immunoglobulin (Ig) production (40). Even in the presence of mitogens and IL-2 or IL-4, MAX.16H5 IgG_1 addition reduced Ig secretion (40). Moreover, the production of IL-2 and IL-4 by T-helper (T_h) cells was minimally influenced by MAX.16H5 IgG_1 under various stimulating conditions (40). Thus, cytokines were not responsible for lower Ig secretion after MAX.16H5 incubation. More likely, the reduction of direct cellular contacts between T_h and B cells by MAX.16H5 IgG_1 and its $F(ab')_2$ lead to reduced crosstalk between the two cell types causing reduced Ig secretion indicating that CD4-blockade by MAX.16H5 interferes with early T-B cell collaboration (40). In RA patients, Ig reduction was observed after MAX.16H5 IgG_1 treatment, especially rheumatoid factor (RF) production, indicating that this effect is also present *in vivo* (13).

Effects on Monocytes

In the treatment of RA with MAX.16H5 IgG_1, $CD14^+$ monocytes in the PB were reduced one hour after infusion of MAX.16H5 IgG_1 (13). Continuing the MAX.16H5 IgG_1 treatment kept monocyte levels in normal ranges (13). The authors offer two possible explanations: either the monocyte/macrophage system is responsible for the depletion of antibody-coated T cells or the MAX.16H5 IgG_1 bound to the CD4 molecule present on a monocyte subset results in temporary monocyte reduction in the PB (13). On the other hand, reduced crosstalk between T_h cells and monocytes may play a role in the observed reduction of monocyte activation (13). In different studies, the same RA patients were monitored for monocyte activation indicated by heightened neopterin serum values, MHC class II expression, monocyte counts, and IL-1 production prior to MAX.16H5 IgG_1 application (14). These parameters could be reduced after MAX.16H5 IgG_1 treatment (14). Moreover, elevated levels of soluble CD14 (sCD14) detected in five patients prior to MAX.16H5 IgG_1 treatment were reduced in three patients after antibody application (18). IL-1 and IL-6 serum levels correlated to sCD14 concentrations in RA patients (18). A comparison between therapy responders and non-responders revealed reduced monocyte and T_h cell counts in the responder group, whereas both values increased again in the non-responder group after 1 week (18).

Cytokine Release

In chronic inflammatory diseases such as RA and SLE, the release of pro-inflammatory cytokines plays a crucial role in disease progression. Elevated levels of cytokines produced by $CD4^+$ cells including tumor necrosis factor (TNF)-α, IL-1, IL-6, and IL-17 favor disease pathogenesis [reviewed in Lourenço and La Cava (41) and McInnes and Schett (42)]. Monoclonal antibodies (mAbs) against these molecules or their respective receptors are therapeutic options to treat patients with autoimmune diseases (43). Therefore, the effect of the treatment with MAX.16H5 IgG_1 targeting human $CD4^+$ cells regarding cytokine release was studied in detail *in vivo* and *in vitro*.

IL-6 is known as the most important inducer and regulator of acute-phase response (44). Elevated IL-6 levels were measured in most RA patients before MAX.16H5 treatment (18). The IL-6 levels rapidly declined in four patients during the treatment course, which was observed in parallel with substantial clinical and laboratory improvement (18). On the other hand, one patient showed a slight increase of IL-6 during first treatment course and did not respond to treatment (18). One individual demonstrated a considerable increase of IL-6 and underwent an allergic skin reaction after the first injection (18). In that special patient, the IL-6 levels decreased to the pretreatment values after the treatment was stopped (18).

Besides the positive effect of IL-6 reduction, cytokine release due to MAX.16H5 IgG_1 application was analyzed as potential side effect. During the therapy of RA patients with MAX.16H5 IgG_1, symptomatic patients showed elevated serum levels of TNF-α, IFN-γ, and/or IL-2 (15). Comparison of the modulation efficacy of $CD4^+$ T cells induced by MAX.16H5 treatment did not reveal any difference between patients without clinical adverse effects and those developing systemic side effects (15). The authors hypothesized that the clinical adverse effects were likely a result of lymphocyte activation and/or a monocyte/macrophage interaction with lymphocytes (15). A comparable side effect profile was described for mAb OKT3 treatment (45, 46) but MAX.16H5 IgG_1 induced effects were milder and of much shorter duration which made a further treatment of the patient unnecessary (15). It has to be noted, that only a small cohort of patients systemically received MAX.16H5 IgG_1 therapy for autoimmune disease treatment, which complicates drawing solid conclusions.

The cytokine production in SLE patients was analyzed *in vitro* as well (47). Spontaneous IL-6 secretion was heightened in blood cell cultures from patients with active SLE compared to cultures from inactive SLE patients and healthy controls (47). After incubation with MAX.16H5 IgG_1, cell cultures of active SLE patients demonstrated reduced IL-6 levels, whereas TNF-α levels were not significantly altered (47). When samples from healthy volunteers were stimulated either with phytohemagglutinin (PHA) or lipopolysaccharide (LPS) MAX.16H5 IgG_1 induced IL-6 decrease was found to be antibody dose-dependent (47). In control wells it was shown that the addition of methylprednisolone to the cell cultures of stimulated healthy volunteer samples, stimulated inactive SLE samples and unstimulated active SLE samples not only reduced IL-6 but also TNF-α secretion markedly (47). Summarized it was shown that MAX.16H5 antibody incubation altered stimulated IL-6 secretion of *in vitro* blood cell cultures obtained from SLE patients and healthy individuals. The decrease of stimulated IL-6 secretion was dose-dependent. Other than methylprednisolone MAX.16H5 IgG_1 incubation did not influence TNF-α levels in these assays (47).

Laboratory and Clinical Parameters

In contrast to other anti-CD4 antibodies, MAX.16H5 was the only one improving not only clinical but also laboratory parameters in RA patients [reviewed in Burmester and Emmrich (48)]. Additionally, MAX.16H5 application showed an effect on parameters which were associated with monocyte/macrophage activation (14). In general, a significant decrease of laboratory [erythrocyte sedimentation rate (ESR), RF titer, C-reactive protein (CRP) levels] and clinical parameters (Ritchie articular index and swollen joints) was observed (13). In one of the patients, no impact on ESR and CRP levels was observed during the first cycle of MAX.16H5 infusion (17). Four years before the treatment with MAX.16H5 IgG_1, the patient was diagnosed with RA. Due to a trauma, he underwent splenectomy earlier in life (17). After the second course of MAX.16H5 therapy, ESR and CRP levels were reduced, possibly followed after decreased IL-6 serum values (17). Since the change in laboratory variables did not translate into an improvement of clinical parameters, low dose chlorambucil was implemented into the treatment regimen. The combination of CD4 directed antibody therapy together with chemotherapeutic medication resulted in clinical improvements which also translated in continued reduced levels of certain inflammatory parameters (ESR and CRP) (17). Overall, no adverse effects (especially infections) were observed (17).

The clinical parameters of two children were assessed in another study where MAX.16H5 was given *i.v.* for treatment of refractory juvenile chronic arthritis (JCA) (21). One patient benefited from the antibody therapy clinically within 1 week after the first antibody application. A second antibody application showed an even more improved response compared to the first course of treatment and symptoms like fever and rash were reduced for around 2 months (21). In the second patient, two cycles of treatment were needed to obtain notable improvement of clinical symptoms. Also, these juvenile patients did not show any signs of adverse side effects caused by the MAX.16H5 IgG_1 antibody treatment (21).

Patients suffering from severe acute rejection after kidney transplantation also benefitted from the therapy with MAX.16H5 IgG_1 (22). Histological signs of acute rejection (if present) disappeared as a response to the MAX.16H5 IgG_1 treatment. All patients showed rapid decreasing serum creatinine levels within the first 3 days post injection. However, graft function was impaired in two patients 3–4 weeks after therapy and one patient experienced transplant rejection again after 10 weeks (22). The authors observed a rapid effect of the MAX.16H5 antibody in the treatment of acute rejection after kidney transplantation and concluded that $CD4^+$ T cells seem to play an important role in the rejection process. They further suggested to implement the antibody therapy in established immunosuppression treatment protocols to improve therapeutic efficacy (22).

MAX.16H5 IgG_1 application was also shown to be effective in the treatment of inflammatory bowel disease (IBD) (20).

Especially, when other treatment options are exhausted and conventional therapeutics are ineffective MAX.16H5 IgG_1 can be used as a treatment option in IBD (20). As also discussed in the treatment of autoimmune diseases earlier, single cycle administration of MAX.16H5 was insufficient to reach persistent therapeutic success (20).

Other Effects

Since the exact mechanism of the MAX.16H5 IgG_1 induced effects were not sufficiently explained neither *in vitro* nor *in vivo,* researchers focused on the intracellular signaling after antibody binding to its antigen. By using U937 target cells, the activation of complex inositol polyphosphate responses and Ca^{2+} increase after MAX.16H5 IgG_1 antibody treatment was investigated *in vitro* independently from TCR signaling (49). The authors showed, that MAX.16H5 IgG_1 incubation alone was not sufficient to induce Ca^{2+} increase in CD4-expressing cells (PB-monocytes and the monocyte cell line U937) (49). When goat anti-mouse antiserum was added, clear crosslinking of MAX.16H5 IgG_1 was obtained leading to heightened Ca^{2+} levels (49). The outcome of experiments in U937 cells using $F(ab)_2$ fragments of MAX.16H5 together with $F(ab)_2$ crosslinking agents were not applicable to observations made with whole antibodies (49). The authors concluded that in U937 cells only "[…] crosslinking of CD4 and FcγR, but not cross-linking of CD4 alone specifically activates the inositol polyphosphate/Ca^{2+} signal transduction pathway" (49).

Pharmacokinetics and Pharmacodynamics in Humans

Pharmacokinetic and pharmacodynamic studies with ^{99m}Tc-labeled MAX.16H5 IgG_1 were performed in RA patients (7, 11, 23, 24). In one study, patients received either *i.v.* injection of ^{99m}Tc-labeled MAX.16H5 IgG_1 antibody or *ex vivo* ^{99m}Tc-labeled MAX.16H5 IgG_1 incubated PB-lymphocytes (7). Following images/scans were taken using a gamma camera (7). The use of the CD4-directed MAX.16H5 IgG_1 antibody for medical imaging in order to obtain information about disease progression in RA or also for diagnosis was promising since techniques used at that time, e.g., ^{99m}Tc-early methylene diphosphonate (MDP) bone scans, were rather unsatisfactory (7). The authors concluded that ^{99m}Tc-labeled MAX.16H5 imaged the localization of disease joints more precisely than MDP scans, which made it a promising tool for the diagnosis of autoimmune arthritis (7).

Organ Activity Distribution and Kinetics

In context with their participation in a therapeutic trial for RA [preliminary data after 2 patients enrolled evaluated in (50)], 4 patients received either radio-labeled MAX.16.H5 IgG_1 antibody *i.v.* or PB-lymphocytes labeled with the antibody [1 patient] (11). The "[…] study was mainly concerned with the evaluation of the kinetic behavior of the antibody-labeled cells in the patients" (11). In general, the maximum of activity [100%] of the antibody or the antibody-labeled lymphocytes was reached within a few minutes in the heart and lung (ca. 4.5 min), whereas the maximum of radio-labeled activity was obtained after ca. 12 min in the spleen and about 19 min in the liver (11). However, 90 min after the injection, radiolabeled MAX.16H5 antibody activity reincreased in the patients' hip joints after a first injection peak (11). Interestingly, organ kinetic curves were comparable between patients receiving ^{99m}Tc-labeled MAX.16H5 IgG_1 and the patient who received *ex vivo* antibody-labeled lymphocytes (11). Additionally, study examined the whole-body radioactivity distribution at two different time points [4 and 24 h p.i.] (11). "The splenic uptake decreased by about 39% from 4 h […] to 24 h p.i. […]" (11). A moderate increase of activity measured in the liver was recorded (11). Nevertheless, at both time points, approximately 50% of radioactivity was measured in the bone marrow. Joints overall showed a rather low activity with 0.5 ± 0.09% for not-diseased joints vs. 2 (after 4 h) to 2.5% (after 24 h) for a single affected joint (11).

Another study (23) evaluated the kinetic differences between labeled MAX.16H5 IgG_1 and polyclonal human immunoglobulin (HIG) in RA patients or healthy controls to exclude non-specific accumulation of immunoglobulin. Compared to HIG, ^{99m}Tc-labeled MAX.16H5 IgG_1 showed a higher uptake in the liver and in the spleen of RA patients at 24 h p.i. (23). Since the MAX.16H5 IgG_1 "[…] showed a higher target-to-background ratio in arthritic knee and elbow joints in comparison to polyclonal HIG used for conventional imaging […]" the authors discussed a potential beneficial application of the antibody in the "[…] detection of inflammatory infiltrates rich in CD4-positive cells" (23).

Adverse Effects

The adverse effects observed in studies with human patients or healthy volunteers are summarized in **Table 1**. In different trials using the ^{99m}Tc-labeled MAX.16H5 IgG_1, no adverse reaction was observed after the intravenous application (7, 11, 23, 24).

Several studies examined the treatment of RA patients with MAX.16H5 IgG_1. In general, only occasional and minor side effects were observed which probably resulted from short lived cytokine peaks (15). The infusions were well-tolerated [reviewed in Burmester and Emmrich (48)]. Immediate adverse effects were allergic reactions on rare occasion as well as nausea and fever being symptomatic for the development of a mild to moderate cytokine release syndrome (15). A long-term effect on the topic of laboratory parameters was the development of HAMAs (13, 16, 21). Approximately 25% of the HAMA activity was directed against idiotypic determinants (16). Significant HAMA concentrations were measured between 2 and 12 weeks p.i. (16). Still, in contrast to monoclonal antibodies directed against other T cell epitopes, the amounts of these antibodies were low and never exceeded 2.0 (after 1 cycle) or 2.2 mg/l (after 2 cycles) (16). Thus, patients could be retreated without loss of efficacy (13) as similarly shown with other anti-human CD4 antibodies (51).

The reduced $CD4^+$ T cell numbers did not result in infectious problems in any study (48). Together with an unaltered or even elevated T cell reactivity *in vitro* [4 patients showed heightened T cell reactivity to common antigens and mitogens when $CD4^+$ cell numbers were still reduced (13)], this observation points to low numbers of $CD4^+$ T_h cells being sufficient to maintain the function of the cellular immune system (13, 48).

In the trial using MAX.16H5 IgG_1 in the treatment of refractory JCA in two patients the first treatment course was

tolerated without side effects (21). Application of MAX.16H5 IgG_1 in the treatment of either SLE or IBD patients did not show any side or adverse effect (12, 20). In the therapy of acute rejection in long-term renal allograft recipients, no adverse effects due to the treatment were mentioned in the original article (22).

Although not all trials showed the development of HAMAs as side effect, the possibility of developing immune reactions against the murine IgG_1 Fc-part remains. This risk was considered to be reduced by the development of a chimerized, humanized version of the MAX.16H5 IgG_4 antibody.

DEVELOPMENT OF A CHIMERIZED MAX.16H5 IgG_4 MONOCLONAL ANTIBODY

The use of murine antibody formats for therapeutic interventions was shown to be connected to the development of several side effects, e.g., antibody responses (HAMA) or allergic reactions, which led to the development of optimization protocols in antibody design using recombinant DNA tools already in the 1980's (52, 53). Morrison et al. described a process called "chimerization" where heavy and light chain variable DNA sequences of a murine antibody were connected to DNA sequences encoding human $IgG_{1/2}$ and sequences encoding for the human kappa light chain, respectively (53). Morrison and colleagues discussed the potential of such "near-human" antibody formats with respect to reduced side effect profiles when administered *in vivo* (53). In 1997, the approval of rituximab, a chimeric CD20-directed IgG_1 antibody for the treatment of lymphoma eventually paved the way for modified antibody formats (54). In December 2018, 75 antibodies as therapeutics were approved for the treatment of a variety of diseases including 9 antibodies carrying human Fc domains and murine variable sequence motifs and are therefore defined as chimeric (43). The aforementioned CD20-directed antibody rituximab is listed as well as e.g., obiltoxaximab, which reached US-approval more recently (2016) and is used for the treatment and prophylaxis of inhalational anthrax (55). The chimerization of MAX.16H5 was promoted in order to reduce immunogenicity of the antibody for potential clinical applications. The MAX.16H5 chimerization process was started in 2007. A CD4-directed murine IgG_1 antibody-expressing hybridoma clone was used as starting material. By combining cloning and sequencing techniques together with *in silico* modeling, variable regions of light and heavy chains were extracted, analyzed, modified and connected to human constant regions as commissioned (**Figure 1**) (56). Mammalian cells were used for the production of the chimeric antibody (56). Binding profiles of MAX.16H5 IgG_4 were comparable with the murine MAX.16H5 IgG_1 proving that the correct variable regions were chosen to generate the chimeric antibody (56).

In general, Fc parts of antibodies are known to mediate effector mechanisms which include their interaction with both certain Fc receptors expressed on effector cells and the activation of the complement system (57). Depending on their Fc-binding capacities and mediated effector mechanisms, therapeutic antibodies can be used for different therapeutic purposes. In the setting of HSCT or GVHD, an Fc-mediated depletion of $CD4^+$ cells was not our desired therapeutic approach. Therefore, MAX.16H5 variable domains were specifically connected to human IgG_4 and not to human IgG_1 constant domains since it is known that IgG_4 is a weak activator of ADCC and CDC (58) [reviewed in Davies and Sutton (59)]. Because of the functional features of the IgG_4 isotype, its translation into the clinical application in the field of immune checkpoint blockade was quite successful. To date, pembrolizumab (MK-3475) and nivolumab (MDX-1106), two antibodies harboring IgG_4 backbones, are approved for immune checkpoint blockade in the USA and the EU (43). The molecules effectively inhibited programmed cell death protein 1/programmed cell death 1 ligand 1 (PD-1/PD-L1) interactions but were shown to be inactive eliciting Fc-mediated effector functions [reviewed in Topalian et al. (60)]. Another challenge in the development of IgG_4-based therapeutic antibodies was the isotype's feature of the so-called Fab-arm exchange where single heavy chain-light chain dimers can form bivalent antibodies with other single heavy chain-light chain dimers (61). From a biological point of view it was discussed that the bivalency of IgG_4 molecules may reduce their "pathological potential" (61). For certain applications, therapeutic antibody manufacturing can be challenging due to the Fab-arm exchange of IgG_4. This led to the development of several antibody structure optimization strategies in the past (59, 61). A study published in 2009 showed that bispecific IgG_4 antibodies were detectable in blood samples from patients who received an unmodified IgG_4-based therapeutic antibody (62). The antibody formed half-molecules *in vivo* and furthermore, assembled to bispecific antibodies with patient-specific endogenous IgG_4 (62). The group introduced a S228P amino acid substitution in the hinge-region of the antibody and showed the prevention of Fab-arm exchange impressively (62). The MAX.16H5 IgG_4 sequence was optimized in the same manner in order to prevent Fab-arm exchange (**Figure 1**) (56), to ensure the stability of the antibody, and the reliability of the manufacturing process during clinical development and GMP production.

NON-CLINICAL DEVELOPMENT OF THE CHIMERIZED ANTI-HUMAN ANTI-CD4 ANTIBODY—STUDIES OF MAX.16H5 IgG_1 AND IgG_4 IN MURINE GVHD MODELS

It was sought to examine the properties of the antibody's influence on the immune system using mouse models. To study the effect of the MAX.16H5 antibody against human CD4 expressing cells in mice, a genetically modified murine model with a C56BL/6 background was developed, which functionally expressed human CD4 on T cells while the murine CD4 gene was knocked-out (63–65). Additionally to the murine MHC II the T cells expressed human HLA-DR17 (63–65). In first experiments, spleen and lymph node samples of triple transgenic (TTG) mice previously immunized with tetanus toxoid (TT) showed reduced immune response after MAX.16H5 IgG_1 treatment and re-stimulation with TT *ex*

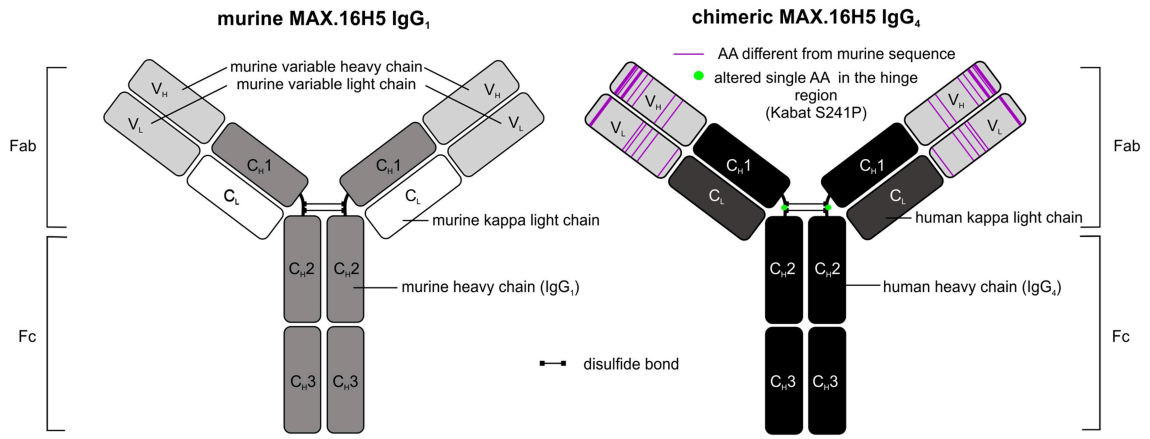

FIGURE 1 | Schematic representation of murine MAX.16H5 IgG_1 and chimeric MAX.16H5 IgG_4 antibodies.

vivo (66). The same effect emerged *in vivo* after injection with 15 µg/g body weight (BW) MAX.16H5 IgG_1 (67) and the $F(ab)_2$ of MAX.16H5 was as potent as the whole antibody (65). Surprisingly, unresponsiveness was preserved after the mice underwent another antigen boost without prior antibody administration, thus indicating a long-lasting but not depleting effect of MAX.16H5, which was moreover antigen-specific and dependent on the ability to form the immunological synapse between CD4 and HLA-DR (65). Therefore, we speculate, that MAX.16H5 does not induce a general immune suppression but only to antigens present simultaneously with or shortly after antibody treatment, most likely while the antibody is still bound to its ligand when the HLA-DR molecule encounters the TCR. This observation led to the idea that MAX.16H5 may conquer an old challenge in immunology: induction of specific tolerance and influencing both host-vs.-graft and graft-vs.-host reactions. The latter severely limit the application of allogeneic hematopoietic stem cell (HSC) and immune cell transplantations for the treatment of, e.g., autoimmune diseases or hematopoietic cancers such as leukemia.

Acute GVHD is the main complication of allogeneic hematopoietic stem cell transplantation (HSCT) and the main reason for early transplantation-associated mortality (68). Conventional immunosuppressive drugs such as corticosteroids are not specific and suppress the entire immune system (69). So far, no therapy manages HSCT or donor lymphocyte infusion without the need for conventional systemic immunosuppressive drugs. Promising *in vivo* data were obtained regarding the feasibility and effectivity of *ex vivo* graft incubation with MAX.16H5 IgG_1 and the antibody's influence on GVHD down modulation after allogeneic full-mismatch immune stem cell transplantation if the graft from TTG mice was preincubated with the antibody (70–72). Of note, removing unbound antibody molecules from the graft did not reduce its effectiveness (70–72). The graft's unresponsiveness to allogeneic $BALB/c^{wt}$ antigens was even preserved if immune and stem cells from transplanted GVHD-free mice were transferred to new $BALB/c^{wt}$ mice without MAX.16H5 preincubation—a phenomenon called "infectious tolerance" (71). This phenomenon is possibly achieved by heightened levels of T_{regs} present in mice receiving MAX.16H5-preincubated grafts suggesting a possible role for long-term unresponsiveness *in vivo* (71). Two studies did not only focus on accelerated GVHD but also on the maintained GVL effect mediated by the transplanted antibody incubated immune cell graft. To investigate the GVL effect mediated by the transplanted graft, $BALB/c^{wt}$ animals did not only receive TTG immune cell grafts but also P815 cells, a murine mastocytoma cell line (70). It was shown that graft preincubation with the murine MAX.16H5 IgG_1 antibody did not influence the GVL effect which was induced by the transplanted immune cells (70, 71). In another murine model, the murine MAX.16H5 IgG_1 also prolonged the survival of recipient C3H/HeN mice (receiving a TTG immune cell graft to induce GVHD) even if they were co-transplanted with myeloblast-like murine cell line 32D Clone 3 (32D) expressing human Fms like tyrosine kinase 3 with the internal tandem repeat duplication ($FLT3^{ITD}$), which constitutes an aggressive acute myeloid leukemia (AML) cell line model (72).

The *ex vivo* graft incubation with MAX.16H5 together with a subsequent washing protocol was found to be an attractive and promising therapeutic setting in HSCT (31, 70, 71). It got even more into focus when patients, who received a therapeutic antibody, suffered from side-effects which were mainly caused by a systemic inflammatory response as a response to systemic application (73). In 2006, unpredicted side-effects emerged during a phase-I (first-in-man) clinical trial with a CD28-directed monoclonal antibody called TGN1412 (73, 74). The antibody was developed to modulate T_{reg} expansion and was praised as "a promising novel tool for the treatment of human autoimmune diseases" (75). As of today, the cytokine release syndrome is a known side-effect of different immunotherapeutic interventions such as therapeutic antibodies (e.g., CD20-directed mAbs, bispecific T cell engagers, and immune checkpoint inhibitors) or chimeric

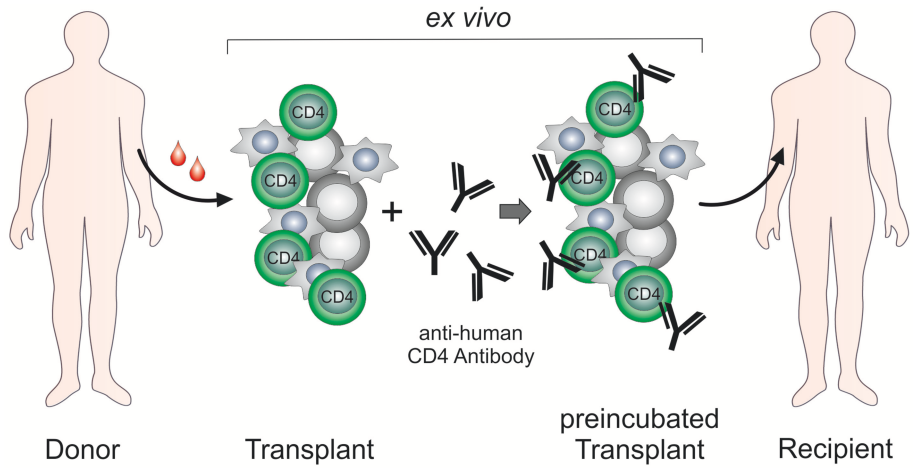

FIGURE 2 | *Ex vivo* treatment of hematopoietic stem cell/immune cell grafts by anti-human CD4 antibody MAX.16H5.

antigen receptor (CAR) T cells [reviewed in Shimabukuro-Vornhagen et al. (76)]. Due to safety considerations, the development and optimization of the MAX.16H5 antibody format for the treatment of GVHD was shifted to *ex vivo* graft incubation rather than systemic administration of the therapeutic antibody.

After the chimerized MAX.16H5 IgG_4 was available, its effectiveness in preventing GVHD was evaluated using a NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mouse model (31). Here, mice received a xenogeneic human PBMC transplant to initiate acute GVHD (31). Both variants, the murine IgG_1 and the chimeric IgG_4 of the MAX.16H5 antibody, were compared regarding their ability to down regulate GVHD development. Unspecific isotype antibodies were used as controls in these experiments (31). The data were published in 2016 and showed that both the murine IgG_1 and the chimerized IgG_4 were able to prevent GVHD in this experimental setting (31). The mice received immune cell grafts which were *ex vivo* incubated with the respective antibodies making a systemic application of MAX.16H5 unnecessary. After 2 h of MAX.16H5 IgG_1 or -IgG_4 preincubation, the grafts were washed with PBS to remove excess, unbound antibodies. Finally 2×10^7 MAX.16H5 or isotype control treated graft cells were diluted in 150 µl 0.9% NaCl and were injected into the tail vein of the animals (31). Mice receiving MAX.16H5 murine IgG_1 or chimeric IgG_4 antibody incubated grafts showed a significant prolonged survival in comparison to mice receiving grafts incubated with isotype control antibodies (31). During these animal experiments, data were obtained collecting several parameters such as general health status (e.g., fur, weight, behavior, mobility), immune cell reconstitution (white blood cell counts, flow cytometric analyses of human T cell, B cell, and lymphocyte markers) and histological data (apoptotic cells in the gut of the mice, TUNEL) (31). Importantly, MAX.16H5 IgG_1 (murine) or IgG_4 (chimeric) incubation of immune cell grafts comparably weakened GVHD development significantly but did not impair the engraftment of the transplanted cells (31).

CONCLUDING REMARKS

It was early known that even the murine IgG_1 istoype of MAX.16H5 does not stimulate CDC or ADCC (13), but the mechanism of action is still not understood. Over the years of development, many theories have been arosen on its mechanism of action to induce tolerance. Despite being of interest, most data were not published as they showed negative results. Due to the quick responses observed in clinical trials, it was speculated that the antibody's mechanism was independent of antigen recognition which would take longer but was more likely caused by inhibition of preactivated IL-2-dependent T cells (32). However, this theory cannot explain why the antibody is effective in GVHD prevention after an *ex vivo* incubation, i.e., before the cells of the transplant had antigen contact. Moreover, the monocyte/macrophage system was thought to be involved, but this idea too was in conflict with reported data (13). Rising T_{reg} amounts in a murine model (71) are probably a mediator of tolerance but do not explain the mechanism how tolerance is achieved.

The majority of the studies performed with the anti-human CD4 antibody MAX.16H5 focused on the treatment of autoimmune diseases such as RA, SLE, IDB and JCA and revealed striking results that identified the MAX.16H5 antibody as a promising alternative for conventional therapeutics. Following initial systemic application of MAX.16H5, a new strategy was developed leading to similar success in therapy and improved safety of patients: graft $CD4^+$ cells were incubated *ex vivo* with the MAX.16H5 antibody and re-infused into the patient (**Figure 2**). This innovative approach extended the scope from the treatment of autoimmune diseases to hematological malignancies. By *ex vivo* incubation of an allogeneic immune- and stem cell transplant with the epitope-specific anti-human CD4 antibody MAX.16H5, a new therapy strategy has emerged for the first time enabling both the preservation of the GVL effect of the transplant and the permanent suppression of GVHD without the need for conventional immunosuppression.

The following essential benefits of this innovative therapeutic approach can be expected: First, the treatment of a human immune cell transplant does not deteriorate the anti-tumor effectiveness with regard to different leukemia types. Second, it is expected that an allogeneic HSC graft treated with MAX.16H5 anti-human CD4 antibodies leads to a general improvement of the survival due to suppression of the GVHD. Furthermore, the dosage and amount of conventional immunosuppressive drugs (toxicity, side effects, and duration of the treatment) can be reduced and the patients' quality of life could be improved. Due to the suppression of the GVHD, patients lacking a suitable donor will be applicable to receive donor cells whose transplantation normally would be associated with a higher risk for GVHD (e.g., more HLA mismatches). Thus, immune cell therapies will become applicable to cure other diseases (e.g., autoimmune diseases and primary immunodeficiencies) whose curative treatment regimen does currently not include this form of therapy because of a high risk for GVHD development. Finally yet importantly, the incubation of the allogeneic HSCT grafts with the epitope-specific anti-human CD4 antibody MAX.16H5 can be performed outside of the body which reduces side effects and therapy costs, antibody amounts as well as improves the safety of the transplantation remarkably.

AUTHOR CONTRIBUTIONS

LS, AD, NH, UT, and SF analyzed the publications, created the figures and tables, and wrote the paper.

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Invariant NKT Cells From Donor Lymphocyte Infusions (DLI-iNKTs) Promote *ex vivo* Lysis of Leukemic Blasts in a CD1d-Dependent Manner

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Allogeneic hematopoietic cell transplantation (allo-HCT) is a curative treatment option for hematologic malignancies but relapse remains the most common cause of death. Infusion of donor lymphocytes (DLIs) can induce remission and prolong survival by exerting graft-vs.-leukemia (GVL) effects. However, sufficient tumor control cannot be established in all patients and occurrence of graft-vs.-host disease (GVHD) prevents further dose escalation. Previous data indicate that invariant natural killer T (iNKT) cells promote anti-tumor immunity without exacerbating GVHD. In the present study we investigated lysis of leukemic blasts through iNKT cells from donor-derived lymphocytes for leukemia control and found that iNKT cells constitute about 0.12% of cryopreserved donor T cells. Therefore, we established a 2-week cell culture protocol allowing for a robust expansion of iNKT cells from cryopreserved DLIs (DLI-iNKTs) that can be used for further preclinical and clinical applications. Such DLI-iNKTs efficiently lysed leukemia cell lines and primary patient AML blasts *ex vivo* in a dose- and CD1d-dependent manner. Furthermore, expression of CD1d on target cells was required to release proinflammatory cytokines and proapoptotic effector molecules. Our results suggest that iNKT cells from donor-derived lymphocytes are involved in anti-tumor immunity after allo-HCT and therefore may reduce the risk of relapse and improve progression-free and overall survival.

Keywords: DLI, GVHD, GVL, hematopoietic cell transplantation, iNKT cells, adoptive immunotherapy

INTRODUCTION

Allogeneic hematopoietic cell transplantation (allo-HCT) is a curative treatment option for many advanced or high-risk hematologic malignancies like acute myeloid leukemia (AML). Overall survival of such patients has improved over the last decades, but relapse remains the most common cause of death after allo-HCT. Allogeneic donor lymphocytes play an important role in disease control after allo-HCT as they may unfold potent graft-vs.-leukemia (GVL) effects. Infusions of donor lymphocytes (DLIs) from the original hematopoietic stem cell donor after allo-HCT

were found to further strengthen GVL effects. This was first observed by Hans-Jochem Kolb and co-workers in chronic myeloid leukemia (CML) patients in the 1990s (1). Today, DLIs are of clinical use in case of mixed donor chimerism, minimal residual disease (MRD), or relapse. However, the administration of donor lymphocytes is complicated by the high risk of inducing acute or chronic graft-vs.-host disease (GVHD).

Invariant natural killer T (iNKT) cells are characterized by a semi-invariant T-cell receptor (TCRα Vα24-Jα18) with high affinity to glycolipids such as α-galactosylceramide (α-GalCer). Upon stimulation of their TCR, iNKT cells release high amounts of immunoregulatory cytokines such as IFN-γ, TNF-α, and IL-4. This enables iNKT cells to rapidly interact with lymphoid (B, T, and NK cells) and myeloid cells (monocytes, granulocytes), therefore, representing key players in the immuno-regulatory network (2–4). In addition, iNKT cells may induce cell death by producing granzyme B and perforin (5, 6), through Fas/FasL interactions (7–12), and TNF-α-mediated cytotoxic pathways (13). It was recently shown that high iNKT-cell numbers in peripheral blood stem cell grafts are associated with a reduced incidence of GVHD (14, 15) and an improved GVHD-free and progression-free survival (16). Therefore, we analyzed the cellular components of DLIs and investigated whether culture-expanded iNKT cells from DLIs (DLI-iNKTs) could be a way of enhancing anti-leukemia cytotoxicity and thus, help control relapse after allo-HCT.

MATERIALS AND METHODS

Research Subjects

Cryopreserved human donor lymphocytes were obtained from the joint stem cell laboratories of the Department of Medicine II and Children's University Hospital Tübingen. These donor lymphocytes were collected by leukapheresis from 2012 to 2019. Primary leukemia cells (purity $\geq$ 90%) were cryopreserved from untreated patients after informed consent was obtained. The study was approved by our institutional review board to be in accordance with the ethical standards and with the Helsinki Declaration of 1975, as revised in 2013 (IRB approvals 137/2017BO2 and 887/2017BO2).

Flow Cytometry Analysis

PBS57-loaded and unloaded human CD1d tetramers were obtained from the National Institutes of Health Tetramer Core Facility (Atlanta, Georgia, USA). DLIs and iNKT-cell cultures were analyzed by staining with the following antibodies purchased from BioLegend (San Diego, California, USA), BD Biosciences (Franklin Lakes, New Jersey, USA), or eBioscience (Waltham, Massachusetts, USA): CD3 (OKT3, PerCP/Cy5.5), CD4 (RPA-T4, BV785 or BV421), CD8a (HIT8a, AF700 or FITC), CD45 (HI30, BV650), CD19 (SJ25C1, APC-Cy7). Anti-human CD1d APC (Clone 51.1, BioLegend) was used to determine CD1d expression on leukemia cell lines and primary leukemia cells. Fixable Viability Dye eFluor506 from eBioscience and 7-aminoactinomycin (7-AAD, BD Biosciences) were used to exclude dead cells. Anti-human CD107a APC (H4A3, Biolegend) was used for CD107a degranulation assays. Data were acquired on an LSR Fortessa flow cytometer (BD Biosciences) and analysis was performed with FlowJo 10.2 (Tree Star, La Jolla, California, USA).

iNKT-Cell Expansion

iNKT cells from human DLIs were expanded in iNKT-cell culture medium consisting of RPMI 1640 GlutaMAX™ Medium (ThermoFisher Scientific, Waltham, Massachusetts, USA), 10% FBS (fetal bovine serum, Biochrom, Berlin, Germany), 100 IU/ml penicillin-streptomycin (Lonza, Basel, Switzerland), 5.5 μM 2-mercaptoethanol (Roth, Karlsruhe, Germany), 0.1 mM non-essential amino acids (NEAA, Gibco, New York, New York, USA), 10 mM HEPES (Gibco) and 1 mM sodium pyruvate (Gibco). Donor lymphocytes were co-incubated with 100 ng/ml α-GalCer (Sigma-Aldrich, St. Louis, Missouri, USA) and 100 IU/ml recombinant human interleukin 2 (rhIL-2, Novartis, Basel, Switzerland). At day 7, rhIL-2 (100 IU/ml) and α-GalCer (100 ng/ml) was added to the culture and iNKT cells were re-stimulated with irradiated (30 Gy, cesium-137 irradiator Gammacell 1000, Atomic Energy of Canada Limited, Chalk River, Ontario, Canada) and glycolipid-pulsed autologous or allogeneic peripheral blood mononuclear cells (PBMCs) for another 7 days. Thereafter, iNKT-cell expansion was completed. At days 7 and 14, viability and percentage of DLI-iNKTs were measured by flow cytometry.

Magnetic-Activated Cell Sorting (MACS)

For purification of DLI-iNKTs, staining with PBS57-CD1d Tetramer PE was performed. Anti-PE-Microbeads UltraPure (Miltenyi Biotec, Bergisch Gladbach, Germany) were used to enrich DLI-iNKTs via QuadroMACS™ Separator (Miltenyi Biotec) and LS Columns (Miltenyi Biotec) according to the manufacturer's instructions.

Tumor Cell Lysis Assay

DLI-iNKTs were co-incubated with leukemia cell lines or primary patient leukemia cells at increasing effector- to target-cell ratios. The following tumor cell lines were used as target cells: Jurkat (Clone E6-1, ATCC, Manassas, Virginia, USA), K562 (CCL-243, ATCC, Manassas, Virginia, USA), THP-1 (TIB-202, ATCC, Manassas, Virginia, USA). Co-culture was performed in iNKT-cell culture medium with and without 100 ng/ml α-GalCer. After 16 h, cell lysis was measured by flow cytometry (LSR Fortessa, BD Biosciences) using 7-aminoactinomycin (7-AAD, BD Biosciences) and iNKT cells were excluded by staining with PBS57-loaded human CD1d tetramer. Specific lysis was calculated by the following formula: percentage of specific lysis = [1—(target cell viability with effector cells/target cell viability without effector cells)] × 100. For blocking experiments, purified anti-human CD1d (51.1, BioLegend) and the respective isotype control antibody were used at 10 μg/ml.

CD107a Assay

DLI-iNKTs were co-incubated with target cells at a 2.5:1 ratio in presence of anti-human CD107a APC (H4A3, Biolegend) and protein transport inhibitor cocktail (Brefeldin A und Monensin, 500X, eBioscience). For blocking experiments, anti-CD1d or the

respective isotype control were added. After 16 h, additional staining with PBS57-CD1d Tetramer and 7-AAD was performed and cells were measured using an LSR Fortessa (BD Bioscience).

Cytokine Analysis

Supernatants from tumor cell lysis experiments were collected after 16 h and stored at −20°C. A multiplex assay (LEGENDplex™ Human CD8/NK Panel (13-plex), BioLegend) was used according to the manufacturer's instructions. LEGENDplex™ Software from BioLegend was used for analysis of acquired data.

Statistical Analysis

Flow cytometry data were analyzed by FlowJo V10 (Treestar). Data were further analyzed with Prism 7.01 (GraphPad Software, La Jolla, CA, USA). Experiments were performed in duplicates and repeated independently at least three times. Student's *t*-test and one-way ANOVA were used for statistical analysis and $p < 0.05$ was considered statistically significant.

RESULTS

DLIs Contain a Small but Distinct Fraction of Mostly CD4$^-$/CD8$^-$ iNKT Cells

In order to analyze the amount of T cells and iNKT cells in human DLIs ($n = 63$) by flow cytometry, the gating strategy was applied as outlined in **Figure 1A**. CD3$^+$ T cells represent 47.3% of living cells (SD ± 16.0%). A small but distinct fraction of iNKT cells was detected in human DLIs, constituting 0.12% of CD3$^+$ T cells (SD ± 0.22%). We then analyzed iNKT-cell subtypes and found that most iNKT cells were CD4$^-$CD8$^-$ (71.1% of iNKT cells, SD ± 13.4%, **Figure 1B**). 18.4% were CD4$^+$ iNKT cells (SD ± 14.1%) and 9.1% were CD8$^+$ iNKT cells (±6.3%). Administration of granulocyte colony-stimulating factor (G-CSF, Lenograstim, 2 × 5 μg/kg/d for 5 days) prior to collection of donor lymphocytes did not affect iNKT-cell numbers, subsets and function (**Supplemental Figure 1**).

Glycolipid Stimulation for 2 Weeks Leads to Stable *ex vivo* Expansion of Human iNKT Cells From Cryopreserved DLIs

Due to the low cell numbers of iNKT cells in cryopreserved DLIs, we established a 2-week expansion protocol to obtain enough cells for further experiments and potential clinical applications. A 281-fold expansion (range 71.4–696.6) of iNKT cells with a purity of 24.7% was obtained after 2 weeks of cell culture using the glycolipid α-GalCer and rhIL-2 (**Figure 1C**). Subsequently, we further enriched culture-expanded DLI-iNKTs by magnetic-activated cell sorting (MACS, **Figure 1D**) and reached a purity of >95%. Absolute iNKT-cell counts from seven independent experiments are shown in **Figure 1E**. Moreover, we observed a preferential expansion of CD4$^+$ iNKT cells (969-fold expansion compared to 297-fold expansion of CD8$^+$ and 122-fold expansion of CD4$^-$CD8$^-$ iNKT cells, $n = 7$, **Figure 1E**).

DLI-iNKTs Lyse Leukemia Cell Lines in a Dose-Dependent Manner and Upregulate the Degranulation Marker CD107a

Next, we were interested whether culture-expanded and purified DLI-iNKTs could exert anti-leukemia activity being crucial for disease control after allo-HCT. Therefore, DLI-iNKTs were co-incubated with Jurkat leukemia cells at increasing effector- to target-cell ratios. We observed a dose-dependent lysis of Jurkat cells (**Figures 2A,B**) that was more pronounced in presence of α-GalCer (**Supplemental Figures 2A,B**).

CD107a (LAMP-1) is a degranulation marker expressed on activated cytotoxic T cells (17–19) and NK cells (20) and has been shown to correlate with cytotoxicity (21). CD107a is also expressed on iNKT cells (22). We found a significantly higher expression of CD107a upon engagement with Jurkat cells compared to DLI-iNKTs alone indicating the release of cytotoxic effector molecules (**Figures 2C,D**). **Supplemental Figures 2C,D** demonstrate an increased upregulation of CD107a on DLI-iNKTs in presence of α-GalCer compared to without glycolipid. Moreover, upregulation of CD107a was most pronounced on the CD4-CD8- subset of DLI-iNKTs (**Supplemental Figure 4**).

The functional hallmark of iNKT cells is the instant release of immunoregulatory cytokines. Therefore, proinflammatory cytokines and proapoptotic effector molecules such as IFN-γ, TNF-α, and perforin were analyzed after co-culture of DLI-iNKTs and target cells. We observed a significantly increased production of IFN-γ (**Figure 2E**), TNF-α (**Figure 2F**), sFasL (**Figure 2G**), and perforin (**Figure 2H**) when DLI-iNKT cells were co-incubated with Jurkat cells compared to DLI-iNKT cells without target cells.

DLI-iNKTs Lyse Leukemia Cells in a CD1d-Dependent Manner

We challenged various leukemia cell lines with DLI-iNKTs and found significant differences regarding the effectiveness of leukemia cell lysis: dose-dependent specific lysis of target cells was most efficient for Jurkat cells followed by THP-1 and K562 (**Figure 3A**). iNKT cells can be activated by TCR stimulation through presentation of glycolipids by the MHC class I-like molecule CD1d. Therefore, we were interested in determining the expression of CD1d on leukemia cell lines. We found that CD1d expression was highest on Jurkat cells, followed by THP-1 and almost no CD1d was expressed on K562 (**Figure 3B**).

Consequently, we assumed that CD1d expression on target cells is required to induce efficient leukemia cell lysis through DLI-iNKTs. Adding the CD1d-blocking antibody 51.1 resulted in a significantly decreased specific lysis of Jurkat cells (**Figures 3C,D**) and a significantly reduced expression of CD107a on DLI-iNKTs (**Figures 3E,F**) compared to the corresponding isotype control. We could also observe CD1d-dependent lysis in absence of α-GalCer (**Supplemental Figure 3**). In addition, we studied the release of proinflammatory cytokines and proapoptotic effector molecules while blocking CD1d on target cells: levels of IFN-γ (**Figure 3G**), TNF-α (**Figure 3H**), sFasL (**Figure 3I**), and perforin (**Figure 3J**) were decreased in presence of anti-CD1d compared to isotype control antibody. We

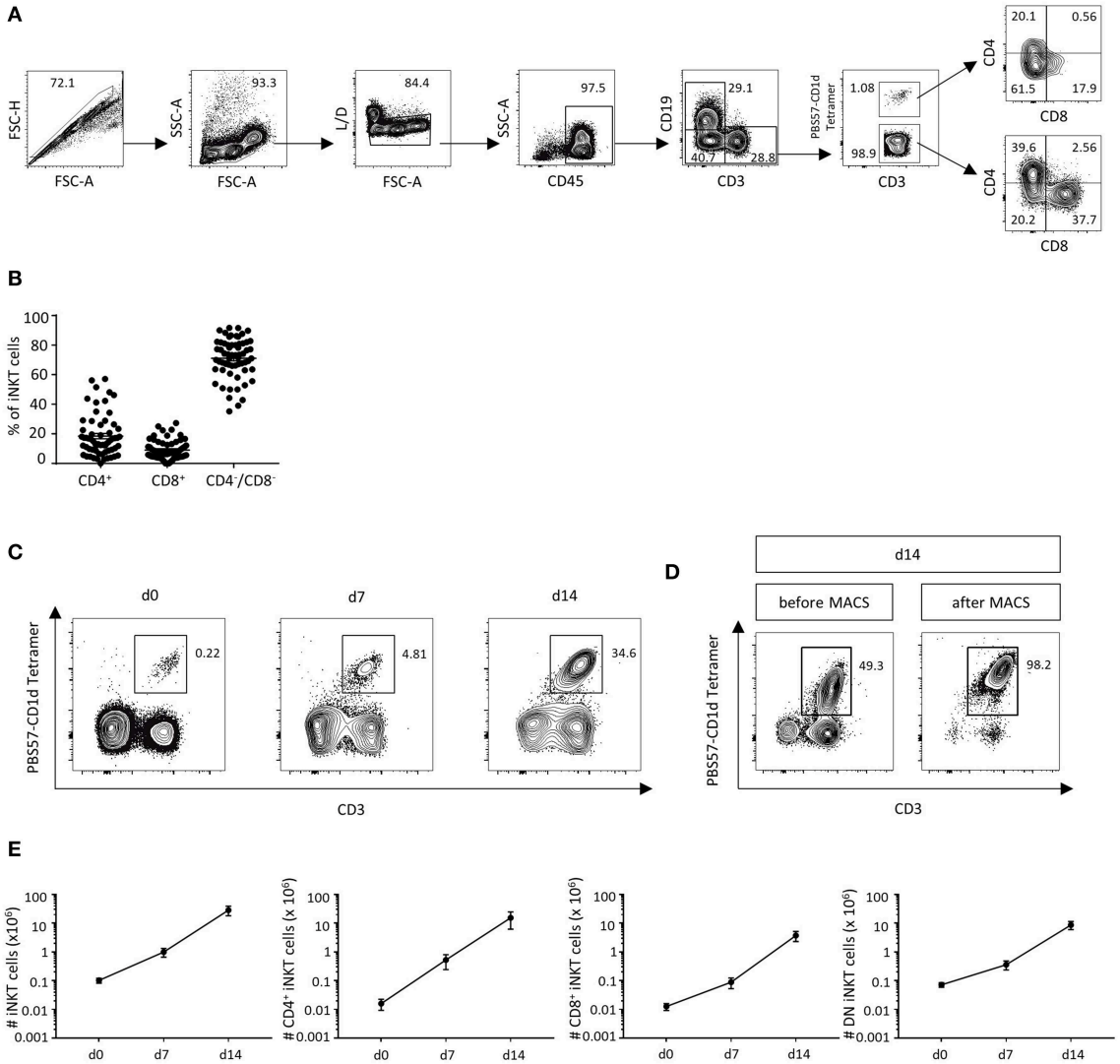

FIGURE 1 | DLIs contain low numbers of iNKT cells that can be expanded *ex vivo*. **(A)** Gating strategy to identify $CD3^+$PBS57-CD1d $Tetramer^+$ iNKT cells and $CD4^+CD8^-$, $CD4^-CD8^+$ and $CD4^-CD8^-$ iNKT-cell subsets in DLIs. **(B)** Percent of iNKT-cell subsets in DLIs prior to *ex vivo* expansion ($n = 63$). Bars represent standard error of the mean (SEM) **(C)**. Representative dot plots showing iNKT-cell expansion following a 2-week cell culture protocol using α-GalCer and rhIL-2 **(D)**. Representative dot plots illustrating further purification of DLI-iNKTs by magnetic-activated cell sorting (MACS) after 14 days of cell culture **(E)**. Absolute numbers of iNKT cells and iNKT-cell subsets at 0, 7, and 14 days of cell culture ($n = 7$). Bars represent SEM.

conclude that DLI-iNKTs release cytotoxic effector molecules in a CD1d-dependent manner resulting in leukemia cell death.

Primary Patient Leukemia Cells Are Lysed by Culture-Expanded DLI-iNKTs in a Dose- and CD1d-Dependent Manner

Next, we investigated whether primary patient AML blasts were also susceptible to DLI-iNKT-induced cytotoxicity. When incubating different primary AML blasts from patients with DLI-iNKTs, we observed efficient and dose-dependent lysis of primary leukemia cells (**Figure 4A**). In line with our previous findings, blockade of CD1d significantly reduced leukemia cell lysis (**Figures 4B,C**). Comparable results were obtained without adding α-GalCer to the culture (**Supplemental Figure 5**). Accordingly, the expression level of CD1d on primary patient AML blasts significantly correlated with their specific lysis through DLI-iNKTs ($r^2 = 0.7$, $p = 0.03$, **Figure 4D**). Therefore, higher expression of CD1d on leukemia cells could be an indicator of improved leukemia cell lysis and a prognostic factor for successful DLI-iNKT cytotherapy. **Supplemental Figure 6** shows representative dot plots of CD1d expression and the immunophenotype of AML blasts used for this study.

DISCUSSION

DLIs are capable of inducing remission and converting mixed into complete donor chimerism after allo-HCT (23). Therefore, DLIs represent a widely accepted therapy for relapse and

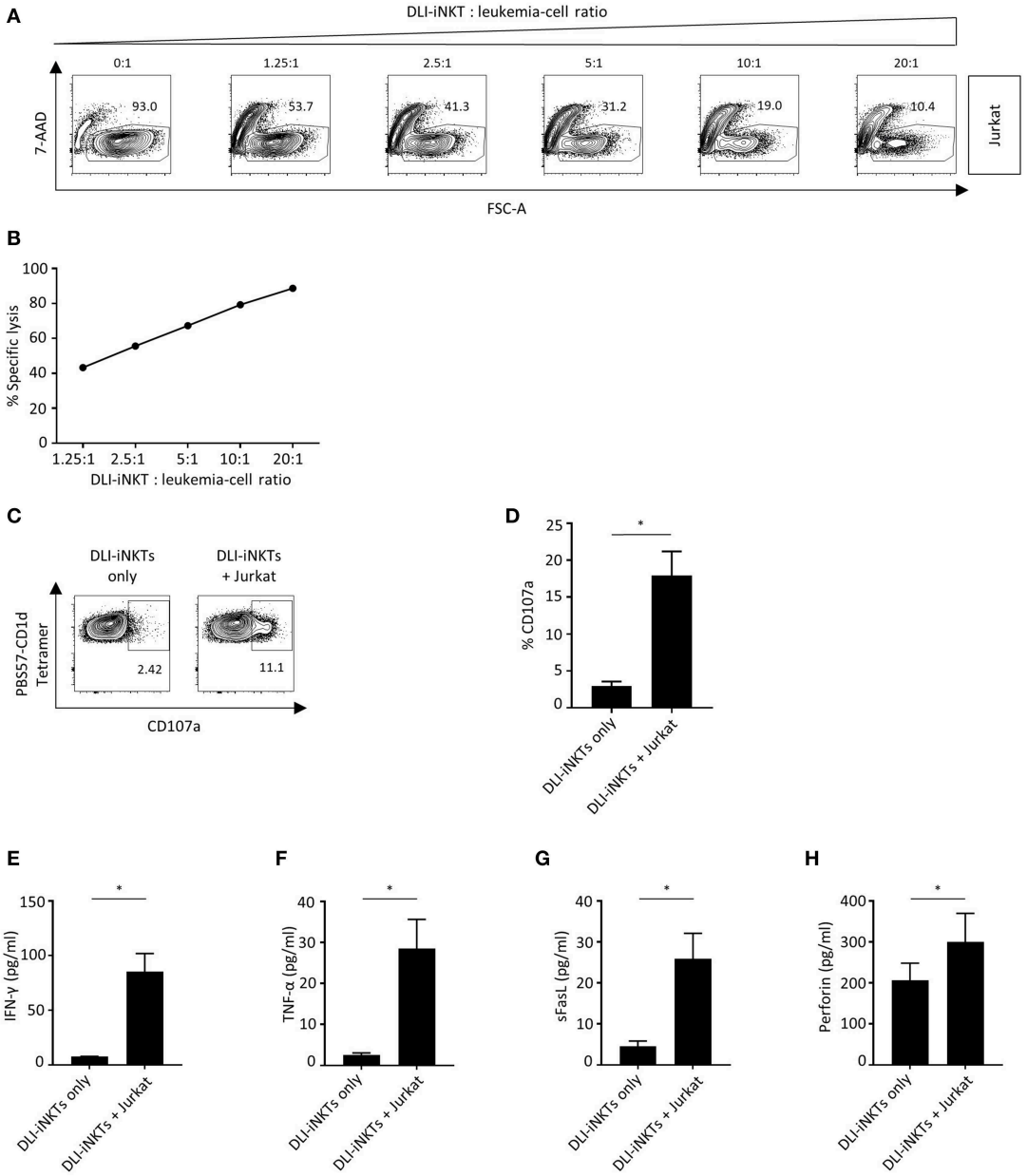

FIGURE 2 | DLI-iNKTs lyse Jurkat cells in a dose-dependent manner. **(A)** Representative dot plots of DLI-iNKT-induced lysis of Jurkat cells in presence of α-GalCer. iNKT cells were excluded by gating on PBS57-CD1d Tetramer$^-$ cells. **(B)** Specific lysis of Jurkat cells co-cultured with increasing numbers of DLI-iNKTs in presence of α-GalCer. Shown is one of seven representative experiments. **(C)** Representative dot plots and **(D)** pooled data illustrating CD107a expression on CD3$^+$PBS57-CD1d Tetramer$^+$ DLI-iNKTs after co-culture with Jurkat cells in presence of α-GalCer ($n = 3$). **(E)** IFN-γ, **(F)** TNF-α, **(G)** sFasL, and **(H)** perforin measured in supernatants after co-culture with Jurkat cells in presence of α-GalCer ($n = 5$). Bars represent SEM. *$p < 0.05$.

prevention of graft rejection. However, loss of immune tolerance and occurrence of GVHD often preclude administration of donor lymphocytes and further dose escalation. Previous clinical studies showed that higher iNKT-cell numbers in the graft or peripheral blood post-transplant were associated with a reduced incidence of GVHD (14, 24). De Lalla and co-workers investigated iNKT-cell reconstitution in pediatric haploidentical transplant patients and found that iNKT cells failed to reconstitute in individuals experiencing relapse (25). Moreover, increased iNKT-cell numbers in peripheral blood stem cell allografts correlated with an improved GVHD-free and progression-free survival indicating that iNKT cells induce immune tolerance while allowing for robust GVL effects (16). In addition, adoptively transferred iNKT cells prevent lethal GVHD without compromising T cell-mediated lysis of leukemia cells in murine models of

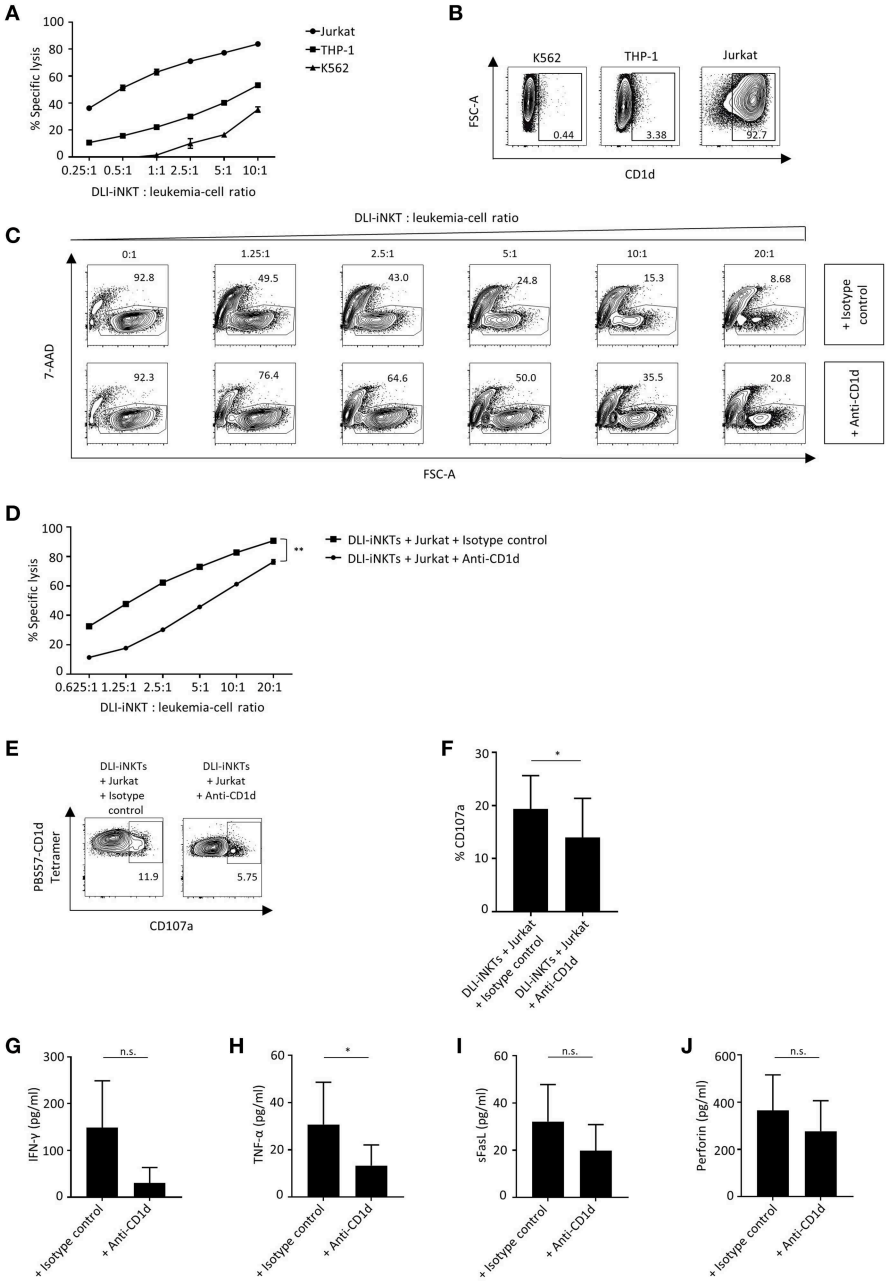

FIGURE 3 | CD1d expression is required for efficient leukemia cell lysis through DLI-iNKTs. **(A)** Specific lysis of Jurkat cells, THP-1 and K562 co-cultured with increasing numbers of DLI-iNKTs in presence of α-GalCer ($n = 2$). **(B)** Representative dot plots illustrating CD1d expression on K562, THP-1, and Jurkat leukemia cell lines. **(C)** Representative dot plots and **(D)** specific lysis of Jurkat cells through DLI-iNKTs in presence of anti-CD1d and isotype control antibody together with α-GalCer ($n = 3$). iNKT cells were excluded by gating on PBS57-CD1d Tetramer$^-$ cells. **(E)** Representative dot plots and **(F)** pooled data illustrating CD107a expression on CD3$^+$PBS57-CD1d Tetramer$^+$ DLI-iNKTs after co-culture with Jurkat cells and anti-CD1d or isotype control antibody in presence of α-GalCer ($n = 3$). **(G)** IFN-γ, **(H)** TNF-α, **(I)** sFasL, and **(J)** perforin measured in supernatants after co-culture with Jurkat cells and anti-CD1d or isotype control antibody in presence of α-GalCer ($n = 5$). Bars represent SEM. *$p < 0.05$; **$p < 0.01$.

allo-HCT (3, 26). In contrast, these iNKT-cell infusions exert potent anti-tumor immunity by themselves (3). Based on these observations we investigated the amount, expansion capacity, and functional properties of human iNKT cells from cryopreserved DLIs.

As iNKT cells are scarce in human peripheral blood and in freshly thawed DLIs, they first need to be expanded *in* or *ex vivo*. This could be done by intravenous administration of exogenous iNKT-cell agonists such as α-GalCer. However, intravenous infusion of α-GalCer may induce overshooting

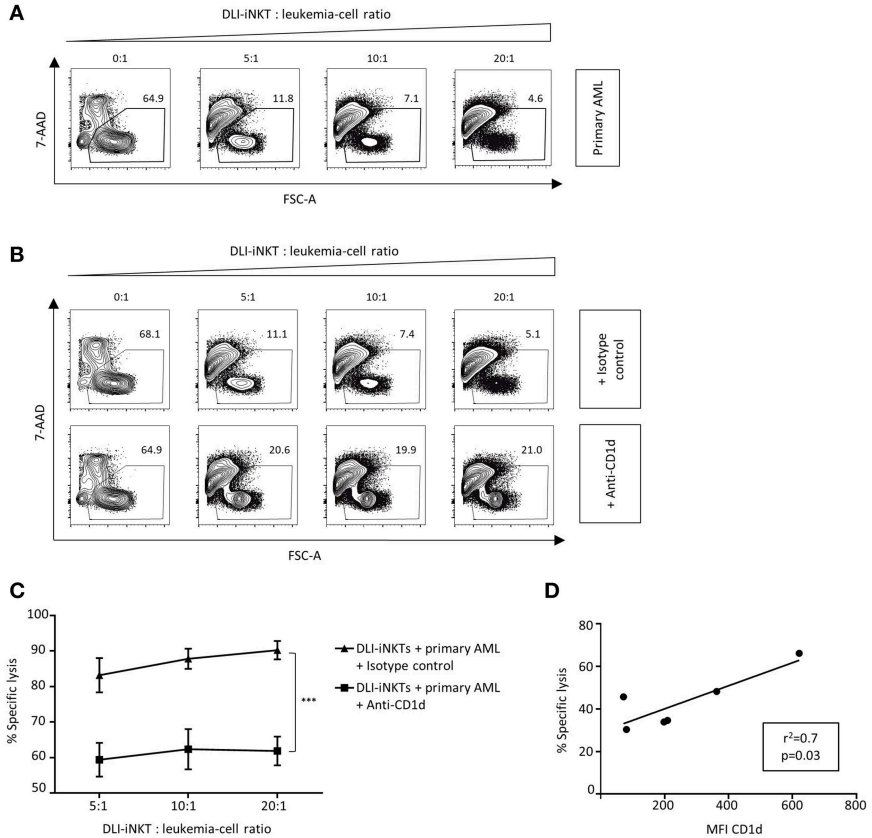

FIGURE 4 | Patient AML blasts are lysed by DLI-iNKTs in a CD1d-dependent manner. **(A)** Representative dot plots illustrating dose-dependent lysis of primary patient AML blasts through culture-expanded DLI-iNKTs. **(B)** Representative dot plots and **(C)** specific lysis of primary patient AML blasts through DLI-iNKTs in presence of anti-CD1d and isotype control antibody together with α-GalCer ($n = 3$). iNKT cells were excluded by gating on PBS57-CD1d Tetramer$^-$ cells. Bars represent SEM. **(D)** Correlation of specific lysis of primary patient AML blasts through DLI-iNKTs with the mean fluorescence intensity (MFI) of CD1d on respective leukemia cells ($n = 6$). ***$p < 0.001$.

donor iNKT-cell activation that can result in iNKT-cell anergy and exhaustion (27), thus failing to show any clinical response. Like freshly thawed DLIs, transplant patients contain low iNKT-cell numbers due to the extensive pretreatment probably further limiting the effectiveness of glycolipid infusions. We therefore expand iNKT cells from cryopreserved DLIs following a 2-week protocol using α-GalCer and rhIL-2: although most iNKT cells were double negative before cell culture, we observed a preferential expansion of CD4$^+$ cells. iNKT cells are a complex cell population with diverse subsets: CD4$^-$ iNKT cells were shown to produce Th1 cytokines such as IFN-γ and TNF-α as well as double-negative iNKT cells that showed a Th1 profile (28). In contrast, CD4$^+$ iNKT cells could produce both Th1 and Th2 cytokines (29). After allo-HCT, stimulation of CD4$^+$ iNKT cells results in secretion of Th2-biased cytokines such as IL-4 and IL-13 that are both critical to restore immune tolerance (30, 31). However, CD4$^+$ iNKT cells are also capable of lysing tumor cells releasing cytotoxic effector molecules (32, 33). Accordingly, we showed that DLI-iNKTs produce proinflammatory cytokines as well as perforin exerting potent anti-leukemia cytotoxicity that is dependent on the expression of CD1d on leukemia cells.

iNKT cells can be activated by direct interaction with tumor cells: CD1d$^+$ tumor cells present endogenous glycolipids via CD1d which is then recognized by the T-cell receptor of iNKT cells leading to perforin/granzyme B or Fas/FasL-mediated cytotoxicity. CD1d is mostly expressed in hematopoietic cells (34, 35) and can be found on myelomonocytic and B-cell lineage malignancies (36). Accordingly, Spanoudakis et al. showed that myeloma progression is associated with decreased CD1d surface expression, linking CD1d with tumor survival in humans (37). Conversely, increasing the expression of antigen-presenting molecules like CD1d by gemcitabine and cyclophosphamide and combining chemotherapy with NKT-cell activation results in enhanced tumor control and survival (38). However, cross-presentation of tumor glycolipids by antigen-presenting cells (APCs) may also play an important role in iNKT-cell activation since some entities show low or no CD1d expression: presentation of glycolipids via CD1d on APCs stimulates iNKT cells to produce cytokines such as IFN-γ and IL-2 that subsequently activate NK cells and tumor-specific T cells (39). In the context of umbilical cord blood transplantation, Beziat et al. showed that iNKT cells efficiently lysed CD1d expressing blasts 6 months after transplant (40).

Importantly, our data indicate that lysis of leukemia cell lines and primary blasts is more efficient in presence of α-GalCer. Glycolipid-loaded tumor cells might be more visible to DLI-iNKTs while the respective ligand induces robust activation of effector cells. To enhance leukemia control after adoptive transfer of DLI-iNKTs, concomitant infusion of such glycolipids should be considered. Chen and colleagues have demonstrated the feasibility and safety of glycolipid infusions in the setting of allogeneic HCT (41).

We reported previously that G-CSF administration prior to donor lymphocyte apheresis results in an improved conversion to complete donor chimerism and a lower incidence of relapse or progression without increasing the risk of GVHD after infusion of donor lymphocytes. We also identified higher numbers of hematopoietic stem and progenitor cells, myeloid-derived suppressor cells and monocytes as independent risk factors for an improved overall survival (42). In the present study, 26 DLIs were derived from donors that were pretreated with G-CSF. However, G-CSF administration did not influence iNKT-cell numbers, subsets, expansion and lysis of leukemic blasts. Taken together, we assume that iNKT-cell numbers in conventional DLIs are too small to directly exert robust anti-leukemic effects. Instead, prior expansion and activation by glycolipids seems to be a reasonable approach to promote sustained GVL effects through iNKT cells themselves.

Therefore, in order to exploit potent GVL effects without exacerbating GVHD, manipulating the cellular composition of DLIs may be beneficial: by expanding iNKT cells *ex vivo*, they could be enriched in DLIs prior to infusion into patients. As GVHD represents a major dose-limiting toxicity and side effect of allo-HCT and DLIs, further dose escalation of donor lymphocytes is often impossible, therefore not allowing clinicians to completely harness the power of DLIs in leukemia control and prevention of relapse. iNKT cells represent a promising opportunity as they suppress GVHD without losing GVL effects (3, 16).

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Helsinki Declaration of 1975, as revised in 2013 (IRB approvals 137/2017BO2 and 887/2017BO2) with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the institutional review board of the University Hospital Tuebingen, Germany.

AUTHOR CONTRIBUTIONS

SJ designed and performed research, analyzed data, and wrote the manuscript. HS, KA-S, JE, S-DS, HK, and RB performed experiments and analyzed data. IS-M performed the leukapheresis procedure and provided cell products. MS, RH, WB, LK, and CS helped interpreting data and assisted in preparing the manuscript. DS designed experiments, wrote the manuscript, and provided overall guidance. All authors edited the manuscript for content.

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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.2019.01542/full#supplementary-material

Supplemental Figure 1 | Application of G-CSF before donor lymphocyte apheresis. Impact of prior G-CSF administration ($n = 26$) compared to steady state apheresis ($n = 35$) on **(A)** iNKT-cell numbers, **(B)** iNKT-cell subsets, **(C)** expansion of iNKT cells ($n = 7$), and **(D)** specific lysis of Jurkat cells by culture-expanded DLI-iNKTs ($n = 8$). Bars represent SEM.

Supplemental Figure 2 | DLI-iNKTs lyse Jurkat cells in a dose-dependent manner. **(A)** Representative dot plots of DLI-iNKT-induced lysis of Jurkat cells without and with α-GalCer. iNKT cells were excluded by gating on PBS57-CD1d Tetramer$^-$ cells **(B)**. Specific lysis of Jurkat cells co-cultured with increasing numbers of DLI-iNKTs without and with α-GalCer. Shown are pooled data of three representative experiments. **(C)** Representative dot plots and **(D)** pooled data illustrating CD107a expression on CD3$^+$PBS57-CD1d Tetramer$^+$ DLI-iNKTs after co-culture with Jurkat cells without and with α-GalCer ($n = 3$). Bars represent SEM. *$p < 0.05$; ***$p < 0.001$.

Supplemental Figure 3 | CD1d expression is required for efficient leukemia cell lysis through DLI-iNKTs. **(A)** Representative dot plots and **(B)** specific lysis of Jurkat cells through DLI-iNKTs in presence of anti-CD1d and isotype control antibody without and with α-GalCer ($n = 3$). iNKT cells were excluded by gating on PBS57-CD1d Tetramer$^-$ cells. **(C)** Representative dot plots and **(D)** pooled data illustrating CD107a expression on CD3$^+$PBS57-CD1d Tetramer$^+$ DLI-iNKTs

after co-culture with Jurkat cells and anti-CD1d or isotype control antibody without and with α-GalCer ($n = 3$). Bars represent SEM. ***$p < 0.001$.

Supplemental Figure 4 | Expression of CD107a on DLI-iNKT subsets. Expression of CD107a on **(A)** $CD4^+CD8^-$ **(B)** $CD4^-CD8^+$ **(C)** $CD4^-CD8^-$ $CD3^+$PBS57-CD1d $Tetramer^+$ DLI-iNKTs after co-culture with Jurkat cells and anti-CD1d or isotype control antibody without and with α-GalCer. For each group $n = 3$. Bars represent SEM. *$p < 0.05$.

Supplemental Figure 5 | Patient AML blasts are lysed by DLI-iNKTs in a CD1d-dependent manner. **(A)** Representative dot plots and **(B)** specific lysis illustrating dose-dependent lysis of primary patient AML blasts through culture-expanded DLI-iNKTs in absence and in presence of α-GalCer ($n = 3$). **(C)** Representative dot plots and **(D)** specific lysis of primary patient AML blasts through DLI-iNKTs in presence of anti-CD1d and isotype control antibody together with and without α-GalCer ($n = 3$). iNKT cells were excluded by gating on PBS57-CD1d $Tetramer^-$ cells. Bars represent SEM. *$p < 0.05$.

Supplemental Figure 6 | Phenotype of patient AML blasts. **(A)** Representative dot plots of CD1d staining. **(B)** Immunophenotype of patient AML blasts. 0, negative; 0/+, low; +, positive; n.a., data not available; pB, peripheral blood.

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REVIEW
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Graft Engineering and Adoptive Immunotherapy: New Approaches to Promote Immune Tolerance After Hematopoietic Stem Cell Transplantation

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Allogeneic hematopoietic stem cell transplantation (HSCT) is a curative therapeutic option for a wide range of immune and hematologic malignant and non-malignant disorders. Once transplanted, allogeneic cells have to support myeloid repopulation and immunological reconstitution, but also need to become tolerant to the host via central or peripheral mechanisms to achieve the desired therapeutic effect. Peripheral tolerance after allogeneic HSCT may be achieved by several mechanisms, though blocking alloreactivity to the host human leukocyte antigens while preserving immune responses to pathogens and tumor antigens remains a challenge. Recently uncovered evidence on the mechanisms of post-HSCT immune reconstitution and tolerance in transplanted patients has allowed for the development of novel cell-based therapeutic approaches. These therapies are aimed at inducing long-term peripheral tolerance and reducing the risk of graft-vs-host disease (GvHD), while sparing the graft-vs-leukemia (GvL) effect. Thus, ensuring effective long term remission in hematologic malignancies. Today, haploidentical stem cell transplants have become a widely used treatment for patients with hematological malignancies. A myriad of *ex vivo* and *in vivo* T-cell depletion strategies have been adopted, with the goal of preventing GvHD while preserving GvL in the context of immunogenetic disparity. αβ T-cell/CD19 B-cell depletion techniques, in particular, has gained significant momentum, because of the high rate of leukemia-free survival and the low risk of severe GvHD. Despite progress, better treatments are still needed in a portion of patients to further reduce the incidence of relapse and achieve long-term tolerance. Current post-HSCT cell therapy approaches designed to induce tolerance and minimizing GvHD occurrence include the use of (i) γδ T cells, (ii) regulatory Type 1 T (Tr1) cells, and (iii) engineered FOXP3^{+} regulatory T cells. Future protocols may include post-HSCT infusion of allogeneic effector or regulatory T cells engineered with a chimeric antigen receptor (CAR). In the present review, we describe the most recent advances in graft engineering and post-HSCT adoptive immunotherapy.

Keywords: immune tolerance, gamma delta (γδ) T cells, Treg - regulatory T cells, CAR (chimeric antigen receptor) T cells, haploidentical allogeneic hematopoietic stem cell transplantation

INTRODUCTION

Successful allogeneic hematopoietic stem cell transplantation (HSCT) requires the development of immune tolerance toward both the donor and host allogeneic antigens. Induction of immune tolerance can prevent T-cell mediated graft-rejection and graft-vs-host disease (GvHD), which cause severe pathology in HSCT recipients. Current approaches to prevent rejection and GvHD after HSCT primarily rely on pharmacological immune suppression, either prior to or after HSCT. These approaches are limited by acute and long-term drug toxicity, lack of antigen specificity, and the requirement for long-term therapy, which often leads to severe complications. Recent progress in understanding the mechanism of action of alloreactive and regulatory cell populations has led to the use of specific cell subsets to prevent/treat graft rejection and GvHD and induce immune tolerance. In hematologic malignancies maintaining effective anti-tumor control while inducing sustained immune tolerance is critical to survival following allogeneic HSCT.

In the past decade, several new graft-engineering approaches have been explored to reduce the risk of life-threatening GvHD, while retaining the effector cells that mediate infection control and graft-vs-leukemia (GvL). Concurrently, post-HSCT adoptive cell therapies have been used with increasing frequency to induce tolerance and reduce the risk of leukemia recurrence. This review will summarize the results of these new approaches in patients with hematological malignancies.

RECENT ADVANCES IN GRAFT MANIPULATION: THE ROLE OF HLA-HAPLOIDENTICAL HSCT

Allogeneic HSCT from an HLA-matched donor, either related or unrelated, has been widely employed to treat patients with both malignant and non-malignant disorders (1). Only 25% of patients who are candidates to receive allogeneic HSCT have an HLA-identical sibling. Suitable, unrelated donors (UD) can be identified for <60% of the remaining patients in need (2). The likelihood of finding an appropriate UD varies among racial and ethnic groups, with the probability of identifying an appropriate donor being highest among whites of European descent (75%) and lowest among blacks or those of South or Central American descent (16%) (3). As such, a related full-haplotype mismatched donor (haploidentical) as an alternative source of hematopoietic stem cell (HSC), is highly attractive, as virtually all patients have a readily available haploidentical family member who can serve as an HSC donor (2, 4, 5). Despite many advantages associated with haploidentical (haplo-) HSCT, in the past the widespread use of this procedure was hampered by severe clinical complications due to bidirectional alloreactivity toward incompatible HLA molecules, including high rates of graft-rejection and severe GvHD. Since donor-derived T lymphocytes contained in the graft are the major mediators of severe GvHD in haplo-HSCT, several approaches have been explored to deplete T cells from the graft prior to or post-infusion. Over the past 10 years, the clinical use of haploidentical donors has gained traction thanks to the use of T-cell depleted peripheral blood stem cells (PBSC) or of unmanipulated (either bone marrow -BM- or PBSC) grafts followed by high-dose post-transplant cyclophosphamide (PTCY) (6–9). The very first *ex vivo* T-cell depleted haplo-HSCTs using soybean agglutinin and rosette formation sheep red blood cells were performed in children with primary immunodeficiencies (10). As of today, hundreds of Severe Combined Immune Deficiency (SCID) patients have been transplanted worldwide using an HLA-haploidentical related donor, with a high rate of long-term, partial or complete, immune reconstitution (11). Initially, these encouraging outcomes were not replicable in leukemia patients, in whom haplo-HSCT was associated with an unacceptably high incidence of graft failure (12). Since then, several preclinical studies have led to a variety of promising techniques to diminish the intense alloreactivity in haplo-HSCT for hematological malignancies. These new approaches have yielded high rates of successful engraftment, effective GvHD control and favorable outcomes. Retrospective analyses of adult cohorts reported in the last decade have demonstrated similar survival after haplo-HSCT, HLA-matched-related, or HLA-matched-unrelated HSCT in leukemia patients (13, 14).

The unmanipulated haploidentical approach, pioneered by the group of Fuchs EJ and Luznik L, relies on the use of PTCY. This drug targets the early proliferation of both donor and recipient alloreactive T cells that occurs in the first few days after HSCT (15). Indeed, cyclophosphamide mediates *in vivo* depletion of both donor and recipient alloreactive cells while sparing quiescent non-alloreactive T cells, when given in the 72 h window after T-cell replete HSCT (either BM or PBSC). This method promotes engraftment and reduces the risk of severe acute GvHD. Pilot studies in adults conditioned with a non-myeloablative (NMA) preparative regimen and transplanted with BM cells showed 90% engraftment with very low incidence of both acute and chronic GvHD (16). Subsequent studies in haplo-HSCT using myeloablative conditioning and PTCY reported better control of leukemia with no significant increase in GvHD or Non-relapse mortality (NRM) (17, 18). The use of PBSC as graft source instead of BM led to some increase in acute GvHD incidence, but similar outcomes in terms of engraftment and NRM (19). Overall, these studies have established PTCY-based haplo-HSCT as a frontrunner for alternate donor HSCT in adults, prompting selection of PTCY-based haplo-HSCT over matched UD (MUD) or umbilical cord blood (UCB) HSCT (14) for many patient.

While this strategy has been extensively investigated in adult patients, results on the use of unmanipulated haplo-HSCT in the pediatric population have only recently been published (20–22). Early results of GvHD prevention are encouraging, though limited information on follow-up results is available.

Ex-vivo T-Cell Depletion in Haploidentical HSCT: The Evolution

Pioneering studies in adults demonstrated that infusion of "*megadoses*" of purified $CD34^+$ cells can prevent both graft

rejection and severe GvHD in adult haplo-HSCT recipients (12, 23). In a pilot study of adults with acute leukemia, Aversa et al. used a combination of donor BM and G-CSF-mobilized PBSC. This allowed for the collection of 7–10 times more hematopoietic progenitor cells compared to BM allografts alone. Allografts were T-cell depleted using soybean agglutination and erythrocyte rosetting. No post-transplant immunosuppression was given. Engraftment rate was above 90%, with a cumulative incidence of both grade II–IV acute and chronic GvHD below 10%. In this study, only two patients relapsed, but NRM occurred in 9 out of 17 patients (23). The method was then further improved by immunomagnetic selection of $CD34^+$ cells, which drastically reduces the T- and B-cell content in the graft, allowing the infusion of more than 10×10^6/kg $CD34^+$ cells, with a mean $CD3^+$ cell infusion of a 3×10^3/kg. In this seminal study, Aversa et al. showed sustained engraftment in 41/43 adult and pediatric patients (age range 4–53 years) with advanced leukemia, without acute or chronic GvHD and a long term disease-free survival (DFS) rate of 28% (12). Although the administration of HSC "*megadoses*" addressed the rejection problem, removal of T cells from the graft entailed prolonged lymphopenia and delayed immune reconstitution for patients, with low $CD4^+$ T cells persisting for more than a year after transplantation. As a result, the NRM was 40%, with two thirds of these deaths due to opportunistic infections. After these initial studies, Martelli et al. reported that freshly isolated donor-derived regulatory T cells (Tregs), coinfused with conventional T cells (Tcons), protect recipients against GvHD (24). In 2014, the same group investigated whether Treg-Tcons adoptive immunotherapy prevents post-HSCT leukemia relapse (24). Forty-three adults with high-risk acute leukemia conditioned with a total body irradiation (TBI)-based regimen received $CD34^+$ cells, Tregs and Tcons without post-HSCT immunosuppression. Ninety-five percent of patients achieved full-donor engraftment but 15% of them developed grade II-IV acute GvHD. The rate of DFS was 56% at a median follow-up of 46 months. Cumulative incidence (CI) of relapse (REL) (5%) was significantly better than in historical controls. These results demonstrate the immunosuppressive potential of Tregs in preventing GvHD without loss of GvL activity. Humanized murine models have since provided insights into the mechanisms underlying the separation of GvL from GvHD, with the effect of GvL being largely due to unopposed Tcons alloantigen recognition in BM.

Aversa et al. have also performed an extensive analysis of using $CD34^+$ positively selected haplo-HSCT combined with rigorous T-cell depletion of the graft and the use of PTCY (25) in both a pre-clinical model and three patients. In this report, the authors demonstrated that, in mice treated with a NMA conditioning regimen, coupling the power of high-dose PTCY with the infusion of T-cell depleted HSC "*megadoses*" can be a suitable option for overcoming graft rejection. This approach was then evaluated in two patients with multiple myeloma and one patient with Hodgkin lymphoma. At a 25 month follow up, the first myeloma patient exhibited full donor chimerism in the myeloid- and B-cell lineages and mixed chimerism in the T-cell lineage. Conversely, the second myeloma patient failed to attain chimerism. Notably, the low toxicity of this protocol enabled a subsequent successful fully myeloablative haplo-HSCT in this patient. The third patient was conditioned with slightly higher TBI, resulting in prompt engraftment. All patients currently remain in remission without GvHD (25). These preliminary data lay the foundation for a novel and safer NMA haplo-HSCT, a potential platform for immune tolerance induction for either cell therapy and/or solid organ transplantation.

Recently, another approach to selective T-cell depletion has been developed, based on removal of $CD45RA^+$ *naïve* T lymphocytes, while retaining $CD45RO^+$ memory T cells. The rationale for this strategy is based on experimental data demonstrating that mouse $CD4^+$ memory T cells, as well as effector memory $CD8^+$ T cells, are devoid of GvHD reactivity (26). A recent study of 17 patients with high risk hematologic malignancies detailed the results of performing $CD45RA^+$ depleted haplo-HSCT following a novel TBI- and serotherapy-free reduced-intensity conditioning (RIC) regimen. Remarkable depletion of $CD45RA^+$ T cells and B cells, with preservation of abundant memory T cells, was achieved in all 17 grafts. No infection-related mortality has been reported. Despite the infusion of a median of $>100 \times 10^6$ haploidentical T cells, no patient experienced acute GvHD. However, 6/17 developed symptoms of chronic GvHD (27). This finding may be explained by the fact that the $CD45RA^+$-depleted fraction contained both T effector-memory (EM) cells and T central-memory (CM) cells which may mediate chronic GvHD (28).

αβ T-Cell/CD19 B-Cell Depleted Haploidentical Transplant: A New Source of Effector and Tolerogenic Cells?

Despite encouraging clinical data using "*megadoses*" of purified $CD34^+$ cells, extensive lymphoid cell depletion results in an increased risk of opportunistic infections, especially in the first months after HSCT. To reduce the risk of infection and leukemia recurrence, a new strategy of graft manipulation has been implemented based on the selective elimination of αβ T cells and CD19 B cells (αβhaplo-HSCT) (29). This refined technique of graft engineering reduces complications associated with delayed immune recovery observed in the purified $CD34^+$ haplo-HSCT approach. With the αβhaplo-HSCT cell selection approach, it is possible to transfer HSCs and committed hematopoietic progenitors to the recipient donor, as well as mature natural killer (NK) and γδ T cells (30, 31). We first reported promising clinical results using αβhaplo-HSCT in children with life-threatening non-malignant disorders (32). More recently, single-center experiences in pediatric patients with malignancies have been published (33–35). These studies show that the risk of NRM and leukemia relapse are comparable to those in HLA-identical siblings or UD-HSCT. Moreover, patients receiving αβhaplo-HSCT have a lower risk of acute and chronic GvHD, leading to better GvHD-free/relapse-free survival (GRFS). These data were confirmed in a multicenter retrospective analysis which compared 98 αβhaplo-HSCT recipients with 245 UD-HSCT (36). This study definitively established that αβhaplo-HSCT is an equally effective option to UD-HSCT for children with acute leukemia lacking a sibling donor.

The NK and γδ T cells which are still present in the αβhaplo-HSCT might facilitate engraftment and reduce the risk of both infections and leukemia recurrence (30, 31). While T cells carrying the αβ TCR are responsible for GvHD (37, 38), γδ T cells have no alloreactive capacity (39), but do have important anti-infectious (40) and anti-leukemia effects (41–44). Thanks to this novel graft manipulation approach, patients can immediately benefit from donor NK cells contained in the graft that are able to fully exert their activity in the 6–8 weeks after transplant, before mature KIR^+ NK cells differentiate from $CD34^+$ cells (45, 46). The infusion with the graft of these different lymphoid cell subsets explains that NRM and LFS are superimposable in αβhaplo-HSCT and allelic matched UD (MUD)-HSCT (36). The large number of effector cells infused with the graft, along with a "*megadose*" of HSCs combined with the fully myeloablative conditioning regimen, might explain the remarkably low incidence of graft failure (2%) observed in αβhaplo-HSCT. The absence of pharmacological post-HSCT GvHD prophylaxis and the use of a fully myeloablative conditioning regimen could also be responsible for the lower incidence of relapse (CI of REL 29%, 95% CI 20–42) when compared with other previously published studies (33, 34, 36).

A randomized, prospective trial between haplo-HSCT (either αβhaplo-HSCT or PTCY) and MUD recipients is expected to start in the next 18 months. Thanks to these recent results, αβhaplo-HSCT is becoming the first choice for T-cell depletion in HSCT for pediatric patients affected by hematological malignancies.

IMMUNOREGULATORY CELLS TO ACHIEVE TRANSPLANTATION TOLERANCE AND GRAFT-VS-LEUKEMIA EFFECT

Anti-infectious, Anti-leukemic, and Tolerogenic Properties of γδ T Cells

γδ T cells are a small subset of T lymphocytes in the peripheral blood, but constitute a major T-cell population in tissues (47). These T cells mediate both adaptive and rapid, innate-like immune response, playing multiple roles in the initiation and effector phases of immune reactions. In contrast to conventional αβ T cells, the available number of germline genes coding for T-cell receptor (TCR) variable elements of γδ T cells is very small. There is a preferential localization of γδ T cells expressing given Vγ and Vδ genes in certain tissues. γδ T cells play an important role in the successful clinical outcome of αβhaplo-HSCT in pediatric patients with high-risk leukemias because they can recognize tumor cells without the need for major histocompatibility complex (MHC) presentation (47) and have potent anti-leukemia activity in the absence of relevant GvHD-inducing effect (35, 36, 48). The results of recent studies suggest advantageous effects of elevated γδ T cell immune recovery after HSCT in terms of infections, GvHD and overall survival (43, 49). Despite this, further clarification is needed to properly assess γδ T-cell potential in HSCT. This includes investigation of tissue biopsies (i.e., gut, liver, skin) and peripheral blood samples from patients with GvHD to better determine and differentiate the effects of various γδ T-cell subtypes.

The main subset of circulating γδ T cells express the Vδ2 chain associated with Vγ9 (i.e., Vγ9Vδ2). γδ T cells bearing the Vδ1 chain are a minor subset. Both subsets share antitumor properties, but Vδ1 cells also reside within epithelial tissues and may undergo selective expansion in transplanted patients upon cytomegalovirus (CMV) reactivation. Human Vγ9Vδ2 T cells recognize phosphorylated metabolites (phosphoantigens) that are secreted by many pathogens, but are also overexpressed by tumor cells, explaining why these cells play a role in both anti-infectious and anti-tumor immune responses. Similarly, the recently reported ability of human non-Vδ2 γδ T cells to recognize endothelial protein C receptor provides a link between immunity against epithelial tumor cells and CMV-infected endothelial cells (50).

To better understand the role of γδ T cells in αβhaplo-HSCT, we recently investigated their reconstitution kinetics in 27 children, 15 of whom had leukemia (51). Immunophenotypic characterization of peripheral blood mononuclear cells at one, three, and six months after HSCT revealed an initial abundance of γδ T cells, followed by a progressive predominance of αβ T cells. As in healthy donors, γδ T cells included three different populations: Vδ2, Vδ1 and, to a lesser extent, $Vδ2^-/Vδ1^-$ T cells. The relative proportions of the different Vδ2 and Vδ1 populations remained stable over time and were similar to those detected in the donor's HSCs. Naïve Vδ2 cells increased significantly between 20 days and three months after αβhaplo-HSCT, suggesting that circulating γδ T cells in transplanted patients consisted of not only mature cells derived from the graft, but also of cells differentiating from donor's HSCs (51). In patients given αβhaplo-HSCT, CMV specific cells were predominant in the Vδ1 T-cell subset, in contrast to healthy donors (51). Patients experiencing CMV reactivation displayed a significant expansion of the Vδ1 T-cell subset with a cytotoxic EM phenotype, which was absent in patients without CMV reactivation. These CMV-driven Vδ1 T cells killed *in vitro* primary acute lymphoblastic (ALL) and acute myeloid (AML) blasts more efficiently than Vδ1 T cells from patients that did not reactivate CMV infection, suggesting that CMV infection promotes both expansion and activation of Vδ1 T cells (51). These findings are consistent with those in kidney transplant, demonstrating expansion of $Vδ2^-$ γδ T cells displaying an EM phenotype and exerting cytotoxic function upon CMV infection (52). Interestingly, the expansion of Vδ1 and $Vδ1^-/Vδ2^-$ T cells with a restricted TCR repertoire was observed during CMV infection, which is indicative of antigen-driven selection (53).

We also showed that Vδ2 T cells from patients who received αβhaplo-HSCT expanded *in vitro* upon incubation with zoledronic acid (ZOL), which promoted the acquisition of an EM phenotype and potentiated the cytotoxic activity against primary leukemic blasts. This activity is dependent on the levels of phosphoantigens expressed by leukemia cells and on TCR Vγ9 mediated recognition (51). Indeed, lytic capacity of γδ T cells is strongly enhanced by sensitizing the leukemic target cells with ZOL. Based on these *in vitro* results, a clinical study investigating the effect of ZOL infusion was performed

in 43 pediatric recipients of αβhaplo-HSCT (44). Here, ZOL was infused every 28 days. The treatment was safe and well-tolerated, and, when administered three or more times, improved overall survival. The first treatment with ZOL induced the differentiation of Vδ2 T cells, switching them from a CM to an EM phenotype. Such maturation corresponded with increased Vδ2 T-cell mediated cytotoxicity against primary leukemia cells irrespective of their phosphoantigen expression. Proteomic analyses identified an anti-proliferative effect of infused ZOL on total γδ T cells consistent with the decrease in Vδ2 T cells, starting three months after HSCT. Such an effect was already evident after the first ZOL infusion and was further boosted by subsequent infusions. The percentage of Vδ1 T cells increased during ZOL infusions regardless of CMV reactivation (44). Altogether, these results suggest that maintenance and activation of γδ T cells after αβhaplo-HSCT improve long-term LFS. This approach may also potentiate the anti-leukemic activity of endogenous Vδ2Vγ9 T cells. Future prospective controlled clinical trials will determine if this approach leads to significant clinical benefit. In addition, future studies aimed at deeply understanding the fine mechanisms whereby γδ T cells, especially theVδ1 subset, recognize malignant and virus-infected cells will assist in uncovering the therapeutic potential of γδ T cells for haplo-HSCT.

Recent studies further suggest that some subsets of γδ T cells can have regulatory activity and antigen-presenting capacity, though their functional plasticity and the extent of γδ T cell ligand diversity have not yet been determined. Although they are FOXP3 negative, γδ T cells strongly suppress T helper cell proliferation in an IL-2-independent mechanism and produce high amounts of TGF-β. We are currently exploring whether γδ T cells can regulate the immune response mediated by αβ T cells after HSCT, especially in the context of immunogenetic disparity, such as in haplo-HSCT.

The suppressive function of human γδ T cells was first described in 1989 by Patel and colleagues (54). Since then, the regulatory role for γδ T cells have been reported in several pre-clinical and clinical studies (55–58). Both Vδ1 and Vδ2 T-cell subsets may exhibit regulatory properties, albeit in different settings (58).

Drobyski et al. showed that the co-administration of γδ T cells and naive αβ T cells at the time of MHC-mismatched HSCT infusion exacerbates GvHD, in comparison to the administration of naive αβ T cells alone. Conversely, when the infusion of naive αβ T cells was delayed for 2 weeks after HSCT, the survival of mice transplanted with BM and with activated γδ T cells was increased compared to that of mice given BM cells alone. These results indicate that only activated γδ T cells have a modulatory ability on effector αβ T cells to prevent GvHD after HSCT (59). The mechanism of suppression used by γδ T cells remains somewhat uncertain. Kuhl et al. claim that the regulatory activity is mediated by the immunosuppressive cytokines TGF-β1 and IL-10, which are secreted by γδ T cells after anti-CD3/CD28 mAb stimulation. They showed that activated γδ T cells secreted significantly more TGF-β1 than conventional $CD4^+CD25^+$ Tregs (60). They also quantified the relative TGF-β1 mRNA content in different γδ T-cell subsets isolated from peripheral blood. The Vδ1 cell subset showed increased TGF-β1 mRNA content compared with Vδ2 T cells in six out of seven donors, suggesting greater suppressive capacity of Vδ1 than Vδ2 and αβ T cells. Subsequently, Li and colleagues demonstrated that TGF-β1-stimulated $CD25^+CD27^+$Vδ1 γδ T cells exert a suppressive effect on naïve $CD4^+$ T cells similar to classical $CD4^+FOXP3^+$ Tregs and that this mechanism is cell-cell contact dependent. Peters et al. demonstrated that co-culturing of Vδ cells with responder cells (CD25-depleted $CD4^+$ αβ T cells) leads to upregulation of CD80, CD86, and PDL-1 on stimulated Vδ2 γδ T cells, which then interact with CTLA-4 or PD-1 on responder cells, leading to their suppression (56). In this study, the immunosuppressive capacity of Vδ2 γδ T cells was abrogated by Toll-like-receptor (TLR) 2 ligands, which correlated with increased phosphorylation of Akt and NFκB in αβ T cells and down-regulation of inhibitory molecules such as PD-1 and CTLA-4. Based on these studies, it can be hypothesized that the elimination of suppressive γδ T cells from cells used for adoptive immunotherapy in cancer patients could be useful in boosting antitumor effects.

Overall, γδ T cells play an important role in the successful clinical outcome of HSCT, but may act as a double-edged sword with both effector and regulatory functions dependent on the subset of cells and the environment they are in. Manufacturing of a cell product containing γδ T-cell subsets with anti-infectious and anti-leukemia activity, but lacking regulatory function, could have future clinical applications in haplo-HSCT.

Regulatory Type 1 T (Tr1) Cells: Clinical Application

Mechanisms underlying tolerance after allogenic HSCT consist of peripheral clonal deletion or active suppression mediated by regulatory cells such as T, B, NK, and mesenchymal stromal regulatory cells. Among Tregs, $CD4^+$ and $CD8^+$ or double negative T cells have been described extensively in literature (59). Tregs are an important component of the immune system involved in dampening immune reactions and inducing tolerance (61). Due to their efficacy as immune modulators in several pre-clinical models, the clinical applications of Tregs have been extensively explored. Several types of $CD4^+$ Tregs have been identified, including the forkhead box P3 (FOXP3)-expressing Tregs ($FOXP3^+$ Tregs) and regulatory type 1 T (Tr1) cells (62). For the purpose of this review, we will focus on Tr1 cells and their clinical applications in the context of allogeneic HSCT, as well as on engineered $FOXP3^+$ Tregs.

Studies in patients with SCID and β-thalassemia who became chimeric post-HSCT demonstrated that, although clonal deletion of alloreactive T cells might occur, peripheral tolerance mediated by Tr1 cells is crucial for active suppression of effector T-cell alloreactivity (62–64). $CD4^+$ Tr1 cells are defined and distinct from Th1, Th2, Th3, and Th17 cells, based on their unique pattern of cytokine production. They produce high levels of IL-10, TGF-β, low levels of IL-2, variable levels of IL-5 and IFN-γ in the absence of IL-4 and IL-17 (65). Tr1 cells have specific metabolic requirements that distinguishes them from $FOXP3^+$ Tregs: Tr1 cells depend on glycolysis and are inhibited by hypoxia

and extracellular ATP (66), while peripheral FOXP3$^+$ Tregs depend on fatty acid oxidation (67). We have demonstrated that CD49b LAG-3 are good markers for a subset of memory Tr1 cells (68). The vast majority of memory CD4$^+$CD49b$^+$LAG-3$^+$ T cells secrete large amounts of IL-10 but not significant levels of IL-4 and IL-17, do not constitutively express FOXP3, and display regulatory activity both *in vitro* and *in vivo*. Both CD49b and LAG-3 are stably expressed on functional human Tr1 cell clones. CD49b is expressed on Tr1 cells regardless of their activation state, whereas LAG-3 is expressed on Tr1 cells upon activation when the cells produce IL-10 and display suppressor activity. CD49b and LAG-3 have been used to identify Tr1 cells in the peripheral blood of healthy donors and tolerant patients. CD49b and LAG-3 can be used to track Tr1 cells in cell products generated *in vitro* (68). Tr1-cell mediated suppression is mainly driven by the secretion of IL-10 and TGF-β. Tr1 cells require activation via their TCR to mediate suppression, but once activated, they mediated bystander suppression against other antigens (62).

Tr1 cells were first identified and characterized in SCID patients who were immune reconstituted after HSCT from HLA mismatched donors (63, 64). These patients developed spontaneous split chimerism, with T and NK cells of donor origin and B and professional APC of host origin in the absence of GvHD. Single cell cloning of T cells from the peripheral blood of these chimeras leads to the isolation of donor derived CD4$^+$ and CD8$^+$ T-cell clones specific for the HLA antigens of the host (64). These data indicate that alloreactive T cells are not deleted from the T-cell repertoire of the patients. A significant number of these T-cell clones produce high levels of IL-10 when activated with the host alloantigens *in vitro* (63). These findings correlate with the absence of GvHD and a state of active tolerance between host and donor cells. Similar data was obtained from β-thalassemic patients who developed persistent mixed chimerism following HSCT (69). The presence of Tr1 cells *in vivo* was confirmed by detection of CD49b$^+$ and LAG-3$^+$ T cells at higher frequency in the peripheral blood mononuclear cells (PBMC) of these tolerant patients compared to normal donors' PBMC (68). Moreover, high amounts of IL-10 production were detected *ex vivo* by PBMCs of patients with persistent mixed chimerism. High levels of IL-10 production and presence of Tr1 cells were not detected in patients with complete donor chimerism, suggesting that chronic allo-antigen stimulation by mismatched host APCs plays a role in Tr1 cell induction *in vivo* (69). Overall, these results indicate that IL-10 and Tr1 cells are associated with long-term, spontaneously established, tolerance, suggesting Tr1-based cell therapy can be used to promote tolerance in HSCT (70). Several protocols have been developed to generate Tr1 cells *in vitro* that are suitable for *in vivo* use. Although IL-10 is indispensable for Tr1 cell induction, efficient *in vitro* production of Tr1 cells also requires APC. Tr1 cell induction *in vitro* is optimal when IL-10-producing human dendritic cells (DC-10) are used as APC (71). DC-10 are monocyte-derived dendritic cells generated *in vitro* in the presence of exogenous IL-10 in addition to GM-CSF and IL-4, which are required for *in vitro* differentiation of mature myeloid derived DC (71). DC-10 are CD14$^+$, CD16$^+$, CD11c$^+$, CD11b$^+$, HLA-DR$^+$, CD83$^+$, CD1a$^-$, CD1c$^-$, express the Ig-like transcripts (ILT)2, ILT3, ILT4, and HLA-G antigen and display high levels of CD40 and CD86 and up-regulate CD80 following differentiation *in vitro*. Stimulation of CD4$^+$ cells with allogeneic DC-10 and IL-10 is efficient in generating a Tr1 -cell product that suppresses antigen-specific proliferative responses of autologous CD4$^+$ T cells (68, 72).

A first clinical trial aimed at prevention of GvHD and establishment of immunological tolerance after haplo-HSCT with purified CD34$^+$ cells in adult with high risk leukemias was carried out using donor Tr1 cells specific for host alloantigens (73). Adaptively infused donor T cells were primed by the host monocytes as APC and IL-10 for a short period of time. The method to "instruct" the Ag-specific T cells to differentiate into Tr1 cells and generate allo-specific IL-10-anergized T cells (IL-10 DLI) was validated in good-manufacturing-practice (GMP) (74, 75). Following donor myeloid engraftment in 12 patients post-CD34$^+$ purified haplo-HSCT, these cells were infused at the dose of 10^5 CD3$^+$ T cells/kg. Patients experienced only mild to moderate GvHD and had a rapid normalization of their lymphocyte counts. Moreover, they showed a normal polyclonal TCR repertoire and presented a good *in vitro* T-cell response against viral antigens and mitogens. Donor T cells remained hyporesponsive to host alloantigens *in vitro,* and cells with the Tr1-cell specific phenotypic markers CD49b and LAG3 were observed to increase over time in the peripheral blood. Four patients benefited from this adoptive immunotherapy and fully recovered from the diseases with uneventful long-term follow-up. These long-term survivors (mean follow-up 7.5 years) have established tolerance, and circulating Tr1 cells are present at high frequency. This study represented the proof-of-concept that Tr1 cells can boost immunotolerance after HSCT, expediting immune recovery while reducing the risk of GvHD. However, higher doses of Tr1 cells may be required to prevent GvHD in a mismatched T-cell replete setting and to obtain a more robust immune reconstitution with infection and relapse prevention. The discovery of DC-10 offered the opportunity to modify the protocol to generate an alloantigen- specific Tr1 cell-rich product. Functional assays demonstrate that stimulation of human PBMCs or CD4$^+$ T cells with allogeneic DC-10 induces the differentiation of anergic alloantigen-specific IL-10-producing Tr1 cells (T-allo10) (74). We recently initiated a Phase I clinical trial (NCT03198234) using this improved T-allo10 cell product in the context of mismatched HSCT for children and young adults with hematological malignancies. Purified donor derived CD4$^+$ T cells are cultured with tolerogenic DC-10 of host origin in the presence of IL-10 for 10 days to obtain allo-antigen specific Tr1 cells. In this setting, donor-derived T cells should react against host allo-antigens to suppress GvHD after HSCT. Tr1 cells *ex vivo* generated are donor-derived and specific for patient allo-antigens. The current T-allo10 product contains up to 15% of CD49b$^+$LAG-3$^+$ Tr1 cells, compared to <5% in the first generation IL-10 DLI product. The first results with the lowest dose indicate that the therapy is safe and well-tolerated, while the effects of GvHD and long-term tolerance are yet to be established.

These results suggest that further optimization of Tr1 cell products is required for future clinical studies. The T-allo10

cell product still contains a large proportion of effector T cells that could potentially limit the *in vivo* efficacy of Tr1 cells. To overcome this limitation, a method to generate a selected population of IL-10-producing Tr1 cells by lentiviral vector (LV)-mediated human IL-10 gene transfer was developed. IL-10-engineered $CD4^+$ ($CD4^{IL-10}$) cells display a cytokine profile and phenotype super-imposable to *bona fide* Tr1 cells and suppress T-cell responses (76). Adoptive transfer of these human $CD4^{IL-10}$ cells in NSG mice did not result in GvHD, but down regulated GvHD induced by human PBMC. $CD4^{IL-10}$ cells showed GvL effect in mice transplanted with human leukemic cell lines, indicating that these cells have clinical potential for prevention of both GvHD and leukemia relapse after HSCT (77). $CD4^{IL-10}$ cells selectively eliminate $CD13^+$ leukemic cells and for optimal killing of target cells, require stable CD54/LFA-1-mediated adhesion and CD112/CD226-mediated activation. This newly identified antileukemic activity of $CD4^{IL-10}$ is a promising area for investigation into identification of treatment regimens that prevent GvHD without affecting the GvL effect.

NEXT STEPS AND FUTURE PERSPECTIVES

αβHaplo-HSCT: The Optimal Platform for Adoptive Immunotherapy?

Despite significant improvement in NRM, leukemia recurrence remains the most important cause of treatment failure in patients with hematological malignancies. This is true for matched-donor HSCT as well as for patients undergoing haplo-HSCT (36). In the latter group, outcomes in children with leukemia not in complete remission at the time of HSCT or beyond second CR have been poor (78). For this reason, a significant challenge in HSCT for hematologic malignancies is the identification of novel strategies for the enhancement of a GvL effect, without increasing incidence of GvHD. Recent development of adoptive immunotherapies for hematologic malignancies, such as the infusion of donor-derived either manipulated or unmanipulated donor lymphocyte infusions, offer some promise for patients after haplo-HSCT (79). Haploidentical transplantation allows immediate donor availability for the collection or generation of additional cells, such as T cells or NK cells, which are capable of enhancing the antitumor effects without being rejected. The absence of pharmacological immunosuppression, typical of T-cell depleted approaches, might facilitate expansion and persistence of cell-based products.

A novel approach to both accelerate the recovery of adaptive immunity and to promote GvL activity simultaneously relies on the use of suicide gene-modified T cells. The administration of donor T cells with a "safety switch" mechanism can help in relapse prevention when administered earlier after HSCT, with the possibility of triggering pharmacologically induced cell apoptosis if severe GvHD occurs. The first method described relating to this approach was based on the insertion of the herpes simplex thymidine kinase suicide gene into T cells (HSV-TK cells) to achieve *in vivo* susceptibility to ganciclovir. A phase I/II multi-center trial (TK007) in adult $CD34^+$-selected haplo-HSCT patients showed that post-transplantation infusion of the modified T cells enabled regulation of GvHD, while promoting immune reconstitution (80). The results of a multicenter randomized phase III clinical trial (the TK008 study) to assess the efficacy of HSV-TK^+ cells in the context of $CD34^+$ selected haplo-HSCT for leukemia confirmed improved survival, faster immune reconstitution and efficient prevention of GvHD by suicide gene induction (81). Subsequently, Brenner M. et al. developed an alternative strategy using T cells engineered to express caspase 9 (iC9-T cells), which can be activated via a dimerizing agent. In these engineered T cells, the caspase recruiting domain of the human caspase 9 was modified with a drug binding domain, allowing T-cell elimination after administration of a chemical dimerizing drug, AP1903. The administration of AP1903 dimerizes and activates the caspase 9, which activates downstream caspases, leading to rapid apoptosis (within minutes to hours) (82–84). These iC9-T cells provided rapid immune recovery in 10 pediatric patients (ages 3–17) who received haplo-HSCT. In 5 patients who developed GvHD, iC9-T cells were eliminated within 2 h after AP1903 administration and the GvHD was rapidly resolved without a significant effect on antiviral immune reconstitution (82). Currently, a multicenter US (NCT03301168) and EU (NCT02065869), prospective phase I-II clinical trial using αβhaplo-HSCT followed by addback of donor T cells genetically modified with iC9 safety switch (BPX-501 cells) in patients with malignant or non-malignant disorders is underway. BPX-501 cells are infused on day14 ± 4 after the allograft. No post-transplant pharmacological GvHD prophylaxis is included in this study. Patients who develop GvHD resistant to conventional steroid therapy will receive up to three doses of AP1903 to activate iC9. Preliminary results on a subset of patients with high-risk acute leukemias show a 2-year LFS rate approaching 90% (2018 ASH Annual Meeting, abstract #307, Locatelli F).

Despite these positive preliminary results, the "switch safety" strategy remains a cumbersome approach, requiring time-consuming and costly manufacturing processes. As a result, we are investigating novel strategies based on the infusion of other cell populations post-αβhaplo-HSCT, which may achieve the same goal but are cheaper and easier from a manufacturing standpoint (**Figure 1**). Specifically, we are focusing on adoptive infusions of *ex vivo* expanded γδ T cells and donor-derived Tr1 cells.

Due to their unique features, γδ T cells are optimally suited for cell therapy in αβhaplo-HSCT when immunogenetic disparity requires both robust anti-leukemia activity and anti-viral protection as well as early immune recovery. As described above, both Vδ1 and Vδ2 γδ T cells can recognize and lyse different type of cancers in a MHC-unrestricted manner, making them good candidates for manufacturing of an off-the shelf cell product. Several clinical trials have highlighted the therapeutic potential of γδ T cells for hematologic malignancies (i.e., Multiple Myeloma and Non-Hodgkin Lymphoma), suggesting that this cell product is both feasible and well-tolerated in addition to its ability to mediate tumor responses (85, 86). Due to the low frequencies of γδ T cells in peripheral blood, an

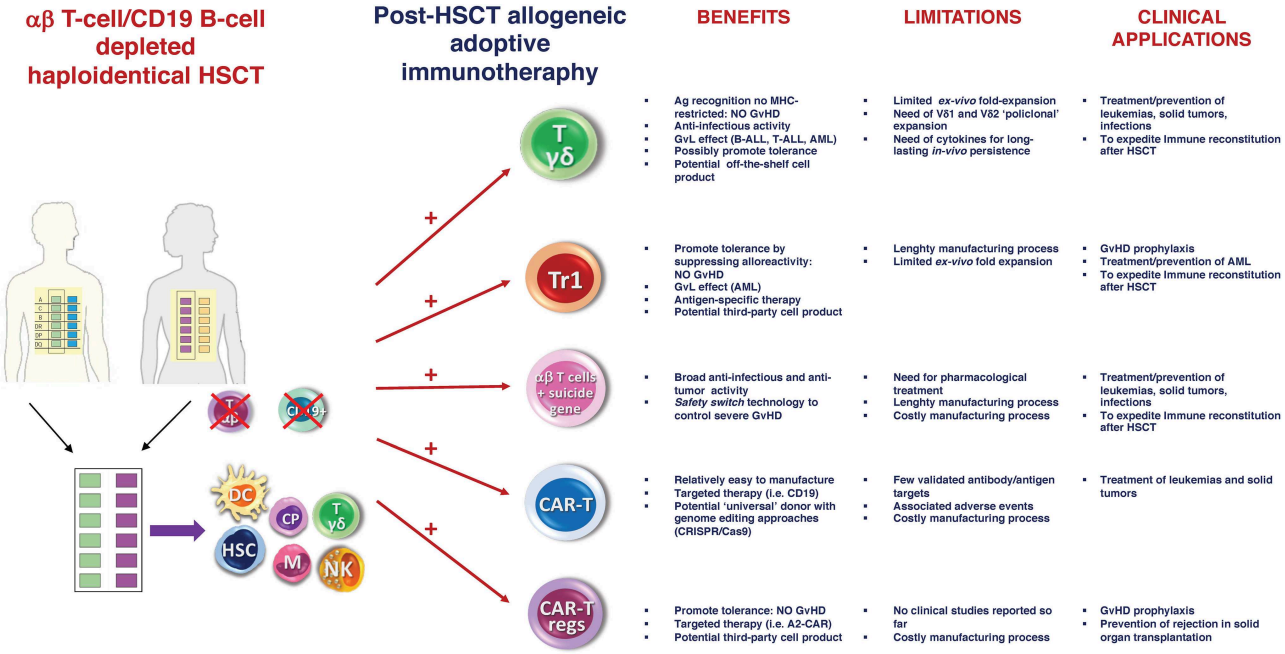

FIGURE 1 | αβ haplo-HSCT as optimal platform for adoptive immunotherapy. Schematic representation of the current and future potential strategies of post-HSCT allogeneic adoptive immunotherapy. Main benefits, limitations and clinical applications of each approach are reported. Ag, Antigen; Tr1, regulatory Type 1 T (Tr1) Cells; MHC, major histocompatibility complex; CAR, chimeric antigen receptor; GvHD, graft-vs-host disease; GvL, graft-vs-leukemia; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; Cas9, Caspase 9.

appropriate method of *ex vivo* expansion is required for effective immunotherapy. Some groups are currently investigating the infusion of *ex vivo* expanded γδ T cells to further reduce the risk of disease recurrence without increasing the risk of GvHD (39). Combinations of IL-2 or IL-15 and aminobisphosphonates (i.e., ZOL) are the principal methods currently used for the expansion and activation of these purified γδ T cells.

We have previously described peripheral Tr1 cells, with alloantigen (Allo-Ag)-specific suppressor functions, as being consistently associated with a state of immune tolerance in chimeric patients after allogeneic HSCT. We are currently testing the safety and efficacy of an optimized Tr1-based cell product, the T-allo10 cells, in a phase I clinical trial. In addition to diminish GvHD, T-allo10 cells may also enhance GvL thanks to the cytotoxic activity of activated Tr1 cells (70). An early addback of T-allo10 cells to patients receiving αβhaplo-HSCT could therefore improve immune recovery and decrease relapse.

Allogeneic T Cells Engineered With a Chimeric Antigen Receptors (CARs) After HSCT: A New Approach?

The engineering of donor lymphocytes to express suicide genes is a security system against the development of severe GvHD. However, it provides a non-targeted antitumor effect. Advances in cell culture and gene transfer technology have resulted in the ability to expand clinically relevant engineered T cells that express chimeric antigen receptors (CARs), which can redirect T cells to recognize a selected target antigen (87). Autologous T cells modified with CD19-targeted CAR constructs consistently demonstrate high antitumor efficacy in children and adults with relapsed $CD19^+$ ALL when infused both before and after allogeneic-HSCT (79, 88, 89). Recently, the administration of donor derived CD19-specific CAR T cells early after haplo-HSCT as adjuvant therapy to prevent disease relapse proved to be safe and showed promising results in adult patients with advanced $CD19^+$ non-Hodgkin lymphoma or ALL (90). It is reasonable to hypothesize that a similar approach will be applied in the pediatric setting.

The use of autologous cells for manufacturing CAR T cells can be a challenge in patients with malignancies who are often under intensive chemotherapy. One or multiple rounds of apheresis may be required, in addition to the considerable processing time required to isolate, transduce, expand and release the clinical grade products. Patients with active malignancies also often have decreased numbers and reduced functionality of T cells (91). Furthermore, gene transfer leads to a heterogeneous cell population with varying vector copy numbers and expression levels of the CAR. Retroviral or lentiviral engineered T cells also potentially carry the risk of transformation from insertional mutagenesis (92). Therefore, use of CAR T cells both as a bridge before HSCT or as a relapse prevention tool needs to be further investigated.

The advent of genetic engineering using guided endonucleases allows for the introduction of precise modifications in the

genome of immune cells by creating specific DNA double-strand breaks, and introducing a homologous DNA template to the targeted region that stimulates DNA repair through homologous recombination. This makes it possible to efficiently disrupt genes and integrate whole transgene cassettes through introduction of a DNA template (93). Use of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated guided endonuclease Cas9, complexed with a chemically modified single guide RNA (sgRNA) to form a ribonucleoprotein (RNP), combined with recombinant adeno-associated virus (rAAV) can lead to targeted integration of transgenes in 50–80% of primary human T cells (93).

Gene editing can be used to overcome allo-recognition, which otherwise limits allogeneic T-cell therapies. Initial proof-of-concept applications have included generation of "universal" T cells expressing CARs against CD19 target antigens combined with transient expression of DNA-targeting nucleases to disrupt the T cell receptor alpha constant chain (TRAC) (94). The use of "universal donor" allogeneic T cells to produce CAR T cells might address the challenges associated with the use of patient-derived cells, like decreased T-cell function (91) and contamination with leukemic cells (95, 96). June C. H. et al., recently reported the case of a 20 year-old male with B-ALL treated with tisagenlecleucel (Kimryah®, Novartis) who relapsed after 261 days. Refined immunophenotypic evaluation of the circulating leukemic cells of this patient revealed that they were CAR-transduced B-cell leukemia (95). This finding clearly demonstrated the need for improved manufacturing technologies. Proof-of-principle studies have shown that knock-out of the TCR can prevent T cells from alloreactivity, thereby making it possible to use allogeneic, donor-derived T-cell products without the risk of GvHD (92, 93). Furthermore, targeted integration of the CAR into the constant region of the TRAC in-frame with the endogenous gene's open reading frame has the potential to increase CAR T cell functionality and persistence (97). Ultimately, this technology could enable potent CAR T-cell immunotherapy in combination with allogeneic HSCT, thus abrogating the risk of GvHD.

FOXP3$^+$ Tregs-Based Adoptive Cell Therapy

FOXP3$^+$ Tregs are key players in the maintenance of peripheral tolerance in physiological and pathological conditions. FOXP3$^+$ Treg-based cell therapies to restore tolerance in T-cell mediated disorders have been extensively investigated (98). Translation to the clinic is difficult, as Tregs represent only 2–10% of CD4$^+$ T cells in peripheral blood. The transfer of meaningful numbers requires *in vitro* expansion protocols that are expensive and technically challenging. Nevertheless, GMP grade, large-scale Tregs expansion has been achieved (99), and studies infusing third-party, UCB-derived Tregs as a component of GvHD prophylaxis demonstrate promising results (100). The infusion of Tregs (with or without IL-2) directly isolated from donors, has been tested for the treatment of chronic GvHD. These expansion protocols primarily rely on polyclonal anti-CD3/28 antibody-based expansion. The mechanisms by which Tregs may attenuate GvHD include release of regenerative cytokines (i.e., amphiregulin) (101), APC function inhibition (i.e., via CTLA4), and the inhibition of T effector cells by the release of inhibitory molecules (i.e., adenosine, TGF-β, IL-35, and IL-10) (102) and/or IL-2 consumption (103). Several hurdles, including insufficient number of FOXP3$^+$ Tregs and lack of stability and antigen specificity of *in vitro* expanded FOXP3$^+$ Tregs, have made clinical use of this cell subset challenging. Recent advances such as LV-mediated gene transfer of the transcription factor FOXP3 in conventional CD4$^+$ T cells, to convert effector T cells into Treg-like cells (104), and genetic manipulation to confer antigen specificity and enhance the potency of FOXP3$^+$ Tregs (105), offer new avenues to make adaptive Treg cell therapy more feasible and effective.

We developed a LV-based strategy to ectopically express high levels of FOXP3 that do not fluctuate with the state of T-cell activation. This method produces suppressive cells that are as potent as *ex vivo* isolated FOXP3$^+$ Tregs and can be propagated as a homogeneous population. Using this system, both naïve and memory CD4$^+$ T cells can be efficiently converted into Tregs (106). We further explored the adoptive transfer of *in vitro* engineered autologous Tregs to control autoimmunity in patients with immune-dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome caused by mutations in FOXP3 (107). The human FOXP3 coding sequence was cloned under the control of a constitutive promoter in a bidirectional LV construct allowing simultaneous expression of full-length FOXP3 and of a cell-surface marker (ΔNGFR) for the identification/selection of transduced T cells (LV-FOXP3). CD4$^+$ T cells converted into FOXP3$^+$ Treg cells by LV-mediated FOXP3 gene transfer (CD4^{FOXP3}) display a stable phenotype and suppressive function and are stable in inflammatory conditions *in vitro* and in a model of xenogeneic GvHD (107). These findings pave the way for the treatment of IPEX patients by adoptive cell therapy using genetically engineered Treg cells. These data also lay the foundation for future use of CD4^{FOXP3} T cells to prevent or treat GvHD after HSCT. The fact that CD4^{FOXP3} T cells can be obtained from naïve or memory CD4$^+$ T cells renders the manufacturing process easier and more cost effective compared to other methods. CD4^{FOXP3} T cells do not require extensive *in vitro* expansion with high cytokine concentration. Once safety and proof-of-concept studies are completed in IPEX patients, use of these Treg-like cells could be investigated as a treatment for severe GvHD in the context of allogeneic HSCT.

The successful use of effector T cells carrying specific CARs suggests that a similar approach can be applied to generate alloantigen-specific Tregs. Levings M. K. et al., recently reported the creation of HLA-A2-specific CAR (A2-CAR) Tregs (105). *In vitro*, A2-CAR-expressing Tregs maintain their expected phenotype and suppressive function before and after A2-CAR mediated stimulation. In a xenogeneic GvHD animal model, human A2-CAR-expressing Tregs were superior at preventing xeno-GvHD caused by HLA-A2$^+$ T cells to Tregs expressing an irrelevant CAR. These results suggest that the CAR technology

can be used to generate alloantigen-specific human Tregs, enhancing their therapeutic potential in HSCT.

Recently, the concept of third-party Treg cell therapy has also emerged with the aim of improving safety, quality, accessibility and cost. As for effector T cells, wide application of adoptive Treg cell therapy using autologous T cells might be limited due to its nature as a patient-specific cell product, which is time-consuming and expensive to manufacture. The use of third-party Tregs also offers the opportunity to isolate cells from sources other than peripheral blood. Bone marrow or UCB could be used instead. However, third-party cells express allogeneic HLA molecules, which render them susceptible to rejection by the recipient's immune system. To overcome this hurdle, several research groups have proposed using genome editing technology to knockout β-2-microglobulin or FASL to generate HLA class I negative T cells or FAS-resistant T-cells, respectively (108). Furthermore, the use of the CRISPR/Cas9 system to knockout the endogenous TCR and generate TCR negative cell products could represent a solution to the rejection issue.

CONCLUSIONS

Allogeneic HSCT has dramatically changed the natural course of hematological malignant and non-malignant diseases and provided a definitive cure for many patients. Transplanted patients have offered a unique opportunity for investigating tolerance establishment across the donor/recipient allogeneic barrier. Limited availability of fully matched related and unrelated donors has promoted the search for new strategies of graft engineering to overcome the HLA mismatch of haploidentical donors. In this setting, αβ T-cell/CD19 B-cell depletion has shown the most robust and promising clinical results in pediatric patients. Due to the absence of post-HSCT pharmacological prophylaxis, αβhaplo-HSCT might represent the optimal platform for adoptive immunotherapy. In addition to the established strategy using "safety switch" cells, new approaches using post-HSCT infusion of *ex vivo* expanded T-cell subpopulations (i.e., γδ T cells and Tr1 cells) or engineered autologous or allogeneic T cells (i.e., CAR T cells) represent the future of immunotherapy for the optimization of hematological malignancies treatment. To facilitate and accelerate the clinical implementation of T effector- and Treg-based cell therapy, effective and cost-efficient GMP-grade protocols must be established in order to generate the large numbers of cells required for successful patient treatment. Methods of efficient homing and entry of effector T cells into tissues (109) will also be critical to the clinical success of these adoptive immunotherapy approaches. This will require further understanding of the mechanistic cellular and molecular components impacting trafficking of T effector cells (109).

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AB and MR equally contributed in conceiving, writing, and editing this manuscript.

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REVIEW
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Targeting Regulatory T Cells by Addressing Tumor Necrosis Factor and Its Receptors in Allogeneic Hematopoietic Cell Transplantation and Cancer

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An intricate network of molecular and cellular actors orchestrates the delicate balance between effector immune responses and immune tolerance. The pleiotropic cytokine tumor necrosis factor-alpha (TNF) proves as a pivotal protagonist promoting but also suppressing immune responses. These opposite actions are accomplished through specialist cell types responding to TNF via TNF receptors TNFR1 and TNFR2. Recent findings highlight the importance of TNFR2 as a key regulator of activated natural FoxP3^{+} regulatory T cells (Tregs) in inflammatory conditions, such as acute graft-vs.-host disease (GvHD) and the tumor microenvironment. Here we review recent advances in our understanding of TNFR2 signaling in T cells and discuss how these can reconcile seemingly conflicting observations when manipulating TNF and TNFRs. As TNFR2 emerges as a new and attractive target we furthermore pinpoint strategies and potential pitfalls for therapeutic targeting of TNFR2 for cancer treatment and immune tolerance after allogeneic hematopoietic cell transplantation.

Keywords: GVHD, graft vs. host disease, cancer, Tregs (regulatory T cells), TNFR family costimulatory receptors, TNFR2 agonists, TNFR2 antagonism

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INTRODUCTION

Tumor necrosis factor-alpha (TNF) regulates innate as well as adaptive immune processes and controls tissue homeostasis in various ways. TNF reached prominence as a prototypic proinflammatory cytokine, however, more recently, the TNF-TNF receptor system gained attention for its immunomodulatory and even anti-inflammatory functions. Here, we review important activities of TNF and its receptors crucial for T cell and Treg function under pathologic conditions such as acute graft-vs.-host disease (GvHD). The implications of the molecular basis of TNF receptor signaling are then discussed for the rational development of therapeutic TNFR-receptor-targeting reagents for clinical applications.

GENERAL ASPECTS OF TNFR1 AND TNFR2 SIGNALING

TNF is a single spanning type II transmembrane protein and the name giving member of the TNF superfamily (TNFSF) (1). TNF and the other ligands of the TNFSF share a conserved C-terminal

homology domain, the TNF homology domain (THD), which mediates self-assembly into trimeric molecules and receptor binding. A short stalk region connects the THD of TNF with the transmembrane and the cytoplasmic domain. Membrane TNF (memTNF) can be cleaved in its stalk region by the metalloprotease TNFα converting enzyme (TACE, ADAM17) resulting in the release of trimeric soluble TNF (sTNF) (2). Both forms of TNF are able to bind to two receptors, TNF receptor-1 (TNFR1) and TNFR2, which belong to the TNF receptor superfamily (TNFRSF). A trimeric TNF molecule interacts with three molecules of either TNFR1 or TNFR2 (3). Importantly, memTNF activates both TNF receptors, while only TNFR1 responds strongly to sTNF (**Figure 1**) (4). Triggering of TNFR2-associated signaling pathways requires secondary clustering of initially formed trimeric TNF-TNFR2 complexes. This occurs spontaneously for memTNF-induced TNF-TNFR2 complexes but not sTNF-liganded TNFR2 complexes (**Figure 1**) (5). Lymphotoxin-alpha (LTα), another soluble ligand trimer of the TNFSF, also interacts with the two TNF receptors triggering similar effects as sTNF.

TNFR1 and TNFR2 can be assigned to two different subgroups of the TNFRSF. TNFR1 belongs to the TNFRSF death receptor subgroup. Death receptors are characterized by a cytoplasmic protein-protein interaction domain called death domain (DD), which enables these receptors to trigger cytotoxic signaling (1). TNFR2, on the other side, is a representative of the TNF receptor associated factor (TRAF)-interacting receptor subgroup of the TNFRSF. Therefore, TNFR2 lacks a death domain and instead directly interacts with TRAF family members, which form homo- or heterotrimers (6). Although TNFR1 can trigger apoptotic and necroptotic signaling via its DD, these cytotoxic activities are not prevalent. They are typically inhibited by Fas-associated death domain (FADD)-like IL-1β-converting enzyme-inhibitory proteins (FLIPs) and/or complexes of a TRAF2 trimer and a single cellular inhibitor of apoptosis-1 (cIAP1) or cIAP2 E3 ligase molecule (7, 8). Accordingly, TNFR1 stimulation results primarily in the engagement of cell death-independent proinflammatory pathways activating NFκB transcription factors and MAP kinases. Notably, TRAF2 and the cIAPs not only antagonize cytotoxic TNFR1 signaling but also contribute to TNFR1-induced proinflammatory signaling (9). The TRAF2-cIAP1 and TRAF2-cIAP2 complexes are indirectly recruited to trimeric TNF-TNFR1 complexes by the DD-containing adapter protein TNF receptor associated death domain (TRADD). In context of the TNFR1 signaling complex, the TRAF2-cIAP1/2 complexes K63-ubiquitinate the DD-containing serine/threonine kinase receptor interacting protein (RIP), which is recruited via its DD to the DD of TNFR1 independently from TRADD. K63-ubiquitinated TNFR1-bound RIP creates docking sites

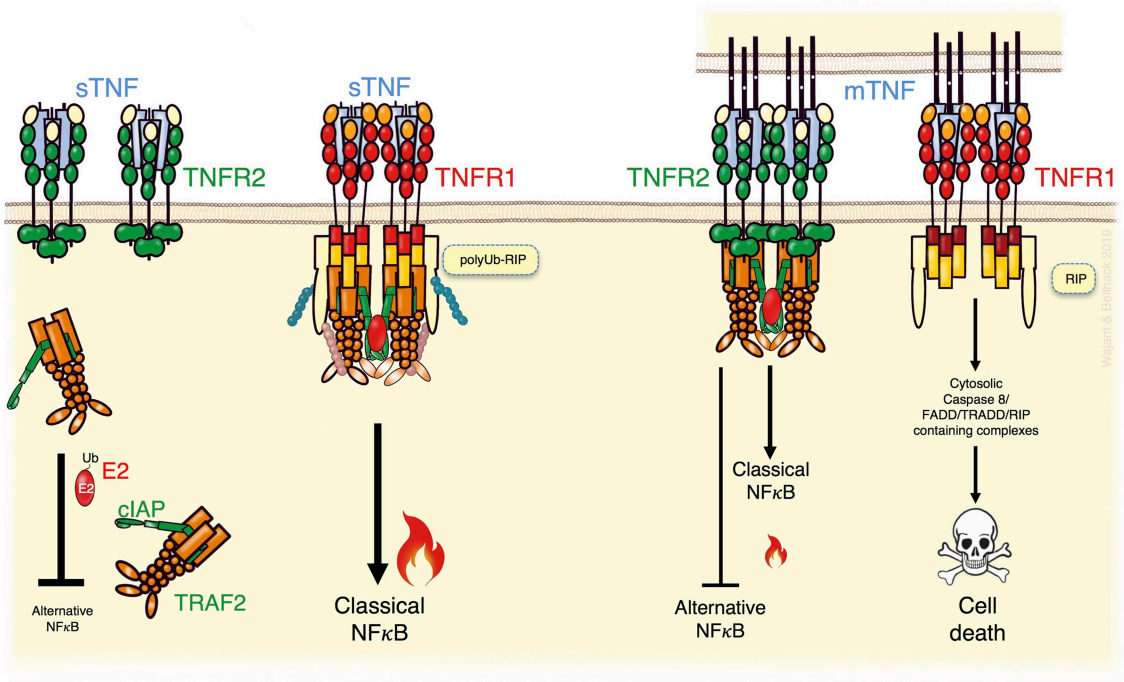

FIGURE 1 | TNFR2 can modulate TNFR1 signaling. TNFR1, activated by soluble TNF (sTNF) or membrane TNF (memTNF), recruits TRAF2 adapter protein trimers enabling transactivation of the TRAF2-associated E3 ligases cIAP1 and cIAP2 and activation of the classical NFκB pathway but also other proinflammatory signaling pathways not indicated here (**Left**). In addition, in TNFR1 signaling the TRAF2-cIAP1/2 complexes inhibit triggering of cell death by K63 ubiquitination of RIP (**Left**). TNFR2 activation by memTNF recruits TRAF2-cIAP1/2 complexes, too, and triggers classical NFκB signaling (**Right**). Due to the higher expression levels of TNFR2 and its ability to trigger TRAF2 degradation, however, TNFR2 activation can result in a substantial depletion of cytosolic TRAF2-cIAP1/2 complexes (**Right**). This entails enhanced alternative NFκB signaling and sensitizes for TNFR1-induced death signaling. For details see text.

for the linear ubiquitin chain assembly complex (LUBAC) and for various K63 or linear ubiquitin binding-domain containing signaling intermediates, such as the NFκB essential modulator (NEMO) subunit of the inhibitor of kappaB kinase (IKK) complex and the TGF-beta activated kinase-1 (TAK1) binding protein-2 (TAB2) subunit of the IKK-activating TAB2-TAK1 complex (7, 9). Thus, K63-ubiquitination of RIP strongly enhances the TRAF2-dependent ability of the TNFR1-TRADD-TRAF2-cIAP1/2 core complex to recruit the IKK complex and the TAB2-TAK1 complex. This leads to IKK-mediated phosphorylation of the inhibitor of kappaB-alpha (IκBα), its proteasomal degradation and the subsequent nuclear translocation of previously IκBα-sequestered dimers of the NFκB transcription factor family. Furthermore, K63-ubiquitination of TNFR1-associated RIP antagonizes the ability of the latter to trigger apoptosis and necroptosis. The initiation of these cell death responses is based on the release of RIP from the TNFR1 signaling complex and its subsequent interactions with caspase-8 and/or RIP3 in cytosolic complexes (7, 8). TNFR1-induced RIP-mediated caspase-8 activation results in apoptosis, while the interplay of RIP with RIP3 may stimulate necroptosis. Since caspase-8 actively suppresses necroptotic signaling, e.g., by cleavage of RIP and RIP3, TNF-induced necroptotic signaling typically becomes only relevant in cells with a compromised ability to activate caspase-8 (7, 8).

Interestingly, TNFR2 recruits very efficiently TRAF2-cIAP1/2 complexes (**Figure 1**). Indeed, TRAF2 and the cIAPs were originally identified as TNFR2 signaling components (10, 11) and are relevant for TNFR2-induced classical NFκB signaling, too. Since TNFR2 is typically much higher expressed as TNFR1, recruitment of TRAF2-cIAP1/2 complexes to TNFR2, but not to TNFR1, reduces the freely available cytoplasmic pool of these molecules (**Figure 1**) (12). Moreover, TNFR2-mediated depletion of cytosolic TRAF2-cIAP1/2 complexes can be enhanced by TNFR2-stimulated TRAF2 proteolysis. Since the cytosolic TRAF2-cIAP1/2 complexes contribute to constitutive MAP3K NFκB inducing kinase (NIK) degradation, sequestration, and degradation of TRAF2-cIAP1/2 complexes by TNFR2 result in NIK accumulation. NIK has a high basal activity and, therefore, NIK accumulation already triggers phosphorylation of NIK substrates. NIK's best investigated substrate is the p100 precursor protein of the p52 NFκB transcription factor subunit. NIK-mediated p100 phosphorylation promotes limited proteolysis to p52. This triggers the conversion of cytoplasmic p100-containing NFκB dimers to p52-containing dimers, which can translocate into the nucleus. In contrast to TNFR1, TNFR2 can therefore not only stimulate nuclear translocation of NFκB dimers by the IKK complex-dependent classical pathway but also by an alternative pathway based on IKK complex-independent p100 processing (5). TNFR2-mediated sequestration/degradation of TRAF2 complexes not only affects the inhibitory effect of the TRAF2-cIAP1/2 complexes on the alternative NFκB pathway but also limits their availability for TNFR1 (**Figure 1**). Consequently, TNFR2 activation can attenuate TNFR1-induced classical NFκB signaling and sensitize cells for TNFR1-induced cytotoxicity (12–19).

EXPRESSION OF TNF AND ITS RECEPTORS TNFR1 AND TNFR2

TNF is mainly produced by immune cells, e.g., monocytes, macrophages, and T- and B-cells (20). Non-immune cells, such as keratinocytes, astrocytes, endothelial, and epithelial cells but also cancer cells can also express TNF (20). TNF production is highly inducible (up to 10.000 fold). Members of the NFAT-, NFκB-, and basic region-leucine zipper transcription factor families control TNF production on the transcriptional level and ERK1/2, p38MAPK and JNK signaling at the posttranscriptional level by modulation of mRNA stability and translation efficacy (20, 21). As TNF activates the MAP kinase signaling cascades and transcription factors of the NFκB family, TNF can induce its own transcription via both types of TNF receptors (16, 22–28). While TNFR1 is expressed in almost any cell type, TNFR2 expression is limited to myeloid cells, T- and B-cells and endothelial cells (29, 30). Although, sometimes several thousand molecules can be detected, TNFR1 expression levels are typically below 1,000 molecules per cell, especially in T cells (**Table 1**), limiting its detection with flow cytometry. Thus, lack of flow cytometric TNFR1 detection does not exclude functionally relevant TNFR1 molecule numbers. TNFR2 expression varies more and can reach $\geq 10^5$ molecules per cell in tumor cell lines (40).

TNF AND ITS RECEPTORS IN T CELL BIOLOGY

After its molecular cloning, TNFR2 was discovered to promote proliferation of thymocytes and peripheral T cells (41, 42).

TABLE 1 | TNFR1 and TNFR2 expression in primary cells.

Cell type	TNF binding sites per cell			References
	Total	TNFR1	TNFR2	
Human umbilical cord vein (HUVEC) cells	1,500	n.d.	n.d.	(31)
Human SAC[a]-activated B-cells	6,000	n.d.	n.d.	(32)
Human neutrophils	3,000	n.d.	n.d.	(33)
Human neutrophils	6,000	n.d.	n.d	(34)
Peripheral T cells, healthy subjects	130/140	n.d.	n.d.	(35–37)
Peripheral T cells, MS patients	950/840	n.d.	n.d.	(35, 36)
Peripheral T cells, myasthenic patients	660	n.d.	n.d.	(37)
OKT3/IL2 activated T cells	600	n.d.	n.d.	(38)
PHA activated PBMCs	5,600	10–20%	80–90%	(39)

[a] *Staphylococcus aureus Cowan strain I.*

Subsequently, TNFR2 was recognized as a costimulator of naive $CD8^+$ T cells *in vitro* and *in vivo* (43–46). Accordingly, TNFR2-mediated T cell costimulation is impaired in patients suffering from common variable immunodeficiency (47). At the molecular level, the costimulatory activity of TNFR2 has been associated with an increased expression of survival proteins such as survivin and Bcl-2 (44). However, the role of TNFR2 in $CD8^+$ T cell regulation is presumably more complex, context-dependent, and goes beyond sole improvement of $CD8^+$ viability. For example, in mice infected with respiratory influenza virus or acute lymphocytic choriomeningitis virus TNFR2 contributes to the contraction of the antigen-specific $CD8^+$ T cell population (48, 49). In accordance with the counterintuitive proapoptotic TNFR2 activity in these models, TNFR2 deficient $CD8^+$ T cells were less sensitive for TNFR1-dependent cell death and activation induced cell death *in vitro* (50, 51). As discussed above, TNFR2 can sensitize cells for TNFR1-induced cell death by depletion/degradation of protective TRAF2-cIAP/2 complexes but also activates the alternative and classical NFκB pathways, which upregulate antiapoptotic proteins and proliferation promoting factors. Thus, it is tempting to speculate that the balance of these two effects determines the outcome of TNFR2 activation in $CD8^+$ T cells. Particularly, in situations where $CD8^+$ T cells are protected TRAF2-cIAP1/2-independently from TNFR1-induced killing, the proliferation promoting effects of TNFR2 might dominate.

TABLE 2 | *In vivo* evidence for TNFR2-dependent Treg functions.

Model	Method	Effect	References
TNFR2 KO mice	Cecal ligation and puncture	Reduced Treg expansion	(60)
TNFR2 KO nTregs	T cell transfer induced colitis	Loss of suppressive Treg activity	(65)
TNFR2 KO	EAE	Reduced Treg numbers and exacerbated disease	(66)
TNFR2 KO Tregs of EAE mice	Transfer in EAE mice	Loss of EAE inhibitory activity	(66)
TNFR2 KO bone marrow reconstitution	B16F10 metastasis	Reduced tumor Tregs and metastasis	(67)
TNFR2 KO mice	Friend virus-induced $V\beta5^+$ Treg expansion	Loss $V\beta5^+$ Treg expansion	(68)
Wt mice	TNFR2 agonist	$V\beta5^+$ Treg expansion	(68)
Wt mice	TNFR2 agonist	Treg expansion	(69)
Wt mice	TNFR2 agonist priming	Treg expansion and protection from GvHD	(69)
TNFR2 KO Tregs	Treg transfer-mediated GvHD protection	Loss of GvHD protection	(70)
Wt Tregs	TNFR2 blocking antibody in Treg transfer-mediated GvHD protection	Loss of GvHD protection	(70)
Wt mice	TNFR2 agonist in collagen induced arthritis	Increased Treg number and reduced disease score	(71)
Wt and TNFR2 KO mice	TNFR2 agonist	Treg expansion in naïve mice	(72)

THE RELEVANCE OF TNF AND ITS RECEPTORS FOR TREG BIOLOGY AND TREG FUNCTION

Early on, it had been reported that administration of soluble TNF to neonatal non-obese diabetic (NOD) mice enhanced diabetes onset while reducing $CD4^+CD25^+$ T cell numbers in thymus and spleen. Treatment with anti-TNF antibodies resulted in opposite effects (52). Moreover, T cell transfer experiments of $CD4^+CD25^+$ T cells from TNF-treated neonatal mice displayed diminished inhibitory activity (52). Again in the NOD model, TNF inhibited Tregs via TNFR1 (53). Accordingly, TNF contained in the synovial fluids of rheumatoid arthritis (RA) patients was reported to impair Treg function by upregulation of protein phosphatase 1 and dephosphorylation of Foxp3 (54). Notably, the latter was restored in RA patients treated with the TNF neutralizing antibody Infliximab (54). Already earlier and in accordance with a Treg inhibitory effect of TNF, several reports showed a moderate but significant increase in Treg frequency in the peripheral blood of RA patients treated with the TNF neutralizing antibodies Adalimumab and Infliximab (55–57). Furthermore, exogenous soluble TNF inhibited the suppressive activity of Tregs derived from HBV patients (58). Likewise, TNF alone, or in combination with IL6, inhibited the suppressive activity of Tregs isolated from naïve mice *in vitro* (59).

However, by 2007 Chen et al. not only showed that TNFR2 is highly expressed on murine and human Tregs but also that TNFR2 supports Treg proliferation and maintenance of their suppressive activity (60–64). Indeed, $TNFR2^+$ expression marks the most suppressive subset of Tregs (63). Consequently, various animal models, including models of inflammatory diseases and cancer, confirmed the relevance of TNFR2 for Treg proliferation and Treg activity (**Table 2**).

Noteworthy, adoptive transfer experiments with antigen-specific Teffs and Tregs revealed that effector T cells promote the expansion of sub-optimally proliferating antigen-stimulated Tregs in a TNF-dependent manner (62, 73). Similarly, $V\beta5^+$ Tregs, recognizing mouse mammary tumor virus encoded superantigen, expand after Friend virus infection due to TNFR2 activation by $CD8^+$ expressed membrane TNF (68). Thus, the capability of T cell expressed TNF to promote Treg proliferation and activation via TNFR2 may represent a negative feedback mechanism to terminate inflammation.

The seemingly conflicting data on the proliferation and activity of Tregs via targeting TNF or TNFR2 might be related to two obvious scenarios:

First, neutralization of TNF might inhibit without discrimination detrimental and beneficial effects of TNF on Tregs that separates with the two TNF receptors (**Figure 2**). Evidence supports TNFR1 mediated negative effects on Tregs. TNFR1 deficiency increased Treg activity (53) and Tregs deficient for FLIP, a major inhibitor of TNF-induced apoptosis, have extremely low Treg numbers and develop a scurfy-like

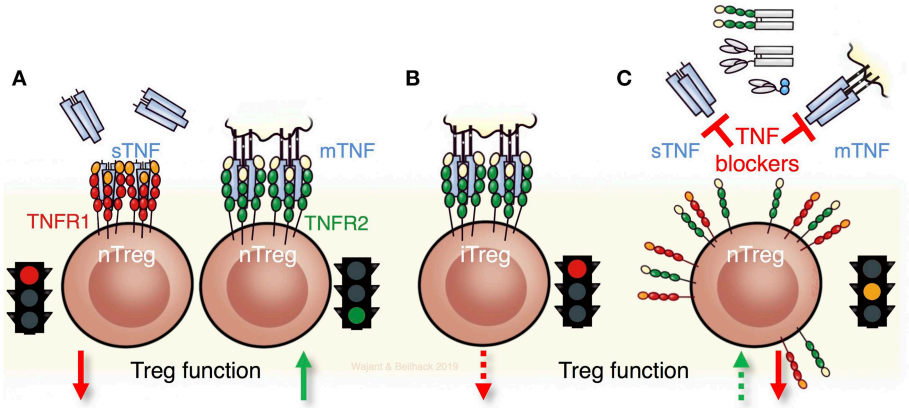

FIGURE 2 | TNF and its receptors for Treg biology and Treg function. **(A)** Soluble TNF (sTNF) can impair the maintenance and function of thymic derived naturally occurring Tregs (nTregs) via TNFR1. In contrast, stimulation of TNFR2 expands and fosters the function of nTregs. **(B)** Notably nTregs and induced Tregs (iTregs) respond differently to TNF. Triggering of TNFR2 in iTregs diminishes their stability and function. **(C)** The seemingly contradictory results obtained with anti-TNF biologicals that are in current clinical use such as antibodies, antibody-fusion proteins, or Fab' fragments can be ascribed to the different effects of TNF on the two receptors TNFR1 and TNFR2. Consequently, neutralizing TNF and not directly targeting its receptors can result in complex scenarios by exerting detrimental and beneficial effects on Tregs, dependent on which receptor is being engaged and whether nTregs or iTregs, or both, are implicated.

phenotype (74). Notably, TNFR2 markedly improves myeloid derived suppressor cell survival via FLIP upregulation (75). Opposing effects of the two TNF receptors have also been reported regarding the suppressive effect of Tregs on effector T cell proliferation *in vitro* (76). While TNFR1 deficiency in Tregs resulted in enhanced suppressive activity, TNFR2 deficient Tregs almost completely lost their suppressive potential.

Another factor contributing to the seemingly inconsistency in the available literature on the role of TNF in Treg biology is that nTregs and iTregs respond differently to TNF (**Figure 2**). Indeed, TNF neutralization in an EAE model increased Treg levels due to the reversal of an inhibitory effect of TNF on TGFß-induced iTreg differentiation (77), while nTregs remained unaffected. Noteworthy, TNF inhibited iTreg differentiation also via TNFR2 (77). Accordingly, restoration of Treg function in RA patients treated with Infliximab has been traced back to an emerging and unusual $CD62L^-$ Treg population that after TNF blockade differentiates via TGFβ from $CD4^+CD25^-$ cells of RA patients but not of healthy individuals (78).

TNFR2 SIGNALING IN REGULATORY T CELLS

Already in 2002, high TNFR2 expression was reported on human $CD4^+CD25^+$ thymocytes, which showed T cell suppressive activity after polyclonal expansion (79). The first reports demonstrating the importance of TNFR2 for Treg functions, however, were published only 5–6 years later (60, 61, 80). Although regulation of Tregs has meanwhile become the most intensively studied *in vivo* activity of TNFR2, limited knowledge exists about the molecular mode of action in Tregs. Based on what is known about TNFR2 signaling in other cell types, without claim of completeness, three possible mechanisms appear plausible:

First, TNFR2-induced activation of NFκB transcription factors (**Figure 3**). Activation of the classical and alternative NFκB pathway by TNFR2 has been demonstrated in a variety of cell types and these pathways are also stimulated by the TNFR2-related TNFRSF receptors CD27, OX40, and GITR, all been implicated in Treg development or survival. Therefore, it is tempting to speculate that TNFR2-induced NFκB signaling is also involved in the control of Treg expansion/activity. In fact, the NFκB subunit cRel, with its well-established role in thymic Treg development and which has also been implicated in iTreg generation (81) has just recently been identified, along with p65 (RelA), as a crucial factor for the maintenance and functionality of mature nTregs and iTregs (82, 83). Activation of cRel- and p65-containing NFκB dimers is typically triggered by the classical NFκB pathway. Despite normal or slightly increased Treg numbers in spleen and lymph nodes, mice with cRel or p65 deficient $Foxp3^+$ Tregs showed mild (cRel deficient Tregs) or significant but slowly progressing (p65 deficient Tregs) lymphoproliferative disease (83). This points to a role of cRel and p65 for the suppressive activity of Tregs. Indeed, in contrast to wild type Tregs, Tregs lacking cRel or p65 were unable to rescue mice from T cell transfer-induced colitis (83). Mice double deficient for p65 and cRel in Tregs succumbed early to a scurfy-like (Foxp3 defective) phenotype (83). Although, cRel deficiency did not impair iTreg formation, cRel is also here relevant, because absence of cRel and p65 in $CD4^+$ T cells impaired iTreg induction (83, 84). Tregs can also be categorized in two distinct functional subsets, resting Tregs (rTregs) in lymphoid tissue, and activated Tregs (aTreg) with reduced Foxo1 expression, migrating to inflamed tissues including cancer (85). Now, cRel but not p65 turned out as important for aTreg differentiation and tumor development (82). Inducible p100 processing, which results in the conversion of p100-RelB complexes to a p52-RelB NFκB dimers (**Figure 3**), is the central step in the alternative NFκB pathway. Mice

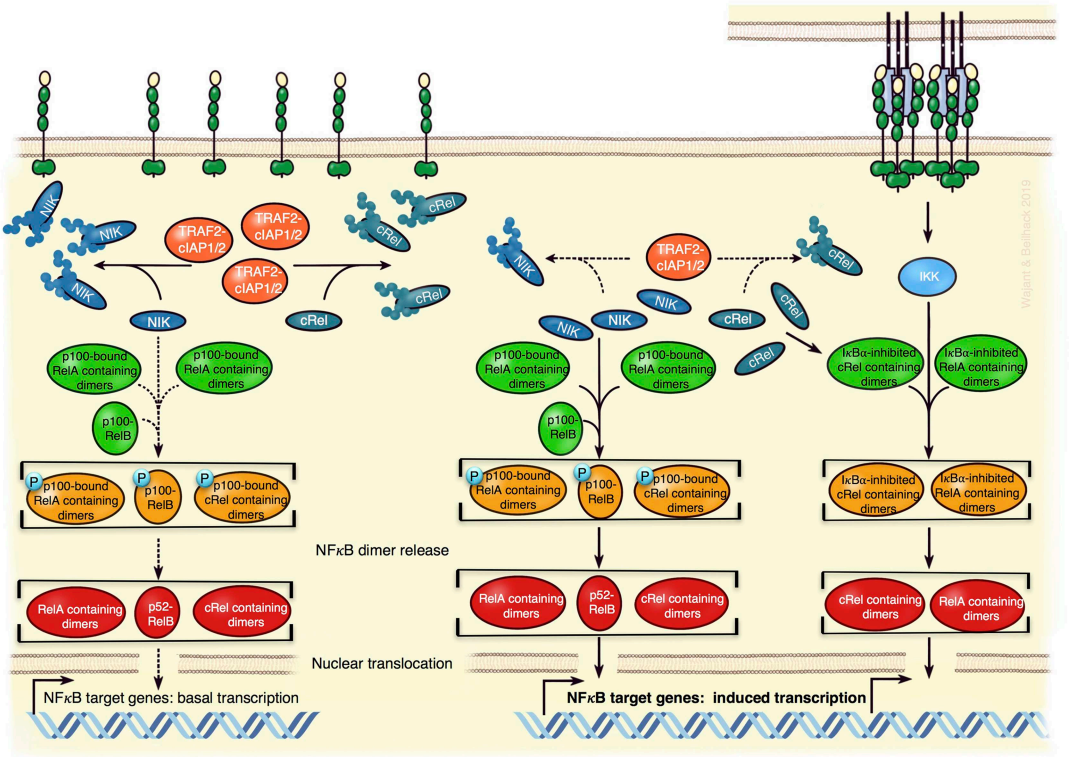

FIGURE 3 | Model of TNFR2-mediated regulation of NFκB transcriptions factors in Tregs. In absence of appropriate exogenous stimuli, the classical NFκB pathway is not active (**Left**). Constitutively active cytosolic TRAF2-cIAP1/2 complexes K48-ubiquitinate NIK and cRel triggering so the proteasomal degradation of these proteins (**Left**). This not only dampens the activity of the NIK-dependent alternative NFκB pathway to low basal levels but also diminishes the amount of cRel-containing dimers, which can be activated via the classical NFκB pathway. Since p100 can inhibit cRel- and RelA-containing NFκB dimers, it might also reduce the responsibility of the classical NFκB pathway. TNFR2 activation by memTNF results in the recruitment of TRAF-cIAP1/2 complexes and activation of the IKK-dependent classical NFκB pathway (**Right**). The depletion of the cytosolic TRAF2-cIAP1/2 complexes associated herewith leads to reduced degradation of NIK and cRel, thus (i) to enhanced alternative NFκB signaling and (ii) more cRel-containing NFκB dimers that can respond to the classical NFκB pathway (**Right**). The model is based on what is known about the specific functions of NFκBs in Tregs and the mechanisms of TNFR2 signaling in general.

with p100 deficient Tregs also develop a mild autoimmune syndrome, which depends on RelB and correlated with increased Treg numbers with reduced suppressive activity (86). Notably, interaction with the ankyrin domain of p100 can also inhibit cRel- and p65-containing NFκB dimers (87), whereby p100 binds cRel-containing dimers more preferential than RelA-containing dimers (88). Thus, TNFR2 via activation of the alternative NFκB pathway has the potential to crosstalk into the classical NFκB pathway. Considering that TNFR2 seems to be more important than TNFR1 in Tregs and because TNFR2, in contrast to TNFR1, triggers not only the classical NFκB pathway but also the alternative NFκB pathway, the following scenario appears plausible: TNFR2 (or other p100 processing-triggering TNFRSF receptors) triggers/maintains Foxp3 expression and Treg suppressive activity by stimulating both NFκB pathways yielding in the coordinated activation of cRel, RelB, and RelA-containing NFκB dimers (**Figure 3**).

A second possible mode of TNFR2 signaling in Tregs is based on the ability of TNFR2 to sequester and degrade TRAF2. Depletion of cytoplasmic TRAF2-cIAP1 and TRAF2-cIAP2 pools may not only result in the accumulation of NIK and activation of the alternative NFκB pathway but might also promote other signaling events, which are inhibited in unstimulated cells by TRAF2-mediated degradation. In fact, TRAF2 and cIAPs antagonize proinflammatory signaling in myeloid cells by promoting proteasomal degradation of cRel and IRF5 (89). Therefore, TNFR2-induced depletion of cytosolic TRAF2-cIAP1/2 complexes has the potential to increase cRel levels (**Figure 3**).

Thirdly, it has been suggested that TNFR2 elicits its effect on Tregs not directly by triggering intracellular signaling pathways but indirectly after shedding from the plasma membrane and inhibiting soluble TNF (80). A functional relevant robust TNF neutralizing effect of the soluble TNFR2 ectodomain, however, is hard to reconcile with the very low affinity of monomeric TNFR2 for TNF (90).

PRECLINICAL AND CLINICAL EVIDENCE FOR THE USEFULNESS OF THERAPEUTIC TREG TARGETING VIA TNFR2

Adoptive transfer of Tregs is a straightforward strategy to exploit the overwhelming immunotherapeutic potential of this

cell type, which is being explored in clinical studies (91). Purification/enrichment and *ex vivo* expansion of stable and functional Tregs are crucial factors complicating the applicability and success of therapeutic adoptive Treg transfer (92). In accordance with the crucial role of TNF-TNFR2 signaling in Treg biology, two recent studies demonstrated beneficial effects of targeting of the TNF-TNF receptor system in *ex vivo* Treg expansion protocols. Using a not further specified agonistic TNFR2 antibody, Okubo et al. demonstrated that the additional activation of TNFR2 in standard Treg expansion protocols conferred improved suppressive activity while reducing Treg heterogeneity (93). Furthermore, using the TNFR2-specific mAb MR2-1 as an agonist, TNFR2 signaling promoted the expansion of low purity MACS-isolated Treg preparations to stable homogenous Treg populations (94). Therefore, TNFR2 agonists may potentially improve *ex vivo* Treg expansion methods for clinical applications.

First decisive evidence for the *in vivo* drugability of the TNF-TNFR2 interaction in nTregs stems from mouse experiments of GvHD and collagen-induced arthritis (CIA). Employing a TNFR2-selective nonameric variant of murine TNF, several groups found that exogenous TNFR2 stimulation suffices to expand Treg numbers in mice (68, 69, 71, 72). TNFR2 agonist-induced Treg expansion protected mice from subsequent allogeneic hematopoietic cell transplantation (HCT)-induced GvHD, while preserving graft-vs.-leukemia activity (**Figure 4**) (69). Inhibiting the TNF-TNFR2 interaction blocked Treg activity in GvHD (70). TNFR2-promoted Treg expansion also attenuated the clinical score of mice suffering from CIA (71). In accordance with these findings, Pierini et al. reported that *in vitro* TNF priming in the presence of IL2 enhances TNFR2-dependent murine Treg activation and proliferation resulting in Tregs providing superior protection from GvHD (95).

The microenvironment of most tumors is highly enriched with TNF producing cells such as macrophages, T cells, and fibroblasts and often contains increased Treg numbers that crucially contribute to tumor immune escape and tumor progression (**Figure 4**). Based on the compelling evidence that TNF-TNFR2 interaction stimulates Treg activity, various studies addressed the feasibility of blocking TNFR2 therapeutically in animal cancer models. In one study, loss of tumor immunity against secondary tumors was traced back to $CD103^+$ effector Tregs with high TNFR2 expression (96). *In vitro*, TNF induced TNFR2-mediated effector Treg expansion and their transfer suppressed antitumoral $CD8^+$ T cell responses (96). Likewise, increased effector Treg frequencies were found in peripheral blood samples of colon rectal carcinoma and hepatocellular carcinoma patients. Again, these Tregs were significantly enriched *in vitro* in response to TNF (96). Moreover, soluble hTNFR2-Fc enhanced the antitumor activity of cyclophosphamide and further reduced effector Treg numbers in mice bearing CT26 tumors without affecting $CD8^+$ T cell activation (96). Also in CT26 bearing mice, blockade of TNFR2 signaling with the antagonistic antibody M861 reduced $TNFR2^+$ Treg frequency within the tumor microenvironment and enhanced the immune stimulatory activity of CpG oligodesoxynucleotides (97). Notably, the antagonistic TNFR2 antibody TR75-54.7 inhibited growth of 4T1 tumors more efficiently than the antagonistic CD25 mAb PC61 (97). Using a novel antagonistic TNFR2 antibody, Torrey et al. demonstrated that TNFR2 blockade in ascites of ovarian cancer patients result in reduced Treg numbers and increased effector T cell frequency (98).

Patients suffering from acute myeloid leukemia (AML) display increased Treg numbers in the peripheral blood and bone marrow (99), which correlate with poor prognosis (100). The majority of these Tregs strongly express TNFR2 and efficiently migrate into the bone marrow (101). In AML patients subjected to epigenetic therapy, a reduction of $TNFR2^+$ Tregs have been observed in the bone marrow of responders compared to non-responders whereas there was no difference in $TNFR2^-$ Tregs before and after treatment (101).

PRECLINICAL DEVELOPMENT OF TNFR2-TARGETING REAGENTS

The goal of TNFR2 targeting clearly depends on the considered disorder. While inhibiting TNFR2 activities in Tregs or even destroying Tregs may be the goal in cancer, stimulating TNFR2 may be the aim to treat inflammatory conditions or inflammation-associated cancer to improve immune suppression by Tregs.

PRECLINICAL DRUGS WITH TNFR2-INHIBITORY ACTIVITY

In principle, TNFR2 activation might be prevented pharmacological by use of one of the various approved TNF-neutralizing biologicals for the treatment of autoinflammatory diseases. However, this would also inhibit TNFR1 signaling counteracting the beneficial effects of reduced TNFR2 activity. In the immunotherapy of cancer, for example, inhibition of TNFR2 might help to break tumor-associated immune tolerance by reducing Treg activity. The intended stimulation of anti-tumor immunity, however, would suffer from inhibiting proinflammatory TNFR1 activities, too. Selective inhibition of TNFR2 is obviously possible with TNFR2-specific antibodies blocking TNF binding and lacking intrinsic TNFR2-stimulating activity (**Figure 5**, upper panel). The development of antagonistic anti-TNFR2 antibodies appears, at first glance, simple. Indeed, various reports described the use of antagonistic TNFR2-specific antibodies *in vitro* on non-hematopoietic cells. *In vivo*, however, the situation might be complicated by the presence of immune cells and immune cell-associated expression of Fcγ-receptors (FcγRs). Various preclinical *in vivo* studies demonstrated that FcγR-binding can act as an all-dominant factor that determines the agonistic activity of TNFRSF receptor-specific antibodies and even converts antagonistic antibodies into strong TNFRSF receptor agonists (3). The FcγR binding-dependent agonistic activity of TNFRSF receptor-targeting antibodies presumably reflects the fact that membrane-anchoring of antibodies promotes the secondary oligomerization of initially formed antibody-TNFRSF receptor complexes, which is needed for

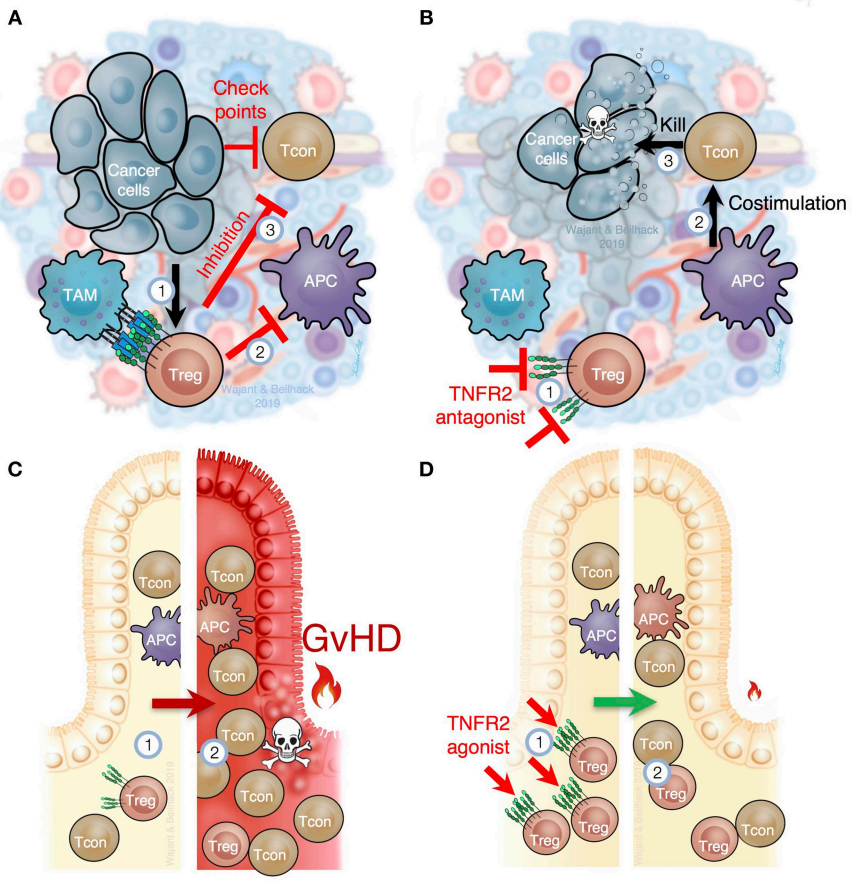

FIGURE 4 | TNFR2 is critical for Treg function in cancer and inflammation. **(A)** In the tumor microenvironment, immune cells, such as tumor associated macrophages (TAM), stroma, and tumor cells, produce TNF, which (1) attracts and stimulates Tregs and myeloid derived suppressor cells (MDSC). TNFR2-mediated Treg activation prevents optimal costimulation by antigen presenting cells to trigger cytotoxic tumor-specific T cell responses (2) and, importantly, prevents T cell mediated tumor lysis through several immune checkpoints (3). **(B)** Selective inhibition of TNFR2 or depletion of TNFR2^{+} Tregs in tumors (1) would improve costimulation by tumor antigen presenting cells (APC) to activate cancer specific immune responses (2), abolish the blockade of cancer specific immune responses within the tumor tissue (3) and, thus, reactivate cytotoxic T cells to destroy cancer cells. **(C)** In recipients of allogeneic hematopoietic cell transplantation (HCT), the underlying disease, intensive therapy and host conditioning systemically reduce Tregs (1). After allogeneic HCT Tregs are overwhelmed to control alloreactive T cells (2), which cause acute graft-vs.-host disease (GvHD). **(D)** TNFR2-specific agonists stimulate Tregs in secondary lymphoid organs and peripheral tissues (1). Increased Treg numbers and function support tissue homeostasis and can contain excessive T cell responses (2) as they occur in acute GvHD or other inflammatory diseases.

full receptor activation (3). In any case, this issue should be evaluated in course of the development of antagonistic anti-TNFR2 antibodies and could necessitate the use of antibody isotypes/variants devoid of FcγR binding.

Inhibition of TNFR2 activity might also be achieved by non-antibody based drugs. For example, Tang et al. identified progranulin as a high-affinity competitor of TNF binding to TNFR2 (102). However, progranulin also competes with TNF for TNFR1 binding and with TL1A for DR3 binding (102, 103). Thus, progranulin based TNFR2 blockers let expect similar limitations as discussed above for TNF-neutralizing reagents. Moreover, several independent groups failed to observe inhibition of TNF-TNFR1/2 interaction by progranulin (104–106). In fact, it has been reported that progranulin rather enhances, than blocks, TNF-induced TNFR2-mediated proliferation of Tregs (107). In the absence of ligand, TNFR1, TNFR2 and several other receptors of the TNFRSF undergo homotypic interaction without activating cellular signaling pathways (108–110). This is mediated via the N-terminal pre-ligand assembly domain (PLAD) and is required for efficient ligand binding. Accordingly, dimeric Fc and GST fusion proteins of the TNFR1 PLAD inhibit TNFR1-promoted pathologies in preclinical models (110–113). Because of the homotypic PLAD-PLAD interaction, fusion proteins of the TNFR2-PLAD may act as TNFR2-specific antagonists leaving TNFR1 signaling intact (**Figure 5**, upper panel).

PRECLINICAL DRUGS FOR SELECTIVE TNFR2-STIMULATION

Activation of TNFR2 can be achieved by recombinant variants of its natural ligands TNF and LTα or by agonistic antibodies or antibody mimetics. Two aspects require consideration for

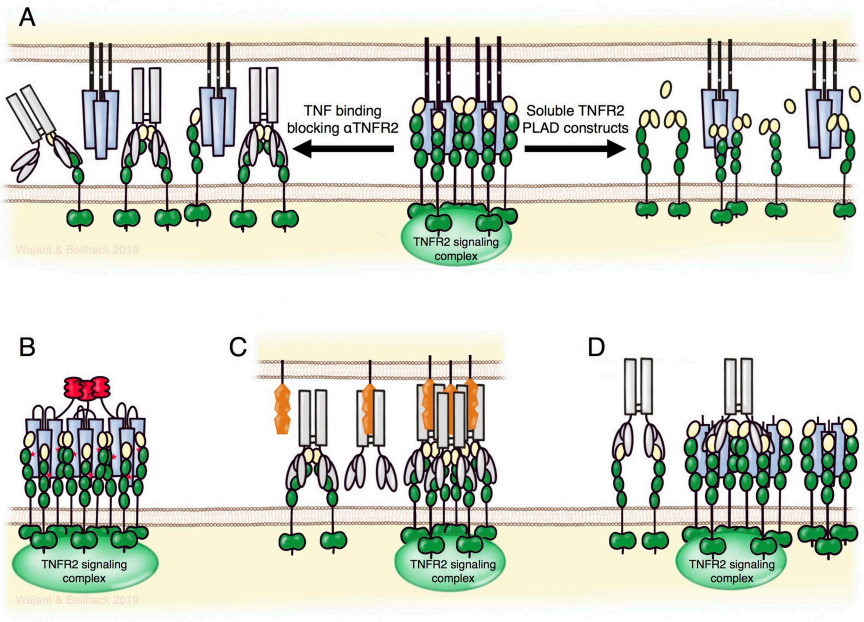

FIGURE 5 | TNFR2 targeting biologicals. **(A)** TNFR2-specific antibodies blocking TNF binding (left part) or fusion proteins of the TNFR2 PLAD domain (right part) can block the formation of TNFR2 signaling complexes without directly affecting TNFR1-related activities. Lower panel: nonameric variants of TNFR2-specific TNF mutants **(B)** and FcγR-bound agonistic anti-TNFR2 antibodies **(C)** allow specific activation of TNFR2. *Per se* non-competitive non-agonistic TNFR2-specific antibodies that oligomerize poorly active soluble TNF-TNFR2 complexes can potentiate TNFR2 signaling **(D)**. For details see text.

recombinant ligand variants for TNFR2 stimulation: first, receptor selectivity, as TNF (and LTα) interacts with TNFR2 and TNFR1; second, limited ability of soluble TNF trimers to stimulate TNFR2 signaling. The challenge of TNFR2 selectivity had been solved early, by various groups identifying mutations conferring selectivity for one of each of the two TNF receptors. Furthermore, oligomerization of soluble TNF trimers potentiates their ability to stimulate TNFR2 (5). This knowledge triggered our rational design of human and mouse TNF fusion proteins that comprise three ligand trimers and act as very potent TNFR2 agonists. To obtain three covalently linked TNF trimers the small trimerization domain of tenascin-C was genetically linked to a triplet of TNFR2-specific TNF protomers separated by peptide linkers long enough to allow intramolecular self-assembly (5, 69). *In vitro* binding and functional studies proved high selectivity of these agonistic fusion proteins for TNFR2 and, accordingly, in no toxicity in mice treated repeatedly with high doses of the murine TNFR2-specific variant (69).

Besides recombinant soluble TNFSF ligand variants, agonistic antibodies are the classical approach to activate receptors of the TNFRSF (**Figure 5**, lower panel). Based on superior pharmacokinetics and the broad experience in antibody production and development, agonistic antibodies remain the first choice to accomplish therapeutic activation of TNFRSF receptors. Indeed, various agonistic antibodies targeting immune stimulatory or cell death-inducing members of the TNFRSF are currently under investigation in clinical trials for cancer therapy. As discussed above for the development of antagonistic TNFR2-specific antibodies, one has to consider again the possibility of antibody binding to FcγRs and the possible agonism-boosting and immune cell-stimulating effects of these interactions. Since agonistic TNFRSF receptor-specific IgG antibodies frequently achieve only maximum activity upon FcγR-binding, such antibodies risk to trigger destruction of targeted cells instead of receptor activation. Thus, TNFR2 targeting with such antibodies *in vivo* could rather deplete Tregs instead of promoting Treg expansion. Therefore, antibodies with a high intrinsic, FcγR binding-independent agonistic activity or Fc domain-mutated antibodies preferentially binding to inhibitory FcγRs may account for the best strategy to achieve TNFR2 activation with agonistic antibodies *in vivo*.

An interesting option to achieve TNFR2 activation *in vivo* is the use of non-competitive antibodies modifying the receptor response to soluble ligand trimers. It has been found that some non-competitive and *per se* non-agonistic antibodies against TNFRSF receptors can strongly enhance receptor activation upon soluble ligand binding presumably via aggregation of otherwise poorly active trimeric ligand-receptor complexes (**Figure 5**, lower panel) (114, 115). This mode of action has also been demonstrated for the TNFR2-specific mAb 80M2 (4). Clinical development of a TNFR2-specific antibody of this type may have two advantages: first, the "agonistic" activity would be fully independent from FcγR-binding and second, the "agonistic" activity would be closely spatiotemporally linked to sites where TNF is actively expressed.

TARGETING TNFR2 TO ENHANCE TREG FUNCTION IN GVHD

The pathophysiologic sequelae of acute GvHD follows a spatiotemporally orchestrated pattern of disease initiation and

an ensuing effector phase (116–120). TNF plays a crucial role in all these events through several mechanisms. Host conditioning triggers an instant TNF release by host macrophages (121) which might enhance maturation of host type antigen presenting cells (APCs), the expression of MHC molecules (122) and T cell adhesion to APCs (123). TNF furthermore provides costimulatory signals to naïve $CD4^+$ T cells and $CD8^+$ cytotoxic T lymphocytes (44, 124–126). TNF, together with IL1β, also enhances TNF expression by freshly activated alloreactive T cells constituting a feed-forward-loop of TNF release (127–129). However, only in recent years it has become clear that TNF also triggers anti-inflammatory feedback loops, e.g., by stimulation of Tregs and myeloid derived-suppressor cells via TNFR2 (see previous paragraphs). After initial priming in secondary lymphoid tissues, alloreactive effector T cells home into GvHD target tissues (116, 117, 130, 131). Upon allorecognition, tissue infiltrating donor T cells release TNF, which can cause epithelial damage (120, 132). Fn14, a tissue damage-induced receptor of the TNFRSF, sensitizes intestinal epithelial cells and renders them particular susceptible to TNF-dependent apoptosis (133). This may also explain, at least in part, why the intestinal tract is a primary target for GvHD tissue damage. The consequent disruption of the barrier function of the gut epithelium results in a vicious cycle of exacerbating GvHD (134).

In patients, systemic TNF release of >100 pg/mL in the first 3 months after allo-HCT strongly correlated with acute GvHD, veno-occlusive disease, endothelial leakage syndrome, and interstitial pneumonitis (128, 129). Also, it was found that levels of shed TNFR1 and TNFR2 correlate with systemic TNF concentrations and allo-HCT related complications (135, 136). Subsequently, it has been furthermore found that a strong increase of soluble TNFR1 (sTNFR1) 7 days after allo-HCT correlated with GvHD incidence and severity and patient survival (137, 138). These results lead to the integration of sTNFR1, together with interleukin-2-receptor-alpha, interleukin-8, and hepatocyte growth factor, into a proposed serum biomarker panel for GvHD diagnosis and prediction of survival (139).

The detrimental effects of TNF on GvHD pathogenesis provided a clear rationale to test TNF-inhibitors in allo-HCT. Indeed, TNF blockade prevented acute GvHD in most mouse models but may also affect graft-vs.-leukemia activity as transplantation of TNFR1 deficient donor CD8 T cells resulted in an increased leukemia relapse after allo-HCT (120, 121, 127, 140). Based on these data, several clinical studies were initiated to test TNF inhibitors for the treatment of acute GvHD or as a preemptive therapeutic approach to prevent the onset of acute GvHD. Importantly, the TNF blocking antibody infliximab failed in clinical trials, both in a treatment setting and in a preemptive therapy approach, and might even increase bacterial and fungal infections (141, 142). Although etanercept, a Fc fusion protein of the TNFR2 ectodomain, in combination with high-dose steroids showed initially promising response rates in GvHD patients, it neither improved survival in comparison to control subjects nor showed it a beneficial activity in a prophylactic setting (143–145).

Blocking TNF does not only inhibit the primarily TNFR1-mediated proinflammatory TNF activities but also the predominantly TNFR2-mediated protective effects. The ambivalence of therapeutically targeting TNF is emphasized by the experience with TNF inhibitors in the treatment of autoimmune diseases. Clearly, TNF blockers have been a game-changer for the treatment of inflammatory diseases such as rheumatoid arthritis and colitis showing high response rates in many patients making them the commercially most successful biologicals on the market. However, many patients do not respond to TNF inhibitors and TNF blockers may even exacerbate inflammation in other diseases, e.g., heart failure or multiple sclerosis (146, 147). This emphasizes that, despite the prominent perception of TNF as a potent proinflammatory cytokine, TNF can exert important immunosuppressive functions, likely depending on the underlying disease and the involved immune regulatory cells.

As pointed out above, an important mechanism explaining these opposing outcomes of TNF-inhibition is the dichotomy of TNFR1- and TNFR2-mediated effects. Therefore, directly addressing TNFR1 or TNFR2 as therapeutic targets through TNFR1 antagonists or TNFR2 agonists appears as an attractive strategy to improve current clinical practice of GvHD treatment. So far, this strategy has been tested in preclinical mouse models employing TNFR2-selective agonists. TNFR2-mediated *in vivo* expansion of Tregs could prevent acute GvHD (69). Notably, fostering Treg numbers and their function may not only counterbalance excessive inflammation but may also improve tissue regeneration (148, 149). Restoration of tissue homeostasis in GvHD target tissue may prove as a key mechanism to improve outcomes in patients undergoing allo-HCT.

Conclusively, therapeutically targeting of TNFR2 in patients appears as a highly promising approach to either propagate donor Tregs *in vitro* or, importantly, to enhance Treg activity by expanding $TNFR2^+$ Tregs in patients before allo-HCT to prevent GvHD. This attractive approach promises to reduce the risk for GvHD while allowing for alloimmune responses against remaining leukemia cells or to allow for efficient immune control of opportunistic infections. More caution will be warranted to employ TNFR2-agonists at the time of donor lymphocyte infusion or at the onset of GvHD. Clearly, the stimulatory effects of TNFR2 on Tcons require careful assessment in preclinical *in vivo* models before TNFR2 agonists will enter clinical trials.

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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Conflict of Interest Statement: Through the department of technology transfer of Würzburg University, HW and AB have filed patent applications regarding the development and generation of TNFR superfamily-addressing reagents.

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