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CELLULAR THERAPIES - PAST, PRESENT AND FUTURE

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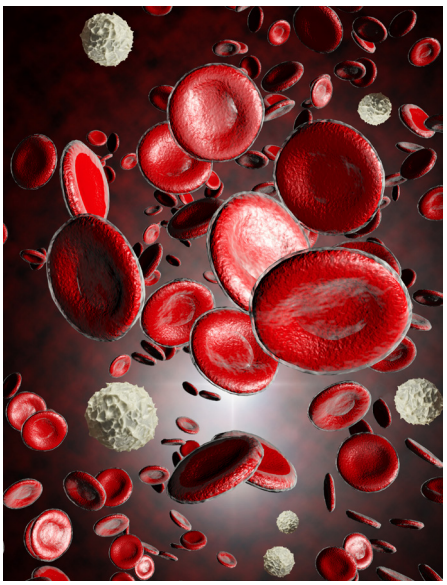

Healthy Human Red and White Blood Cells.
Image: Ezume Images/Shutterstock.com.

Haematopoietic stem cell transplantation was initiated in the early 1970's by pioneers studying radiation-induced bone marrow damage and blood transfusion. Since that time there have been over one million transplants and over 34 million donors registered with the world marrow donor association. This special edition of Frontiers in Immunology highlights the research achievements which led to the curative therapy of haematopoietic stem cell transplantation (HSCT) but also reviews the ongoing complications such as graft versus host disease (GvHD) and infection caused by the procedure. Early animal and human studies are reviewed as well as those which led to the development of changes in transplant protocols such as peripheral blood stem cell and cord blood transplants and the harnessing of graft versus leukaemia (GvL) effects by donor lymphocyte infusions. The eBook covers immunogenetics, the role of biomarkers, and future developments of the therapy which will aim to further improve the outcome for HSCT patients. The eBook is divided into 8 chapters dealing with animal studies; a history of early human studies; the pathophysiology of HSCT; graft versus host disease; graft versus leukaemia effects; immune reconstitution; non-HLA immunogenetics and future developments, including use of mesenchymal stem cells, virus specific T cells and chimeric antigen receptor (CAR) therapy.

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CHAPTER 8

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EDITORIAL
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Editorial: Cellular Therapies: Past, Present and Future

Ralf Dressel[1], *Hildegard T. Greinix*[2], *Ernst Holler*[3] *and Anne M. Dickinson*[4]*

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Keywords: animal models, biomarkers, genetic association, graft-vs.-host disease, graft-vs.-leukemia effects, hematopoietic stem cell transplantation, immunotherapies, reconstitution

Editorial on the Research Topic

Cellular Therapies: Past, Present and Future

This collection of review articles on hematopoietic stem cell transplantation (HSCT) has been compiled by enthusiastic post-graduate students within the Marie Curie Initial Training Network CELLEUROPE. It starts with two reviews of current research with a historical perspective and focus on either animal or human HSCT studies.

Boieri et al. summarize the importance of early animal experiments from the 1950's including graft-vs.-host disease (GvHD) a major complication of HSCT, initially termed "runt disease." The article focuses on the contribution of animal experiments to the understanding of the pathophysiology of acute and chronic GvHD and the development of therapies to overcome these complications. It ends with a discussion of general advantages and limitations of animal models for studying GvHD.

The historic milestones of HSCT in man are reviewed by Juric et al. They describe the development of conditioning therapies enabling engraftment of HSC. The pivotal role of human leukocyte antigen (HLA) typing technologies for the introduction and continuous improvement of HSCT is explained. The shift from serological to molecular methods is discussed in view of their importance for the utilization of matched unrelated donors. Further parameters affecting HSCT outcome are introduced focusing on stem cell sources ranging from bone marrow to peripheral HSC and cord blood. The article closes with an outlook on new developments, such as the adoptive transfer of T cells engineered to express chimeric antigen receptors (CARs) directed against antigens present on leukemic cells.

Ghimire et al. discuss in detail the pathophysiology of GvHD explaining initiation and course of both the acute and chronic forms and introducing new strategies to limit these complications. Despite these efforts, especially chronic GvHD remains a major challenge that warrants further research.

The risk to elicit GvHD by transplantation of allogeneic T cells is balanced by their potential for profound graft-vs.-leukemia (GvL) effects, giving rise to curative therapies for malignancies. Dickinson et al. summarize clinical observations and experimental results demonstrating that allogeneic T cells in the graft reduce the risk of relapse of malignancy after HSCT. They describe the development of donor lymphocyte infusion (DLI) as a means to treat relapse. In addition, newer strategies are explained including the infusion of allogeneic mismatched natural killer (NK) cells or tumor antigen-specific T cells.

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The reconstitution of the immune system after HSCT is reviewed by Ogonek et al.. It is of the utmost importance for the success of HSCT, that the various immune cell subsets recover and regain function in a timely manner. Neutrophils are the first cells that usually reappear within the first month after the conditioning therapy. They are followed in the first 3 months by NK cells and then by T cells. B cell recovery takes longer and may need up to 2 years post-HSCT. The reconstitution of regulatory T cells is explained focusing on factors which influence reconstitution, such as immunosuppressive treatment. Finally, the reconstitution of virus-specific T cells is reviewed in view of the clinical importance of these cells for avoiding complications such as CMV reactivation.

Biomarkers have gained much attention as they may predict the occurrence of GvHD and allow for risk-adapted treatment. Juric et al. review the recent developments and start with a discussion of cellular biomarkers, such as $CD19^{+}CD21^{low}$ B cells that are promising in predicting chronic GvHD. Besides cells, serum molecules have been reported to predict GvHD. In addition to proteins, miRNAs are potentially promising biomarkers for GvHD. Moreover, recent developments to identify biomarkers in urine by proteomic approaches are presented. Juric et al. close by discussing the challenge to validate and integrate the great variety of biomarkers that have been suggested during the last few years.

Gam et al. discuss the genetic associations of HSCT outcome focusing on non-histocompatibility genes and three specific examples. Firstly, it is discussed how polymorphisms of *FOXP3* and *FOXP3*-regulating microRNAs affect the risk of GvHD. The miR-155 and miR-146a regulatory network, their polymorphisms and role after HSCT is outlined as a second example. Polymorphisms of the *MICA* gene, which encodes a ligand of the activating NK cell receptor NKG2D, are introduced as a third example. Furthermore, mRNA and miRNA expression profiling studies aiming at the identification of HSCT associated risks are summarized. The review ends with a discussion of the few genome wide association studies, which have been performed so far to elucidate the role mainly of non-HLA polymorphisms in controlling the outcome of HSCT.

The final review by Reis et al. outlines recent developments in cellular immunotherapies for HSCT-associated complications. These include the transfer of mesenchymal stromal cells (MSCs) or MSC-derived extracellular vesicles to treat GvHD. Further complications are infections occurring in the immunocompromised patients. In recent years, strategies to employ anti-virus-specific T cells in the therapy of viral infections have been developed. Relapse is another outcome that can be targeted by cellular immunotherapies, e.g., with CAR T cells and in this chapter the challenges of these new and fascinating therapeutic strategies is discussed.

This collection of articles is dedicated to Professor Bent Rolstad (1947-2016), an enthusiastic and committed supervisor of two of the project's post-graduate students and Professor Jon van Rood (1926-2017), who was encouraging and supportive to the students in writing and revising the articles.

AUTHOR CONTRIBUTIONS

RD drafted the manuscript, which AD, HG, and EH revised. All authors approved the final version.

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REVIEW
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The Role of Animal Models in the Study of Hematopoietic Stem Cell Transplantation and GvHD: A Historical Overview

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Bone marrow transplantation (BMT) is the only therapeutic option for many hematological malignancies, but its applicability is limited by life-threatening complications, such as graft-versus-host disease (GvHD). The last decades have seen great advances in the understanding of BMT and its related complications; in particular GvHD. Animal models are beneficial to study complex diseases, as they allow dissecting the contribution of single components in the development of the disease. Most of the current knowledge on the therapeutic mechanisms of BMT derives from studies in animal models. Parallel to BMT, the understanding of the pathophysiology of GvHD, as well as the development of new treatment regimens, has also been supported by studies in animal models. Pre-clinical experimentation is the basis for deep understanding and successful improvements of clinical applications. In this review, we retrace the history of BMT and GvHD by describing how the studies in animal models have paved the way to the many advances in the field. We also describe how animal models contributed to the understanding of GvHD pathophysiology and how they are fundamental for the discovery of new treatments.

Keywords: animal models, HSCT, aGvHD, cGvHD, pathophysiology

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The use of animal models to study human diseases is considered essential for understanding underlying pathophysiological and molecular mechanisms (1). Here, we will review how animal models have contributed to understanding the complexity of hematopoietic stem cell transplantation (HSCT) and graft-versus-host disease (GvHD). HSCT is the treatment of choice to cure many types of malignant and non-malignant hematological diseases. Despite continuous improvements in the pre- and post-transplantation procedures, the survival rate of transplanted patients is still poor. Acute GvHD (aGvHD) or chronic GvHD (cGvHD) represents major complications after HSCT with high mortality rates, in addition to other complications, such as relapse of the malignancy, engraftment failure, or opportunistic infections. GvHD is evoked by immunocompetent cells present in the graft that recognize and attack host tissue in an immunosuppressed environment.

THE HISTORY OF BONE MARROW TRANSPLANTATION

The advent of the atomic age in the early 1950s led to a strong interest in developing means to protect or cure the potentially lethal effects of radiation. Exposure to high doses of radiation

was recognized to have deleterious effects on hematopoiesis and immune cell functions. By using different animal models including mice, rats, and guinea pigs, researchers soon discovered that injection of bone marrow or fetal spleen cells into lethally irradiated animals could reconstitute the hematopoietic system (1–5).

At the time, it was not clear how reconstitution occurred. At first, all evidence suggested the presence of "humoral factors" that stimulated regeneration of the endogenous hematopoietic system (6), but several studies in the following years showed that the newly formed hematopoietic system was in fact originating from the donor. In one study, biochemical techniques were used to track rat bone marrow cells transplanted into lethally irradiated mice. The authors postulated that the intravenously injected cells were able to migrate to the bone marrow where they survived and maintained their ability to proliferate and form a new hematopoietic system (7). In another study, Ford and colleagues used chromosomal markers to track the donor cells in the recipient. Their experiments provided the final evidence that reconstitution was originating from donor-derived cells (8). The responsible cells in the graft were identified almost 10 years later when Till and McCulloch in 1963 described a single progenitor cell type in the bone marrow with the potential to expand clonally and to give rise to all lineages of hematopoietic cells. This represented the first characterization of the hematopoietic stem cell (9).

In 1956, Barnes and Loutit proposed that an irradiation-transplantation approach could be used to treat fatal hematopoietic malignancies, such as leukemia (10). They speculated that irradiation followed by injection of bone marrow could treat leukemia if leukemic cells were as sensitive to radiation as normal cells. They also hypothesized, in the same paper, that if the entire population of leukemic cells was not eliminated by radiation, a cure could perhaps be achieved with the injection of cells capable to induce an immune response toward the residual leukemic cells. With this central paper, they introduced the concepts of therapeutic bone marrow transplantation (BMT), graft versus leukemia (GvL), and cell therapy.

At that time, it was already well known that grafts between individuals of different genetic backgrounds were rejected, while transplantations between inbred animals or identical twins were successful. The first successful human BMT was performed in 1959 by Thomas and co-workers who treated two leukemic patients with irradiation followed by infusion of bone marrow from their homozygous twins ("autologous" transplantation) (11). Despite successful transplantation, both patients experienced relapse. Further animal experiments and human transplantations demonstrated that irradiation followed by autologous BMT was not enough to eradicate leukemia. As an alternative approach, transplantation of immune cells derived from an individual or animal with a different genetic background was proposed ("allogeneic," formerly termed "homologous"). This approach was experimentally tested in different mouse models (10, 12), resulting in successful eradication of the malignancies. Unfortunately, the mice died a few weeks later from what was then referred to as secondary or homologous disease. This disease was later defined as GvHD.

GRAFT-VERSUS-HOST DISEASE

The definition of GvHD is the result of a great number of accumulated observations since the 1940s. However, it was in particular the work of two independent researchers that elucidated the details of this phenomenon. Simonsen studied the acquisition of tolerance using chick embryos, and observed that injection of adult spleen or blood cells resulted in splenomegaly and severe hemolytic anemia in the recipient embryo. The rationale behind his experiments was that immunological competence is acquired after birth and, therefore, any immune effect in the adult to embryo transplantation setting is ascribable to the injected cells (13). During the same years, Billingham and Brent (14) performed similar studies in mice, describing splenomegaly, defects in growth, and early deaths when newborn mice were injected with allogeneic ("homologous") adult lymphoid tissues. The phenomenon was termed the "runt disease" due to the growth retardation of the mice. In 1959, the same authors concluded that runt disease resulted from a graft-versus-host reaction (GVHR). Their observations were similar to those of experimental BMT (15–18). In addition, several other research groups at the same time described a reaction of grafted immune cells against the host (19–22), and by the beginning of the 1960s the GVHR was an established caveat for successful BMTs.

The nature of the GvHD reaction was ascribed to immunocompetent cells present in the bone marrow graft. Initial the first experiments showed that different hematopoietic cell populations could be fractionated by centrifugation on discontinuous albumin gradients (23). Factions with low content of lymphocytes and high content of blasts were shown to induce less GvHD (24). The lymphocytes responsible for inducing GvHD was identified as T cells, demonstrated by depletion experiments, first with the use of anti-lymphocyte serum (ALS) (25, 26), and later confirmed by the use of various methods to specifically remove T cells from the graft (27–30). These findings represented an important step forward in improving the success of BMTs. The removal of T cells from the bone marrow graft was soon applied in the clinic, and the depletion methods were substantially improved. Unfortunately, while a reduction in GvHD was achieved, patient survival was not improved, since the absence of T cells led to increased relapse, higher risk of infections, and diminished engraftment. To overcome the detrimental effects related to T cell depletions and to boost the GvL effect, donor T cells was re-introduced after BM transplantation. The infusion was delayed to allow establishment of tolerance toward the host. Murine and canine models served well in testing the timing and protocols for T cell infusions, now termed donor lymphocyte infusion (DLI) (31–34).

The success of allogeneic transplantation depends on the degree of histocompatibility match between donor and recipient. Research on outbred canine models has been vital to study genes involved in histocompatibility, and the importance of

tissue typing and donor selection. In intial experiments, antisera were produced by cross immunization of dog littermates. These antisera were used in cytotoxicity tests in order to establish matched donor/recipient pairs that proved to be effective in reducing, but not eliminating, GvHD occurrence. The number and nature of the allo-determinants were still unknown; however, it was already clear that histocompatibility antigens were allocated to different loci and that the potential presence of different alleles would make the selection of donor–recipient pairs difficult, especially in unrelated animals (35–37). In the following years, several studies, especially on canine models, were vital for understanding the mechanisms related to histocompatibility. Histocompatibility was shown to be linked to a particular genomic region called the major histocompatibility complex (MHC).

Differences in the genes of the MHC region between donor and recipient are the major cause of T cell allo-activation and GvHD induction, but there are also other genes involved. The first observations of allo-antigens encoded outside the MHC complex came from the above-mentioned studies on BMT in dogs. In some instances, dogs developed GvHD when transplanted with MHC-matched bone marrow (37). Subsequent studies in the mouse provided more evidence on the involvement of non-MHC antigens (38–40), called minor histocompatibility antigens (41). GvHD induced through mHA was also T-cell mediated as demonstrated by a series of T-cell depletion experiments (42, 43), but the manifestation of the disease was delayed compared to classical GvHD.

Graft-versus-host disease can develop in two different forms that differ in pathogenesis, symptoms, and organ involvement. aGvHD affects up to 50% of the patients and accounts for 15% of post-transplantation mortality (44). Classically, acute GvHD (aGvHD) develops during the first 100 days after transplantation, but late acute aGvHD has also been described. Typical target tissues for aGvHD are the gastrointestinal tract, skin, and liver, but other atypical tissues include kidneys (45), salivary glands (46), oral epithelium (47, 48), and thymus (49). cGvHD develops later, and it occurs in ~50% of long-term survivors (50). Chronic GvHD (cGvHD) is associated with significant morbidity and mortality, and is still the leading cause of death in long-term survivors of HSCT (51). The organs involved are mainly skin, mouth, eye, and liver, and less frequently the gastrointestinal tract and lung. The pathogenesis of cGvHD is not clearly understood and the manifestations resemble more an autoimmune disease characterized by autoantibody production, chronic inflammation, and collagen deposition in target tissues.

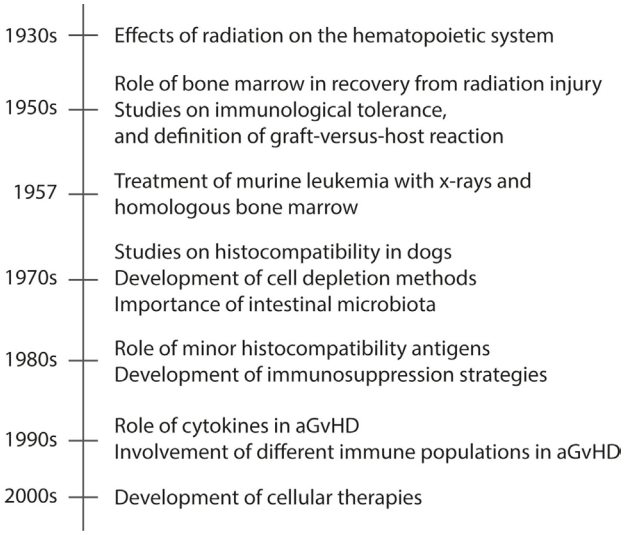

FIGURE 1 | A timeline presenting seminal events in animal models of aGvHD. Acute GvHD is caused by activated alloreactive donor T cells that directly cause tissue damage in target organs, such as skin and gut. The timeline shows the seminal findings in animal models that have led to the current understanding of aGvHD pathology.

ACUTE GvHD

Pathophysiology of Acute GvHD

Understanding the complexity of the process leading to aGvHD requires in-depth mechanistic studies to identify the involvement of the different components of the immune system. For this reason, a great deal of the knowledge on the pathophysiology of aGvHD is derived from animal models. In this section, we will review the seminal findings from animal models that have led to the current view of how aGvHD develops, which is acknowledged to progress through three phases: (i) activation of antigen-presenting cells (APCs), (ii) allo-activation of donor T cells, and (iii) tissue destruction by alloreactive T cells. A summary of these findings are found in **Figure 1**, while an overview of rodent aGvHD models is found in **Table 1**.

The Conditioning Regimens Lead to Activation of APCs

In the first phase, both the conditioning regimen and the underlying disease play central roles. Together, they create the tissue damage responsible for the production and the release of pro-inflammatory cytokines and chemokines that activate macrophages and APCs. Of particular importance is the damage to the intestinal epithelium caused by the conditioning regimens, and the subsequent release of microbial products, such as lipopolysaccharide (LPS) by the resident gut bacteria (82–84). After HSCT, we face the uncommon situation in which APCs from both host and donors are present. Using mouse recipients whose APCs were unable to cross-present class I restricted peptides, Shlomchik and colleagues demonstrated how host, rather than donor APC, are presenting allo-antigens to donor T cells (85). APCs are activated by many signals released during this early phase of inflammation, where cytokines, such as TNF-α, IL-1, and IL-6, are central. These cytokines, apart from activating APCs, can also promote antigen presentation by non-professional APCs in the tissue and cause direct tissue inflammation that allows T cells to access their target tissues (86).

Total body irradiation (TBI) was the standard immunoablative procedure in the first years of BMT, and it is a widely used pre-conditioning method in animal models of HSCT. TBI is a very harsh procedure and causes significant damage to the fast-replicating tissues, such as skin and intestinal mucosa. Through research in murine and canine models, it was shown that the conditioning intensity and GvHD severity were directly

TABLE 1 | Overview of rodent models for acute GvHD.

Species	Model	MHC haplotype	Conditioning	MHC mismatch	Reference
Mouse	C57BL/6 → BALB/c	$H2^b \rightarrow H2^d$	TBI	Complete	(27, 30, 52, 53)
	C3H/HeJ → C57BL/6	$H2^k \rightarrow H2^b$			
	C57BL/6 → B10.BR	$H2^b \rightarrow H2^k$			
	C57BL/6 → B6C3F1	$H2^b \rightarrow H2^{k/b}$	TBI	Haploidentical	(54–58)
	C57BL/6 → B6D2F1	$H2^b \rightarrow H2^{b/d}$			
	C57BL/6 → B6AF1	$H2^b \rightarrow H2^{b/a}$			
	C57BL/6 → B6.C-$H2^{bm1}$	$H2^b \rightarrow H2^{bm1}$	TBI or none	MHC-I	(59, 60)
	C57BL/6 → B6.C-$H2^{bm12}$	$H2^b \rightarrow H2^{bm12}$		MHC-II	(59, 60)
	B10.D2 → DBA/2	$H2^d \rightarrow H2^d$	TBI	miHA	(42, 43, 61–63)
	B10.D2 → BALB/c	$H2^d \rightarrow H2^d$			
	B10 → BALB.b	$H2^b \rightarrow H2^b$			
	C57BL/6→ BALB.b	$H2^b \rightarrow H2^b$			
	DBA/2 → B10.D2	$H2^d \rightarrow H2^d$			
Rat	BN → LEW	$RT1^n \rightarrow RT1^l$	TBI or CYP or anti-CD25/ CD154/CTLA4 Ig	Complete	(45, 64–71)
	PVG → BN	$RT1^c \rightarrow RT1^n$			
	DA → LEW	$RT1^{av1} \rightarrow RT1^l$			
	LEW → BN	$RT1^l \rightarrow RT1^n$			
	Wistar Furth → LEW	$RT1^u \rightarrow RT1^l$			
	LEW.1AR1 → LEW.1AR2	$RT1A^a$, $RT1B/D^u$, $RT1C/E^u \rightarrow$ $RT1A^a$, $RT1B/D^a$, $RT1C/E^u$	TBI	MHC-II	(72)
	BN → (BN × LEW) F_1	$RT1^n \rightarrow RT1^{n/l}$	TBI or none	Haploidentical	(47, 73)
	LEW → (LEW × BN) F_1	$RT1^l \rightarrow RT1^{n/l}$			(48, 74–81)
	LEW → (LEW × DA) F_1	$RT1^l \rightarrow RT1^{l/av1}$			
	PVG → (PVG × DA) F_1	$RT1^c \rightarrow RT1^{c/av1}$			

correlated (83, 87, 88). Therefore, the development of milder conditioning regimens with less damage of the gut were rapidly developed in animal models and then brought to the clinic. At first, canine models showed that reduced intensity conditioning (RIC) led to graft rejection, but introduction of immunosuppression protocols post transplantation led to successful engraftment and reduced GvHD (87).

The role of the gut microbiota in the development of GvHD was first described in the early 1970s when experiments using germ-free mice showed that elimination of the gut microbiota reduced symptoms and mortality related to aGvHD (89, 90). In the following years, gut decontamination using broad-spectrum antibiotics was applied in clinical BMT. Results from clinical trials gave contrasting results and ultimately showed no increase in survival (91, 92). The reason is that the use of broad-spectrum antibiotics for gut decontamination does not take into consideration that the mutualistic relationship between patient and microbiota can be also protective in some instances. In more recent years, studies focusing on the composition of the microbiota have shown how the abundance of some bacterial species over others can protect or promote aGvHD. In particular, immunosuppressive treatments and aGvHD lead to loss in microbiota diversity, and the prevalence of members from the *Enterobacteriales* and *Enterococcus* order together with a loss in *Clostridiales* bacteria can promote aGvHD (93). The loss of *Clostridiae* species has important functional consequences since this population is thought to be an important promoter of regulatory T cell (Treg) proliferation and activity (94). The re-establishment of gut microbiota diversity through the introduction of probiotic therapy has been successful in reducing experimental aGvHD in mice (95).

Alloreactive T Cells Are Activated in Secondary Lymphoid Tissues

During the second phase, host APCs cross-present host auto-antigens to donor T cells, which will be activated and start proliferating. The interaction between APCs and T cells is further enhanced by cytokines produced in the first phase (96). Furthermore, co-stimulatory molecules, including CD80 and CD86 expressed by APCs and CD28 expressed on T cells, give the classical second signal required for full T cell activation. Their expression is upregulated by the ongoing inflammation. The secondary lymphoid tissues of the gut are thought to be the primary site of T cell activation, as shown by experiments demonstrating failure to develop aGVHD in mice lacking Peyer's patches (PP) or where donor T cells lack the ability to migrate into PP (97).

The complex heterogeneity of T cell populations in humans makes it difficult to study the specific role of each subset, and how they may either promote or suppress aGvHD. Animal models are and have been essential for in-depth studies of the function of different T cell populations. For example, involvement of naïve rather than memory T cells in aGvHD has been investigated in mouse models. Several studies have shown how the transfer of purified effector memory $CD44^+CD62L^-$ T cells did not induce GvHD while retaining a GvL effect (98, 99).

$CD4^+$ T helper (Th) cells can differentiate into diverse subsets depending on the cytokines and microenvironment they are exposed to, and different Th subsets may be involved in aGvHD pathogenesis in distinct organs (100): Th1 cells, producing IFN-γ, IL-2, and TNF-α, are mostly involved in the pathogenesis of gastrointestinal GvHD (101), while Th17 cells, producing IL-17A, IL-17F, IL-21, and IL-22, are thought to be the major pathogenic subset in skin GvHD (102). Only the simultaneous depletion of

both these T helper populations is effective in controlling GvHD in mouse models (103).

The role of B cells in aGvHD is still controversial and under investigation. Host B cells have been shown, in mouse models, to be induced by TBI to produce IL-10 and contribute to reduce aGvHD occurrence (104). In previous studies in the rat, Renkonen and colleagues showed that, in lymphoid organs, there is increased B cell activation, proliferation, and antibody production early after BMT before the appearance of aGvHD symptoms. At later stages, the number of B cells decreased in the lymphoid compartment, but remained at high levels in the liver, suggesting a pathogenic role at least in this organ (105).

Alloreactive T Cells Migrate to Target Organs and Mediate Tissue Destruction

The third and last phase of aGvHD pathophysiology is the effector phase with migration of lymphocytes to their target tissues as one of the key steps. Chemokines and chemokine receptors specifically guide T cells in this process [reviewed in Ref. (106)]. CCR5 seems to have a broad effect as it has been described to mediate the recruitment of effector T cells, as well as Tregs, to many different target organs (97, 107, 108). In gastrointestinal aGvHD CXCR3 (109), CX_3CL1 (110), and CCR6 (111) has been shown to play additional important roles. Blocking the interaction between chemokine receptors and their ligands is one of the therapeutic strategies that are currently under investigation. Once T cells reach their target site, the tissue destruction occurs by direct induction of apoptosis mediated by TNF-α and IL-1 (112), and/or by killing mediated by cytotoxic $CD8^+$ T cells through perforin/granzyme and Fas–FasL interactions. The suppression of $CD8^+$ T cell function is crucial in the control of aGvHD (113).

The effector mechanisms in aGvHD have been studied in several mouse models. Donor spleen cells lacking both perforin and FasL failed at inducing aGvHD (114). Using different genetic combinations of donor and host mice, Graubert and colleagues showed that the perforin/granzyme pathway is mostly involved in MHC class I restricted aGvHD, while the Fas–FasL interaction is involved in MHC class II restricted aGvHD (115). A more recent study showed that $CD8^+$ T cells deficient for both perforin and FasL can still induce aGvHD in a donor–recipient combination that differs at a single MHC class I antigen. In this model the serum levels of IFN-γ and TNF-α were increased, and $CD8^+$ T cells showed increased activation and proliferation. The authors concluded that both perforin and FasL are important during the contraction phase, and can contain the expansion of $CD8^+$ T cells (116). T cell expressed TNF-related apoptosis-inducing ligand (TRAIL) induces pro-apoptotic signals upon binding to the TRAIL receptor on target cells, and is a commonly used killing pathway. Interestingly, this pathway has not been involved in tissue destruction in aGvHD, but it mediates anti-tumor responses. Murine T cells overexpressing TRAIL have been shown to suppress GvHD by inducing apoptosis of alloreactive T cells and mediating anti-lymphoma responses. The mechanism of action is thought to be through the interaction of the $TRAIL^+$ T cells with host APCs bearing the TRAIL receptor DR5, but also fratricide of alloreactive T cells (117).

Treatment of aGvHD

The current standard treatment for aGvHD is the use of steroids in combination with calcineurin inhibitors. This treatment induces general immunosuppression, but has side effects. In addition, many patients with aGvHD are resistant to this treatment. There is, therefore, a need to improve treatments and to target specifically aGvHD, without affecting GvL. The complex pathogenic mechanisms described in the previous sections offers a variety of pathways as potential targets for new therapeutic protocols. Also in the development of treatments for aGvHD, animal models have been and will be extremely important, although the translation from the pre-clinical to clinical setting is not always straight forward as human pathology is more complex due to many varying environmental factors as we will discuss in more detail below. Nevertheless, the possibility of studying the mechanisms involved in the efficacy of different treatments in animal models is instrumental for designing safe and effective protocols in humans. We will review some treatment strategies, where the use of animal models has been essential.

Immunosuppression

Canine models have been essential for testing post-transplantation immunosuppressive therapies to ameliorate GvHD. Together with immunohistocompatibility matching and T cell depletion, the use of immunosuppressive drugs in the post-transplantation phase represents one of the major advances for GvHD-free BMT. This is especially the case for partial MHC-mismatched transplantation or for non-myeloablative RIC regimens prior to transplantation. Methotrexate (MTX) is an immunosuppressive drug that targets the production of folic acid, which is essential for the synthesis of nucleic acids and proteins. MTX was first tested in dogs and proved to be effective at reducing GvHD occurrence (118, 119). A few years later, the discovery of calcineurin inhibitors (e.g., cyclosporine A and tacrolimus) greatly improved the prophylaxis protocols. When used early after transplantation, these drugs, alone or in combination with MTX successfully reduced GvHD occurrence in animal models (64, 74, 75, 120–122), and were soon after introduced to the clinic, where the combined use of MTX and cyclosporine showed an advantage over cyclosporine alone (123, 124). Although protocols vary between clinical centers, a combination of calcineurin inhibitors, MTX, and antithymocyte globuline (125) is still the gold standard (126).

Targeting of Cytokines

TNF-α is one of the most important cytokines involved in the pathogenesis of aGvHD, implicated in many steps during the disease progression. The importance of this cytokine in aGvHD was first described in a mouse model (127). Since then several studies have shown how neutralization of TNF-α can lead to reduced symptoms of aGvHD, and different means to target TNF-α and its receptor interaction either post- (128) or pre-transplantation are currently being explored.

IL-6 has a broad effect, activating many different immune cells. It has been associated with various inflammatory diseases, and has a predominant role in the early phases of aGvHD. Mouse studies have shown that IL-6 and its receptor (IL-6R) are upregulated during aGvHD (129), and that the addition of exogenous IL-6 can exacerbate the disease (130). Blockade of IL-6R were shown to reduce GvHD without affecting GvL (129, 131). Interestingly, mice treated with an anti-IL6R antibody also showed increased Treg reconstitution, which can effectively contribute to the reduction of aGvHD (131). A recently FDA-approved monoclonal anti-IL-6R antibody (Tocilizumab) has been shown to have beneficial effects in the treatment of steroid-refractory aGvHD (132).

The classical role attributed to IL-2 is to stimulate T cell proliferation. After many years of both experimental and clinical research, it is now clear that IL-2 has a more broad effect, including the maintenance of Treg homeostasis. Due to its effect on this regulatory population, IL-2 has been investigated as a therapeutic agent for treatment or prophylaxis of aGvHD. Administration of low doses of IL-2 alone has produced contrasting results in mouse models showing either beneficial (133) or no effects (134), but co-administration of rapamycin has been beneficial. Rapamycin targets conventional T cell signaling by blocking mTOR signaling. As Tregs use different signaling pathways, they are insensitive to rapamycin. In the presence of rapamycin, Treg do not compete with conventional T cells for IL-2 and this leads to their expansion (135).

The role of IL-18 and IL-22 in aGvHD pathogenesis is more controversial. ILC3 is a subset of innate lymphoid cells involved in maintaining gut homeostasis by producing IL-22, and is suggested to play a role in aGvHD pathogenesis. IL-22 is a cytokine with both protective and inflammatory functions, most likely depending on the microenvironment and the cell types involved (136). IL-22 produced by ILC3 targets epithelial cells, and regulates the production of anti-microbial factors, which are important in controlling the epithelial barrier function (137). In a mouse model, IL-22 depletion or deficiency in the host was shown to increase aGvHD severity, and ILC3 and IL-22 were suggested to protect the intestinal stem cell pool and epithelial barrier function during inflammation (138). Interestingly, in another mouse model, donor-derived IL-22 was shown to have the opposite effect and contribute to the severity of GvHD by promoting Th1 cell infiltration in presence of IFN-α (139).

Targeting of Chemokines and Chemokine Receptors

Blocking chemokine–chemokine receptor interaction is another logical therapeutic strategy that has been tested using animal models. Administration of anti-CXCR3 (140) or anti-CX_3CL1 (110) antibodies in mouse models of aGvHD were shown to reduce gastrointestinal aGvHD. CCR5 is involved in migration of lymphocytes to several target tissue of aGvHD, and for this reason, it appears as an interesting target molecule. However, targeting CCR5 has given contrasting results as this chemokine is thought to be involved also in Treg recruitment to peripheral tissues (141).

Another interesting approach to treat aGvHD has been to take advantage of the upregulation of CXCL10 (ligand for CXCR3) observed in target tissues during disease. By injecting CXCR3-transfected Tregs, Hasegawa and colleagues showed specific migration of these cells to the target organs and subsequent reduction in aGvHD severity (142). Despite the encouraging results in animal models, it is important to keep in mind that the chemokine system is redundant, and blocking a single interaction does not always directly translate to a milder GvHD phenotype. For this reason, the use of agents with a broader effect that target more than one pathway has been tested. Among these, the broad-spectrum chemokine inhibitor NR58-3.14.3 has been successfully proved to reduce murine aGvHD especially in lung and liver (143).

Targeting of Co-stimulatory Molecules

Engagement of co-stimulatory molecules is necessary for full activation of T cells, and blocking these molecules has interesting potentials. Studies in animal models showed that anti-CD80 and anti-CD86 inhibited T cell expansion, and that mice treated with these antibodies experienced milder symptoms of aGvHD. Moreover, T cells isolated from CD28-deficient mice caused less severe GvHD (144, 145). Other studies have focused on targeting the CD40–CD40L pathway. Also in this case, the use of anti-CD40L antibodies reduced the severity of GvHD, which is thought to induce a selective depletion of activated T cells, and at the same time to induce Tregs (146–148). Along the same lines, the OX40–OX40L interactions are important in the pathogenesis of GvHD. T cells from rats with aGvHD upregulate OX40 (149), and administration of blocking antibodies against OX40L reduced aGvHD mortality in a mouse model (150). Other co-stimulatory pathways have been investigated in animals. Blockade of all of the following pathways have shown potential beneficial effects on aGvHD severity: 4-1BB/4-1BBL (151), ICOS/ICOS-L (152), LIGHT/HVEM (153), NKG2D-NKG2D-L (154), DNAM-1/DNAM-1-L (155), and the CD30/CD30L (156) pathways. However, co-stimulatory molecules are also important for the GvL effect, and blocking these molecules may severely compromise the GvL effect and, therefore, their clinical use may be limited. Nevertheless, experimental models suggest that not all molecules are equally involved in GvHD and GvL, and a better understanding of the importance of different co-stimulatory molecules for either GvHD or GvL may help identify new targets that can reduce GvHD while maintaining GvL.

Cell Therapy

Over the last 15 years, focus has been put on the use of cells with immunosuppressive functions to regulate aGvHD, in particular mesenchymal stem cells (MSCs) and Tregs (157). MSCs are found at very low frequencies in the BM and other organs, such as adipose tissue, placenta, and amniotic fluid, and have the potential to differentiate into adipocytes, chondrocytes, myocytes, and osteoblasts. MSC support hematopoiesis in the BM (158), and contribute to embryo implantation by promoting trophoblast invasion in the placenta (159). MSC also have immunosuppressive functions which, together with the ease at expanding them *ex vivo*, have made them promising candidates for immunotherapy for aGvHD [reviewed in Ref. (160)]. Despite the initial success in the treatment of steroid-refractory aGvHD (157, 161), MSC therapy has failed to give consistent results

and animal studies also show contrasting outcomes (65, 66, 76, 162–167). Clinical trials testing the efficacy of MSC in the treatment of GvHD started before thorough investigation in preclinical models was completed (168). The precise mode of action of MSC on the immune system is not well understood, and these cells seem to acquire different functions according to the environment they are exposed to (169, 170). A better understanding of the biology of MSC, together with improved and standardized techniques for their isolation, characterization, and expansion may allow development of improved methods for their use in aGvHD prophylaxis or treatment. Tregs are a subset of $CD4^+$ T cells that represent 5–10% of the total T cell pool in human and rodents (171–173). Tregs express high levels of CD25 and the transcription factor FOXP3, which is necessary and sufficient for their immunosuppressive activity (174). The functional and phenotypical properties of Tregs are conserved in human and rodent species, making animal studies particularly relevant for applications in humans. Treg have immunosuppressive properties, and they are fundamental to induce and maintain peripheral self-tolerance, protecting from aberrant immune responses that can lead to excessive inflammation and autoimmunity. Earlier animal experiments showed that depletion of Treg from the BM graft resulted in severe aGvHD, with mice dying by day 21 after transplantation compared to day 41 in non-depleted transplantations (175). Moreover, addition of donor Treg to the graft at 1:1 ratio with conventional T cells was shown to delay or prevent aGvHD (175, 176). In order to exert their effects, Tregs must migrate to the secondary lymphoid tissues where alloreactive T cells are activated. For this reason, only the $CD62L^+$ and not the $CD62L^-$ population of Tregs have been shown to protect from lethal aGvHD (177, 178).

Regulatory T cells are categorized into two groups, both important for controlling peripheral tolerance: naturally occurring Treg (nTreg) that develop in the thymus and induced Treg (iTreg) that differentiate from conventional T cells in response to TGF-β and IL-2. The second group is therapeutically interesting because iTreg can be generated *in vitro* from conventional T cells (179), and they can be expanded to therapeutically sufficient amounts. Unfortunately, animal studies have shown that this approach does not lead to any protection from aGvHD. The main reason is that iTreg are unstable *in vivo*, and upon transfer they can lose the expression of FOXP3, together with their immunosuppressive activity (180, 181). One of the hypotheses to explain this instability is that the inflammatory environment of aGvHD can induce the conversion of iTreg back to conventional T cells. In favor of this hypothesis are studies showing how blocking inflammatory cytokines, in particular STAT3-dependent cytokines, can improve the iTreg stability (182, 183). The nTreg represent, therefore, a potentially more effective therapeutic tool, but their low frequency in the periphery requires optimization of *ex vivo* or *in vivo* expansion protocols.

Natural killer cells are another therapeutically interesting cell population in context of aGvHD and GvL. Earlier studies in rodent models demonstrated that NK cells are important for successful engraftment after BMT. NK cells are particularly radioresistant and can mediate rejection of allogeneic cells (184–186). The presence of residual NK cells after immune ablation can play a role in the acceptance or rejection of the allogenic graft. Studies in rats showed that differences in both the classical and non-classical MHC class I genes can contribute to NK-cell mediated rejection (187, 188). On the other hand, the infusion of alloreactive NK cells, together with a reduced TBI in a haploidentical transplantation mouse model, caused eradication of leukemia and depletion of the residual host hematopoietic system, thus facilitating the engraftment of donor BM cells. The additional NK-cell mediated killing of host APC prevents activation of alloreactive T cells and, therefore, no aGvHD (189). The use of NK cells to cause a GvL effect is restricted to those combinations of donor–recipient in which NK cell alloreactivity can be fully exploited (KIR–MHC mismatch). Moreover, not all types of tumor cells have the same sensitivity to NK cells due to variable expression of ligands for activating and inhibitory receptors. Additional stimulation of NK cells with cytokines might be required in order to accomplish an effective and long-lasting GvL effect for the NK-cell resistant tumors. A recent study demonstrated how NK cells pre-activated with a combination of IL-12, IL-15, and IL-18 reduced aGvHD while retaining the GvL effect in a fully mismatched BMT mouse model. Injected NK cells retained their activated phenotype and exerted their immunosuppressive activity by inhibiting alloreactive T cell proliferation (190).

CHRONIC GvHD

Pathophysiology of Chronic GvHD

Chronic GvHD in the clinic was initially defined as any symptoms of GvHD that occurred more than 100 days after transplantation, but it became increasingly clear that this definition was inadequate. Due to the heterogeneity of the clinical manifestations of cGvHD, cGvHD was only properly defined a decade ago with the NIH Consensus Project on cGvHD, and cGvHD is now classified as a disease distinct from aGvHD (191, 192). Both aGvHD and cGvHD arise as a complication of allo-HSCT transplantation, but with different pathology and underlying disease-driving mechanisms. Hallmarks of cGvHD in the clinical setting are systemic fibrosis, chronic inflammation, sclerodermatous manifestations, and autoantibody production. These features are similar to several autoimmune diseases; yet do not fully mimic any particular autoimmune disease, being an entity on its own. However, due to the pathological similarities between cGvHD and autoimmune diseases, there has been a close synergy between the two fields; the difference being that cGvHD is mediated by a foreign donor lymphoid graft.

Therapies directed at ameliorating cGvHD have improved little over the past decades. The reason is incomplete knowledge of the underlying mechanisms that drive the disease. This has been mainly due to lack of animal models that completely recapitulate the full clinical heterogeneity of cGvHD. For more than three decades after cGvHD was acknowledged in the clinic, the best described and most utilized animal models for cGvHD addressed only one or a few of the many clinical manifestations of cGvHD, principally autoantibody generation or sclerodermatous disease (193) (**Table 2**). The clinical relevance of these animal models was a concern, as they did not fully mimic the clinical setting in terms of composition of the donor graft, preparative regimens,

TABLE 2 | Overview of rodent models for chronic GvHD.

Species	Model	Conditioning	Manifestation	Reference
Mouse	C57BL/6 → B10.BR	Cy i.p./TBI	Bronchiolitis obliterans	(194)
	DBA/2 → BALB/c	TBI	Scleroderm.	(195)
	B10.D2 → DBA/2 × B10.D2 F1	TBI	Scleroderm.	(186)
	B10.D2 → BALB/c	TBI	Scleroderm.	(188, 189)
	C57BL/6 → BALB/c[a]	TBI	Scleroderm.	(190)
	BALB/c → BALB/c × A/Jax F1	None	SLE	(185)
	DBA/2 → DBA/2 × C57BL/6 F1	None	SLE	(183)
	CBA → CBA × A F1	None	SLE	(193)
	C57BL/6 → C57BL/6 × BALB/c F1	None	SLE	(193, 196)
	B6 → B6 × bm12 F1F1	None	SLE	(197)
Rat	LEW → SD	SD neonates tolerized with LEW lymphoid cells	Fibrosis	(184)

[a]*Low dose donor spleen cells prerequisite.*

post-transplantation immune suppression, and the diverse human genetic background. Still, the animal models were instrumental for investigating subpopulations of donor and host leukocytes in the pathogenesis of cGVHD. In **Figure 2**, we have highlighted seminal findings from animal models. Moreover, during the last decade, improved models were developed that incorporate more of the features of clinical cGvHD, and these have led to further advances in our understanding of the underlying mechanism of cGvHD pathology.

Lupus-Like and Scleroderma-Like Animal Models of cGvHD

Chronic GvHD as a complication after allo-HSCT in the clinic was first acknowledged during the 1970s, with reports on autoimmune-like symptoms developing in patients several months after BMT. An autoimmune form of GvHD was described more than a decade earlier in experimental mouse and rat models. In 1961, Oliner, Schwartz, and Dameshek reported a form of GvHD ("runt disease") in a parent to F1 hybrid transplantation model with autoimmune characteristics similar to systemic lupus erythematosus (SLE) (197). Two years later, Stastny, Stembridge, and Ziff reported in the rat a chronic form of GvHD (termed "homologous disease") with features of sclerosing skin lesions similar to scleroderma (198). These works were followed by studies arguing that the "runting" syndrome of acute allogeneic disease must be separated from chronic allogeneic disease, the latter with symptoms manifesting at a later time point (199). It was suggested that, as for acute allogeneic disease, the chronic form was evoked by an immunological reaction of the donor against host antigens, although the exact mechanisms was not pinpointed at the time.

Throughout the 1970s and 1980s, the SLE-like and the scleroderma-like mouse models of cGvHD were the dominant animal models for cGvHD. These models were also extensively used for studies of autoimmune diseases. The SLE-like models generally involved transfers of lymphoid cells from a parental strain into non-irradiated F1 hybrids [e.g., BALB/c to BALB/c × A/Jax F1 (199) or DBA/2 to C57BL/6 × DBA/2 F1 (197)], resulting in transient or mixed chimerism. The main manifestation in these models is generation of autoantibodies, while skin pathology is less common. The relevance of these models has been questioned, mainly due to absence of bone marrow derived stem cells in the donor inoculum and absence of host immuno-depletion prior to transplantation.

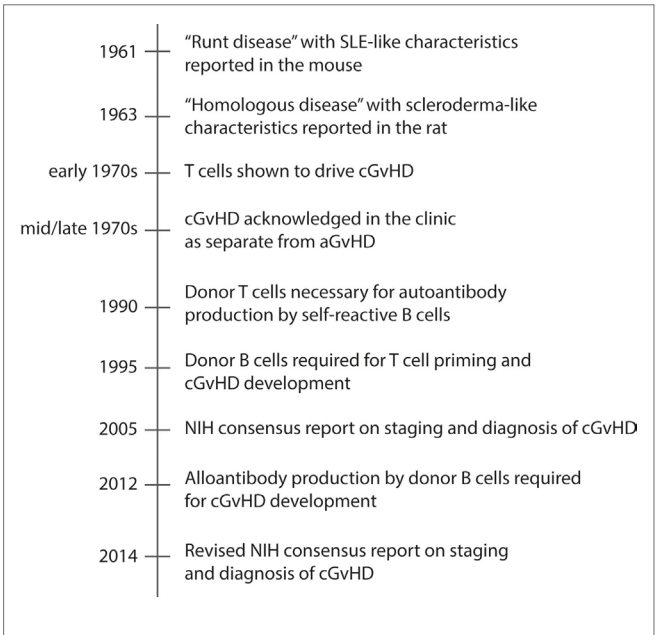

FIGURE 2 | Timeline of major events in cGvHD research using animal models. Chronic GvHD is characterized by autoantibody production and deposition in target tissues, and tissue fibrosis. The timeline presents how animal models have contributed to increased understanding of the pathology of cGvHD, and included is also the NIH consensus reports on staging and diagnosis of cGvHD, which has contributed to development of improved rodent models of cGvHD during the last decade.

Scleroderma-like mouse or rat models involves transplantation of major or minor MHC-matched or mismatched bone marrow into sub-lethally irradiated recipients, resulting in full donor chimerism (200–203). Here, the main manifestations are fibrotic changes in the skin, liver, lung, and salivary glands, while autoantibodies are less common. The scleroderma-like model for cGvHD shares many symptoms with sclerodermatous clinical cGvHD. The incidence of sclerodermatous cGvHD among long-term survivors of allo-HCST is around 3–10%, but the incidence of sclerodermatous cGvHD in the clinic is expected to rise as increasing numbers of unrelated donor transplants are

performed, as well as the increased use of mobilized peripheral blood as stem cell source.

More recently, developed mouse models have better recapitulated human cGvHD. In these models, transplantation of MHC-mismatched T cell-depleted bone marrow together with a low dose donor lymphocytes leads to cGvHD (196). Fibrosis of the skin, salivary gland damage, and serum autoantibodies are observed. Similarly, a mouse model developed in Blazar's laboratory with cyclophosphamide and lethal TBI pre-conditioning followed by allo-BM transplantation and low dose alloreactive T cell infusion, showed cGvHD manifestations in a wide range of cGvHD target organs (52). These models will likely significantly advance our understanding of the underlying immune reactions.

Donor-Derived CD4$^+$ T Cells as Initiators of cGvHD

It was earlier shown that T cells play a central part for autoimmune development by collaborating with B cells for autoantibody generation (204). Fialkow and colleagues suggested that host CD4$^+$ T cells were the drivers of autoantibody production, and that cGvHD was a purely host-derived, but graft-initiated, disease (205). However, it was soon clear that donor-derived CD4$^+$ T cells were the real initiators of the disease, although the antigens recognized by the host-reactive donor T cells were not clear. Several pieces of evidence showed that naïve donor-derived CD4$^+$ T cells were central for inducing cGvHD pathology, e.g., (i) when unfractionated lymph node cells or splenocytes were adoptively transferred into non-irradiated F1 hybrid hosts containing a mutated allele in MHC class I (B6 × bm1), a milder form of cGvHD was observed compared to transfer into F1 hybrid hosts with mutated MHC class II allele (B6 × bm12) (59), (ii) transfer of alloreactive donor CD4$^+$ T cells obtained from mice with aGvHD to lethally irradiated secondary hosts led to cGvHD (206), and (iii) mature donor-derived CD4$^+$ T cells were shown to cause both alloreactive and autoreactive responses using a DBA/2 to BALB/c cGVHD model (207). On the other hand, CD8$^+$ T cells and the pool of CD4$^+$ effector/memory T cells were found insufficient for inducing cGvHD (98, 208–211). Furthermore, depletion of CD8$^+$ T cells from the graft, but not CD4$^+$ T cells, led to autoantibody production. Later, a correlation was made between low CD8$^+$ T cell numbers with cGvHD severity in several parents into F1 hybrid models (212). Thus, there is a notion that the frequencies of donor alloreactive CD8$^+$ T cells may determine whether aGvHD or cGvHD develops. For example, CD8$^+$ T cell anergy can shift the responses from an aGvHD to an SLE-like cGvHD (194, 213). Although CD8$^+$ T cells are not necessary to induce cGvHD, they infiltrate skin and intestines where they contribute to the observed pathology (214).

B Cells as Autoantibody Producers and APCs

In contrast to aGvHD, B cells have a clear role in evoking cGvHD pathology. Although it was presumed that donor helper T cells were needed for production of autoantibodies by B cells, this was not directly shown until 1990, when Eisenberg's group utilizing a mouse model of SLE demonstrated that autoantibody production by self-reactive host B cells, and not donor-derived B cells, was directly induced by donor-derived helper T cells (195, 215). The importance of B cells for inducing cGVHD pathology was subsequently shown by several investigators in SLE-like mouse models, by either blocking co-stimulatory molecules, such as CD40L and CTLA4, important for B-cell crosstalk (146, 216). Furthermore, B cell persistence, obtained by transferring perforin-deficient T cells from an aGvHD model (B6 into B6xDBA/2 F1 hybrids), resulted in a shift to cGVHD symptoms resembling SLE-like cGvHD (217). Later, in a mouse model of RIC, persistence of host B cells was associated with cGvHD lesions and autoantibodies of host origin (218). It was also shown that patients with extensive cGvHD had faster B cell recovery and detectable autoantibodies after allo-HSCT (219). Patients with severe cGvHD also have elevated levels of soluble B-cell activating factor (BAFF), which is evidence for activated B cells (220). Elevated BAFF serum levels were also associated with higher circulating levels of pre-germinal center (221) B cells and post-GC plasmablasts (222). Blockade of germinal centers with lymphotoxin-receptor Ig-fusion proteins was shown to suppress cGvHD, further demonstrating the involvement of mature, activated B cells (223). Interestingly, transplantation of bone marrow incapable of secreting allo-antibodies resulted in less severe cGvHD, demonstrating a role for both auto- and allo-antibodies in cGvHD pathology (223).

In addition to their role as producers of autoantibodies, B cells are potent APCs that stimulate donor T cells to further propagate the cycle that leads to cGvHD. Priming of donor T cells to mHA and subsequent cGvHD development was shown to depend on B cells as APCs (224). Almost a decade later, it was shown for the first time in a clinical setting, that a coordinated B and T cell response to a mHA, with donor B cells mediating the specificity, could be mounted in a setting of cGVHD (225). Further experiments in the mouse demonstrated that donor B cells promoted clonal expansion of autoreactive CD4$^+$ T cells, their differentiation to the Th2 subset, and prolonged survival. In fact, these T cells mediate cGvHD when transferred into secondary recipients (226).

Mouse Models Suggest That cGvHD Is a Th2-Driven Disease

It has been debated whether cGvHD is primarily a Th1 or a Th2-driven disease. Most mouse models suggest that cGvHD is a Th2-driven disease. In the SLE-model, expansion of recipient B cells leading to lymphadenopathy, splenomegaly, and autoantibody production are observed. With this model, Th2 cytokines were shown to stimulate secretion of fibrosis-inducing cytokines (e.g., IL-13 and TGF-β) resulting in sclerodermatous disease (208, 209). When the cytokine balance was manipulated toward a Th1 type, a shift of symptoms to more aGvHD-like pathology was observed (227, 228). Furthermore, increased B cell activity was linked to increased levels of the Th2 cytokines IL-4 and IL-10, with concomitant suppression of IL-2 and IFN-γ by T cells isolated from animals with cGvHD (229). Confirming these observations, were clinical studies showing that a lack of Th1 responses led to early-onset cGvHD, and conversely, an early Th1 response with high IFN-γ production was associated with less cGvHD (230). These observations were later confirmed in

mouse models, demonstrating lower incidence of cGvHD in the presence of donor T cells producing high levels of IFN-γ (221).

Involvement of Thymic Dysfunction for cGvHD Development

The thymus has a central role for both T cell development and for induction of T cell tolerance toward self antigens. Autoreactive T cells are negatively selected in this process. This is illustrated by studies of thymectomized neonatal mice that spontaneously develop multi-organ autoimmune disease (231, 232). Therefore, autoreactive T cells in context of cGvHD could result from defective tolerance induction due to thymic damage as a consequence of pre-conditioning or immune-mediated damage.

A mouse model of thymic dysfunction, where lethally irradiated hosts (C3H/HeN) receive T cell-depleted bone marrow from MHC-mismatched, MHC class II deficient donors (C57BL/6) represent a model where impaired negative selection occurs as a consequence of lack of MHC class II expression by thymic dendritic cells. In this model, many features of clinical cGvHD are observed, including sclerodermatous skin disease, weight loss, fibrosis, inflammation, and immune cell infiltration of salivary glands, while autoantibody generation is not reported (233). A weakness of this model is the fact that host thymic medullary epithelium cells also mediate negative selection. In addition, in this model thymic function is constitutively impaired by lack of MHC class II molecules, and does not address whether there is a temporal window of thymic damage where impaired negative selection occurs. In another model, where sub-lethal irradiation of BALB/c was performed prior to transfer of MHC-matched, mHA-mismatched DBA/2 bone marrow, donor T cells caused lesions characteristic of cGvHD when transferred to secondary allogeneic recipients. These cells were shown to be thymopoiesis dependent, and the authors, thus, concluded that T cells generated in the thymus were responsible for cGvHD development (206). Of note, a previous study using the same animal model could not demonstrate thymic dependence for cGvHD development (234).

A number of other mouse models points against a role for the thymus in the induction of cGvHD, as none of the murine models involving genetically unmodified mice has provided any evidence of impaired negative selection. In particular, no adversities of the thymic architecture or T cell development has been observed in the well-described SLE or Scleroderma-models described in the previous sections. Moreover, transfer of DBA/2 splenocytes and bone marrow to thymectomized BALB/c hosts did not change the incidence or the severity of cGvHD compared to mice with intact thymus (235). However, recent years' research has indicated that alloreactive donor $CD8^+$ T cells may damage thymic epithelial cells, leading to generation of autoreactive T cells (196, 236). The resulting autoreactive T cells were demonstrated to interact with donor B cells resulting in autoantibody production (196). Although recently developed mouse models strongly suggest that dysfunctional thymic negative selection is important for cGvHD pathogenesis, a role for the thymus in human cGvHD pathology is not clear. In addition, one must bear in mind that the thymus involutes by age, and older patients are not likely to have abundant functional thymic tissue.

Treatment of cGvHD

Current treatment of cGVHD is largely based on immunosuppressive steroids, but development of more targeted therapies to replace or to treat steroid-refractory cGvHD are currently tested in pre-clinical animal models and several have now entered clinical trials.

Inhibition of Fibrosis

Platelet-derived growth factor (PDGF) and TGF-β are both pro-fibrotic cytokines inducing fibroblast activation. cGvHD patients are shown to have elevated levels of circulating, stimulating autoantibodies toward PDGFRα. PDGFR signaling leads to enhanced reactive oxygen species generation and subsequent collagen synthesis and deposition. Mouse cGvHD models were instrumental for developing Imatinib, a tyrosine kinase inhibitor that targets PDGFRα (41), and also anti-TGF-β treatment was shown to prevent skin and lung fibrosis (237). Imatinib has shown promising results in clinical trials of steroid-refractory cGvHD patients (238, 239). An enhanced effect was observed by simultaneous targeting of both PDGFRα- and TGF-β signaling pathways using Imatinib and Nilotinib, the latter targeting c-Abl in the intracellular pathway induced by TGF-β (240).

Targeting of B Cells

As donor-derived B cells are central auto- and allo-antibody producers, and significantly contribute to clonal expansion of donor-derived $CD4^+$ T cells, therapies have been directed at depleting B cells from the patients. The well-known B cell-depleting antibody Rituximab (anti-CD20) specifically targets B cells and has been used in the treatment of patients with refractory cGvHD, resulting in objective improvements of symptoms (241–243). However, the antibody rarely results in complete remission of cGvHD. It is also a concern that anti-CD20 antibodies poorly target germinal centers in lymph nodes, in contrast to efficient removal of B cells from peripheral blood (244).

Infusion or Induction of Tregs

As for aGvHD, the use of Tregs in therapy of cGvHD is being exploited in clinical trials, as cGvHD patients have reduced frequencies of Tregs similar to aGvHD patients (245, 246). In mouse models, it was shown that transfer of *ex vivo* expanded Tregs resulted in suppression of cGvHD (247), suggesting that they may be utilized to treat cGvHD. However, the required *ex vivo* expansion of Tregs to obtain sufficient numbers for transfer into patients is technically challenging, and may also be associated with changes in their functionalities as discussed above. Another strategy is the expansion of Treg *in vivo* by injecting low-dose subcutaneous IL-2 leading to increased Treg accumulation that has demonstrated reduced severity of cGvHD (248).

ADVANTAGES AND LIMITATIONS OF ANIMAL MODELS FOR GvHD

As outlined above, animal models have largely contributed to current GvHD prophylaxis and treatment protocols (249).

An overview of the most common animal models are found in **Tables 1** and **2**. Each model has advantages but also their limitations.

In the early days of GvHD research, canine models were important for studying the role of MHC disparities in GvHD (250), and the canine models substantially contributed to advance our understanding of the biological mechanisms at play in HSCT and GvHD. Among the many researchers in this field, Edward Donnal Thomas is often recognized as the father of clinical BMT, for which he earned the Nobel Prize of Medicine in 1990. In addition to his clinical work, he carried out intensive research in canine models of BMT and GvHD. Canine models are still used in studies pertaining to the effectiveness of cellular immunotherapy, such as the utility of an anti-CD28 antibody as therapy to prevent GvHD during allo-HSCT (251).

Although outbred animal models are sometimes required to better mimic several aspects of human HSCT and GvHD, the most preferred animal model in context of GvHD is currently the mouse (252). The advantages of mouse models are the (i) broad availability of transgenic and gene-deficient strains that provide mechanistic insights into the role of individual genes for GvHD (253), (ii) the presence of inbred strains that are well characterized for studying GvHD and GvL, (iii) the availability of many well-characterized reagents, and (iv) the relative low costs of breeding mice (254, 255).

Several well-characterized mouse models of both acute and cGvHD have been established, such as the full MHC class I mismatch C57BL/6 to BALB/c (256) or C3H/HeJ to C57BL/6 (30) for aGvHD, and B10.D2 to BALB/c for cGvHD. The mouse is a particular valuable model to determine the role of individual cell types, genes and factors that affect GvHD. Examples are transgenic mice that have a mutant MHC class I, e.g., B6.C-H2^{bm1} (bm1), or mutant MHC class II, e.g., B6.C-H2^{bm12} (bm12). Both the H2^{bm1} and H2^{bm12} models have been important in understanding the interaction of T cells with recipient and donor APCs (257). Humanized murine models are also interesting models for GvHD and GvL research (258). An example is the Hu-PBL-SCID model, which is based on the NOD-*scid* mice. In this model, HIV-1 envelope protein gp120 delayed GvHD development by activation of human Tregs (259). Similarly, GvHD development was delayed in the Hu-PBL-SCID model based on NOD-*scid IL2*^{null} mice following treatment with a soluble Fas ligand (260). On examination of the kinetics of engraftment and development of GvHD in the latter model, it was observed that mice deficient in MHC class I exhibited a delay in GvHD (261). However, it is difficult to select an appropriate model, as engraftment or the strength GvHD symptoms does not necessarily correlate with the pathophysiology of GvHD in humans (262).

Rats are also used for GvHD studies. Rats are genetically similar to mice, but they are larger in size, have a longer life span, and have more biomaterial that can be used for experiments (263). GvHD models in rats include MHC-mismatched strains between LEW and BN (264, 265), or between PVG and BN (67, 68). Rat models have been used to test immunomodulatory drugs such as Thalidomide (266) and MC1288, an analog for vitamin D (267) as therapeutic strategies for GvHD.

Conditioning prior to transplantation causes tissue damage and pro-inflammatory responses that affect the GvHD outcome (268, 269). Therefore, the timing of transplantation and conditioning regimens will significantly affect the experimental outcome (270). Conditioning regimens in murine models frequently involves TBI, in contrast to the clinical settings where patients are usually given chemotherapy, and where only a few patients are subjected to TBI (271). Sadeghi and colleagues developed a chemotherapy-based GvHD mouse model with busulfan and cyclophosphamide as the conditioning regimen. The mouse model was mismatched for both MHC and mHA [C57BL/6 (H2^{b}) to BALB/c (H2^{d})], and the allogeneic transplanted mice developed clinical and histological symptoms associated with GvHD, such as apoptosis and T cell infiltration into the target organs (272). This model represents a myeloablative-conditioning regimen, which is most commonly used in the clinic. Another mouse model involving the same chemotherapy as conditioning was described using MHC-matched, mHA-mismatched mice [LP/J (H2^{b}) – C57BL/6 (H2^{b})]. This model was developed to more closely mimic the clinical situation, where patients usually are MHC matched. With this model, similar T cell infiltration, GvHD-specific damage, and systemic inflammation were observed in the mice as reported in humans (273). Thus, animal models of selective mHA mismatch may represent human HSCT more closely than MHC-mismatched models (193).

Another important consideration is the fact that the immune cell compositions vary between species. In murine models, mice receive bone marrow and T cells from an allogeneic counterpart to induce severe aGvHD. The T cell expansion is mainly homogeneous in the inbred recipients, in contrast to the heterogeneous T cell response in humans (274). Furthermore, differences in the proportion of lymphocyte subsets (such as CD4^{+}, CD8^{+}, and Tregs) between species can influence pathophysiology of GvHD (193). In addition, the metabolism and pharmacology of animal models can be different and these differences between animal models and humans could explain why some of the findings in mice models have not been successfully translated into clinical trials. For example, IL-11 reduced transplant related mortality (TRM) and prevented GvHD while maintaining GvL effects in mice (275). By contrast, IL-11 included as GvHD prophylaxis caused multi-organ failure in a phase I/II double blinded, placebo-controlled trial for allo-HSCT (276). In another example, experiments in mice showed that GvHD was effectively prevented in animals by therapy with a monoclonal antibody against the IL-2 receptor (IL-2R) (277). However, the use of IL-2R antibodies in two separate clinical trials was only moderately successful in reducing the incidence of severe GvHD (278, 279).

An important difference between mouse and rat animal models and humans is the homogenous genetic composition of inbred rodents, in contrast to the heterogeneous humans (193). Furthermore, the genetic drift that occurs in inbred strains from a particular colony might affect the ability to reproduce data consistently between labs (280). Given that inbred strains are an artificial model, several different inbred strains and/or outbred animals should be used to better represent the genetic complexity in the human population. For this reason, canines are sometimes

preferred to study new regimens in prophylaxis and treatment of GvHD (281).

Moreover, there are important species differences that need to be taken into consideration when extrapolating results found in animal models to humans (282). Differences in the anatomy, physiology, microbiota, play an important role in GvHD pathology (193). In addition, age plays an important role in influencing the efficacy of immune reconstitution post-transplant, as well as susceptibility to GvHD (283). Non-human primates or canine models are better fit for long-term therapies, given their longer life span than rodents. Moreover, the effects of opportunistic infections that affect HSCT outcome that can be observed in humans are not modeled in rodents kept in SPF conditions.

An alternative to study GVHR is the use of the skin explant model. The skin explant model can closely mimic the *in vivo* mechanisms and pathology of human GvHD (284, 285). The skin explant assay for GvHD was initially tested as a method to predict incidence and severity of GvHD in humans (286), and we have previously shown that a rat skin explant assay for GvHD is useful to determine the severity of GvHD between different rat strains (287). Although *in vitro* studies can provide hypotheses and models for research, there is a strong need for testing and validation in an *in vivo* animal model. The important pathophysiological conditions and symptoms of GvHD have been successfully reproduced in a number of animal models (288), and animal models have been very useful in understanding various key mechanisms of GvHD and GvL. However, they still fail to fully compensate for the variable time of onset of the disease, the rate of progression, relapse of primary disease, and other important clinical variables attributed to GvHD pathology and HSC outcome (288). Till date, researchers have failed to create accurately an animal model encompassing all human parameters (289). Identifying suitable models for specific fields would be beneficial.

FUTURE PERSPECTIVES

Overall, substantial progress has been made using animal models to understand GvHD. However, major clinically relevant questions still remain unanswered. It is important to understand the mechanisms involved in the effect of RIC on late-onset aGvHD, for instance, or the mechanisms involved in steroid-resistant disease (290). In spite of distinctive similarities of GvHD pathology between different animal models and humans, the corollary question remains: Do animal models, in absence of immunosuppressive medications post transplantation, adequately simulate GvHD that occurs in humans (291). Designing interventions using animal models involving mimicry of the experience of the patient during their treatment in the clinic could be important. Larger animal models or non-primate humans could be used to investigate steroid resistance, secondary treatments, and also monitor these effects long term.

AUTHOR CONTRIBUTIONS

MB, PS, and MI wrote the manuscript; MB, PS, RD, and MI planned the contents; RD and MI reviewed the contents.

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REFERENCES

1. Jacobson LO, Simmons EL, Marks EK, Eldredge JH. Recovery from radiation injury. *Science* (1951) 113:510–1. doi:10.1126/science.113.2940.510
2. Jacobson LO, Simmons EL, Marks EK, Robson MJ, Bethard WF, Gaston EO. The role of the spleen in radiation injury and recovery. *J Lab Clin Med* (1950) 35:746–70.
3. Patt HM, Tyree EB, Straube RL, Smith DE. Cysteine protection against X irradiation. *Science* (1949) 110:213–4. doi:10.1126/science.110.2852.213
4. Congdon CC, Uphoff D, Lorenz E. Modification of acute irradiation injury in mice and guinea pigs by injection of bone marrow; a histopathologic study. *J Natl Cancer Inst* (1952) 13:73–107.
5. Lorenz E, Uphoff D, Reid TR, Shelton E. Modification of irradiation injury in mice and guinea pigs by bone marrow injections. *J Natl Cancer Inst* (1951) 12:197–201.
6. Jacobson LO. Evidence for a humoral factor (or factors) concerned in recovery from radiation injury: a review. *Cancer Res* (1952) 12:315–25.
7. Nowell PC, Cole LJ, Habermeyer JG, Roan PL. Growth and continued function of rat marrow cells in x-radiated mice. *Cancer Res* (1956) 16:258–61.
8. Ford CE, Hamerton JL, Barnes DW, Loutit JF. Cytological identification of radiation-chimaeras. *Nature* (1956) 177:452–4. doi:10.1038/177452a0
9. Becker AJ, Mc CE, Till JE. Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. *Nature* (1963) 197:452–4. doi:10.1038/197452a0
10. Barnes DW, Loutit JF. Treatment of murine leukaemia with x-rays and homologous bone marrow. II. *Br J Haematol* (1957) 3:241–52. doi:10.1111/j.1365-2141.1957.tb05793.x
11. Thomas ED, Lochte HL Jr, Cannon JH, Sahler OD, Ferrebee JW. Supralethal whole body irradiation and isologous marrow transplantation in man. *J Clin Invest* (1959) 38:1709–16. doi:10.1172/JCI103949
12. Mathe G, Bernard J. [Trial therapy, by x-irradiation followed by the administration of homologous bone marrow cells, of highly-advanced spontaneous leukemia in AK mice]. *Bull Assoc Fr Etud Cancer* (1958) 45:289–300.
13. Simonsen M. The impact on the developing embryo and newborn animal of adult homologous cells. *Acta Pathol Microbiol Scand* (1957) 40:480–500. doi:10.1111/j.1600-0463.2007.apm_698a.x
14. Billingham RE. Studies on the reaction of injected homologous lymphoid tissue cells against the host. *Ann N Y Acad Sci* (1958) 73:782–8. doi:10.1111/j.1749-6632.1959.tb40857.x
15. Trentin JJ. Mortality and skin transplantability in x-irradiated mice receiving isologous, homologous or heterologous bone marrow. *Proc Soc Exp Biol Med* (1956) 92:688–93. doi:10.3181/00379727-92-22582
16. Barnes DW, Ilbery PL, Loutit JF. Avoidance of secondary disease in radiation chimaeras. *Nature* (1958) 181:488. doi:10.1038/181488a0
17. Ilbery PL, Koller PC, Loutit JF. Immunological characteristics of radiation chimaeras. *J Natl Cancer Inst* (1958) 20:1051–89.
18. Schwartz EE, Upton AC, Congdon CC. A fatal reaction caused by implantation of adult parental spleen tissue in irradiated F1 mice. *Proc Soc Exp Biol Med* (1957) 96:797–800. doi:10.3181/00379727-96-23612

19. Woodruff MF, Sparrow M. Further observation on the induction of tolerance of skin homografts in rats. *Transplant Bull* (1957) 4:157–9.
20. Egdahl RH, Roller FR, Swanson RL, Varco RL. Acquired tolerance to homografts and heterografts in the rat. *Ann N Y Acad Sci* (1958) 73:842–7. doi:10.1111/j.1749-6632.1959.tb40864.x
21. Nakic B, Silobrcic V. Tolerance of skin homografts related to fatal disease in separated rat parabionts. *Nature* (1958) 182:264–5. doi:10.1038/182264a0
22. Finerty JC. Parabiosis in physiological studies. *Physiol Rev* (1952) 32:277–302.
23. Harris PF, Harris RS, Kugler JH. Studies of the leucocyte compartment in guinea-pig bone marrow after acute haemorrhage and severe hypoxia: evidence for a common stem-cell. *Br J Haematol* (1966) 12:419–32. doi:10.1111/j.1365-2141.1966.tb05651.x
24. van Dicke KAHJI, van Bekkum DW. The selective elimination of immunologically competent cells from bone marrow and lymphatic cell mixtures. II. Mouse spleen cell fractionation on a discontinuous albumin gradient. *Transplantation* (1968) 6:562–70. doi:10.1097/00007890-196807000-00009
25. Boak JL, Fox M, Wilson RE. Activity of lymphoid tissues from antilymphocyte-serum-treated mice. *Lancet* (1967) 1:750–2. doi:10.1016/0140-6736(67)91366-9
26. van Bekkum DW, Balner H, Dicke KA, van den Berg FG, Prinsen GH, Hollander CF. The effect of pretreatment of allogeneic bone marrow graft recipients with antilymphocytic serum on the acute graft-versus-host reaction in monkeys. *Transplantation* (1972) 13:400–7. doi:10.1097/00007890-197204000-00007
27. Vallera DA, Soderling CC, Carlson GJ, Kersey JH. Bone marrow transplantation across major histocompatibility barriers in mice. Effect of elimination of T cells from donor grafts by treatment with monoclonal Thy-1.2 plus complement or antibody alone. *Transplantation* (1981) 31:218–22. doi:10.1097/00007890-198103000-00015
28. Rayfield LS, Brent L. Tolerance, immunocompetence, and secondary disease in fully allogeneic radiation chimeras. *Transplantation* (1983) 36:183–9. doi:10.1097/00007890-198308000-00015
29. Lonai P, Eliraz A, Wekerle H, Feldman M. Depletion of specific graft-versus-host reactivity following adsorption of nonsensitized lymphocytes on allogeneic fibroblasts. *Transplantation* (1973) 15:368–74. doi:10.1097/00007890-197304000-00004
30. Blazar BR, Carroll SF, Vallera DA. Prevention of murine graft-versus-host disease and bone marrow alloengraftment across the major histocompatibility barrier after donor graft preincubation with anti-LFA1 immunotoxin. *Blood* (1991) 78:3093–102.
31. Weiden PL, Storb R, Tsoi MS, Graham TC, Lerner KG, Thomas ED. Infusion of donor lymphocytes into stable canine radiation chimeras: implications for mechanism of transplantation tolerance. *J Immunol* (1976) 116:1212–9.
32. Sykes M, Sheard MA, Sachs DH. Graft-versus-host-related immunosuppression is induced in mixed chimeras by alloresponses against either host or donor lymphohematopoietic cells. *J Exp Med* (1988) 168:2391–6. doi:10.1084/jem.168.6.2391
33. Tutschka PJ, Hess AD, Beschorner WE, Santos GW. Suppressor cells in transplantation tolerance. I. Suppressor cells in the mechanism of tolerance in radiation chimeras. *Transplantation* (1981) 32:203–9. doi:10.1097/00007890-198109000-00005
34. Kolb HJ, Günther W, Schumm M, Holler E, Wilmanns W, Thierfelder S. Adoptive immunotherapy in canine chimeras. *Transplantation* (1997) 63:430–6. doi:10.1097/00007890-199702150-00017
35. Epstein RB, Storb R, Ragde H, Thomas ED. Cytotoxic typing antisera for marrow grafting in littermate dogs. *Transplantation* (1968) 6:45–58. doi:10.1097/00007890-196801000-00005
36. Storb R, Rudolph RH, Thomas ED. Marrow grafts between canine siblings matched by serotyping and mixed leukocyte culture. *J Clin Invest* (1971) 50:1272–5. doi:10.1172/JCI106605
37. Storb R, Rudolph RH, Kolb HJ, Graham TC, Mickelson E, Erickson V, et al. Marrow grafts between DL-A-matched canine littermates. *Transplantation* (1973) 15:92–100. doi:10.1097/00007890-197301000-00014
38. Halle-Pannenko O, Pritchard LL, Motta R, Mathe G. Non-H-2 antigens can induce high GVH mortality in adult recipients of normal cells. *Biomedicine* (1978) 29:253–5.
39. Hamilton BL, Bevan MJ, Parkman R. Anti-recipient cytotoxic T lymphocyte precursors are present in the spleens of mice with acute graft versus host disease due to minor histocompatibility antigens. *J Immunol* (1981) 126:621–5.
40. Cosgrove GE, Davis ML, Congdon CC. The effect of presensitization of parental donors on graft-versus-host disease in irradiated F 1 hybrid mice. *Proc Soc Exp Biol Med* (1971) 138:210–2. doi:10.3181/00379727-138-35864
41. Abdollahi A, Li M, Ping G, Plathow C, Domhan S, Kiessling F, et al. Inhibition of platelet-derived growth factor signaling attenuates pulmonary fibrosis. *J Exp Med* (2005) 201:925–35. doi:10.1084/jem.20041393
42. Korngold R, Sprent J. Lethal graft-versus-host disease after bone marrow transplantation across minor histocompatibility barriers in mice. Prevention by removing mature T cells from marrow. *J Exp Med* (1978) 148:1687–98. doi:10.1084/jem.148.6.1687
43. Berger M, Wettstein PJ, Korngold R. T cell subsets involved in lethal graft-versus-host disease directed to immunodominant minor histocompatibility antigens. *Transplantation* (1994) 57:1095–102. doi:10.1097/00007890-199404150-00019
44. Qian L, Wu Z, Shen J. Advances in the treatment of acute graft-versus-host disease. *J Cell Mol Med* (2013) 17:966–75. doi:10.1111/jcmm.12093
45. Higo S, Shimizu A, Masuda Y, Nagasaka S, Kajimoto Y, Kanzaki G, et al. Acute graft-versus-host disease of the kidney in allogeneic rat bone marrow transplantation. *PLoS One* (2014) 9:e115399. doi:10.1371/journal.pone.0115399
46. Nagler RM, Laufer D, Nagler A. Parotid gland dysfunction in a murine model of acute graft versus host disease [aGVHD]. *Head Neck* (1998) 20:58–62. doi:10.1002/(SICI)1097-0347(199801)20:1<58::AID-HED9>3.3.CO;2-P
47. Hanada H, Ohno J, Seno K, Ota N, Taniguchi K. Dynamic changes in cell-surface expression of mannose in the oral epithelium during the development of graft-versus-host disease of the oral mucosa in rats. *BMC Oral Health* (2014) 14:5. doi:10.1186/1472-6831-14-5
48. Peszkowski MJ, Fujiwara K, Warfvinge G, Larsson A. Experimental graft versus host disease in the (BN x LEW) F1 rat hybrid: an immunohistochemical study of early disease in oral mucosa. *Oral Dis* (1996) 2:2–10. doi:10.1111/j.1601-0825.1996.tb00196.x
49. Dertschnig S, Hauri-Hohl MM, Vollmer M, Hollander GA, Krenger W. Impaired thymic expression of tissue-restricted antigens licenses the de novo generation of autoreactive CD4+ T cells in acute GVHD. *Blood* (2015) 125:2720–3. doi:10.1182/blood-2014-08-597245
50. Thepot S, Zhou J, Perrot A, Robin M, Xhaard A, de Latour RP, et al. The graft-versus-leukemia effect is mainly restricted to NIH-defined chronic graft-versus-host disease after reduced intensity conditioning before allogeneic stem cell transplantation. *Leukemia* (2010) 24:1852–8. doi:10.1038/leu.2010.187
51. Baird K, Pavletic SZ. Chronic graft versus host disease. *Curr Opin Hematol* (2006) 13:426–35. doi:10.1097/01.moh.0000245689.47333.ff
52. Panoskaltsis-Mortari A, Lacey DL, Vallera DA, Blazar BR. Keratinocyte growth factor administered before conditioning ameliorates graft-versus-host disease after allogeneic bone marrow transplantation in mice. *Blood* (1998) 92:3960–7.
53. van Leeuwen L, Guiffre A, Atkinson K, Rainer SP, Sewell WA. A two-phase pathogenesis of graft-versus-host disease in mice. *Bone Marrow Transplant* (2002) 29:151–8. doi:10.1038/sj.bmt.1703328
54. Blaser BW, Roychowdhury S, Kim DJ, Schwind NR, Bhatt D, Yuan W, et al. Donor-derived IL-15 is critical for acute allogeneic graft-versus-host disease. *Blood* (2005) 105:894–901. doi:10.1182/blood-2004-05-1687
55. Fowler DH, Breglio J, Nagel G, Eckhaus MA, Gress RE. Allospecific CD8+ Tc1 and Tc2 populations in graft-versus-leukemia effect and graft-versus-host disease. *J Immunol* (1996) 157:4811–21.
56. Hildebrandt GC, Olkiewicz KM, Corrion LA, Chang Y, Clouthier SG, Liu C, et al. Donor-derived TNF-alpha regulates pulmonary chemokine expression and the development of idiopathic pneumonia syndrome after allogeneic bone marrow transplantation. *Blood* (2004) 104:586–93. doi:10.1182/blood-2003-12-4259
57. Kanamaru A, Okamoto T, Matsuda K, Hara H, Nagai K. Elevation of erythroid colony-stimulating activity in the serum of mice with graft-versus-host disease. *Exp Hematol* (1984) 12:763–7.
58. Krenger W, Rossi S, Hollander GA. Apoptosis of thymocytes during acute graft-versus-host disease is independent of glucocorticoids. *Transplantation* (2000) 69:2190–3. doi:10.1097/00007890-200005270-00040
59. Rolink AG, Pals ST, Gleichmann E. Allosuppressor and allohelper T cells in acute and chronic graft-vs.-host disease. II. F1 recipients carrying mutations at H-2K and/or I-A. *J Exp Med* (1983) 157:755–71. doi:10.1084/jem.157.2.755

60. Sprent J, Schaefer M, Lo D, Korngold R. Properties of purified T cell subsets. II. In vivo responses to class I vs. class II H-2 differences. *J Exp Med* (1986) 163:998–1011. doi:10.1084/jem.163.4.998
61. Eyrich M, Burger G, Marquardt K, Budach W, Schilbach K, Niethammer D, et al. Sequential expression of adhesion and costimulatory molecules in graft-versus-host disease target organs after murine bone marrow transplantation across minor histocompatibility antigen barriers. *Biol Blood Marrow Transplant* (2005) 11:371–82. doi:10.1016/j.bbmt.2005.02.002
62. Korngold R. Lethal graft-versus-host disease in mice directed to multiple minor histocompatibility antigens: features of CD8+ and CD4+ T cell responses. *Bone Marrow Transplant* (1992) 9:355–64.
63. Zhang Y, Joe G, Hexner E, Zhu J, Emerson SG. Alloreactive memory T cells are responsible for the persistence of graft-versus-host disease. *J Immunol* (2005) 174:3051–8. doi:10.4049/jimmunol.174.5.3051
64. Okayama J, Ko S, Kanehiro H, Kanokogi H, Hisanaga M, Ohashi K, et al. Bone marrow chimerism and tolerance induced by single-dose cyclophosphamide. *J Surg Res* (2004) 120:102–10. doi:10.1016/j.jss.2004.01.011
65. Zinöcker S, Wang MY, Rolstad B, Vaage JT. Mesenchymal stromal cells fail to alleviate experimental graft-versus-host disease in rats transplanted with major histocompatibility complex-mismatched bone marrow. *Scand J Immunol* (2012) 76:464–70. doi:10.1111/j.1365-3083.2012.02758.x
66. Aksu AE, Horibe E, Sacks J, Ikeguchi R, Breitinger J, Scozio M, et al. Co-infusion of donor bone marrow with host mesenchymal stem cells treats GVHD and promotes vascularized skin allograft survival in rats. *Clin Immunol* (2008) 127:348–58. doi:10.1016/j.clim.2008.02.003
67. Nestvold JM, Omdal BK, Dai KZ, Martens A, Benestad HB, Vaage JT, et al. A second prophylactic MHC-mismatched bone marrow transplantation protects against rat acute myeloid leukemia (BNML) without lethal graft-versus-host disease. *Transplantation* (2008) 85:102–11. doi:10.1097/01.tp.0000296856.53493.1f
68. Zinöcker S, Sviland L, Dressel R, Rolstad B. Kinetics of lymphocyte reconstitution after allogeneic bone marrow transplantation: markers of graft-versus-host disease. *J Leukoc Biol* (2011) 90:177–87. doi:10.1189/jlb.0211067
69. Clancy J Jr, Chan P, Schurath R. 3H-deoxythymidine incorporation in graft-versus-host disease in the Norway rat. II. Autoradiographic studies. *Virchows Arch B Cell Pathol* (1976) 22:341–52.
70. Zhu ZX, Fan LY, Wang Q. Simultaneous blockade of costimulatory signals CD28-CD80 and CD40-CD154 combined with monoclonal antibody against CD25 induced a stable chimerism and tolerance without graft-versus-host disease in rat. *Eur Surg Res* (2011) 46:109–17. doi:10.1159/000323011
71. Zhou S, Ueta H, Xu XD, Shi C, Matsuno K. Predominant donor CD103+CD8+ T cell infiltration into the gut epithelium during acute GvHD: a role of gut lymph nodes. *Int Immunol* (2008) 20:385–94. doi:10.1093/intimm/dxm153
72. Jäger MD, Liu JY, Timrott KF, Popp FC, Stoeltzing O, Lang SA, et al. Sirolimus promotes tolerance for donor and recipient antigens after MHC class II disparate bone marrow transplantation in rats. *Exp Hematol* (2007) 35:164–70. doi:10.1016/j.exphem.2006.08.018
73. Sasatomi K, Sakisaka S, Kawaguchi T, Hanada S, Taniguchi E, Koga H, et al. Cholestasis in a rat model of graft-versus-host disease is accompanied by alteration of the expression and distribution of tight-junction-associated proteins. *Int J Mol Med* (2005) 15:431–6. doi:10.3892/ijmm.15.3.431
74. Wolff D, Frei E, Hofmeister N, Steiner B, Kleine HD, Junghanss C, et al. Methotrexate-albumin and aminopterin-albumin effectively prevent experimental acute graft-versus-host disease. *Transplantation* (2006) 82:527–33. doi:10.1097/01.tp.0000229451.40286.64
75. Ohajekwe OA, Hardy MA, Oluwole SF. Prevention of graft-versus-host disease and the induction of transplant tolerance by low-dose UV-B irradiation of BM cells combined with cyclosporine immunosuppression. *Transplantation* (1995) 60:1510–6. doi:10.1097/00007890-199560120-00024
76. Fujino M, Zhu P, Kitazawa Y, Chen JM, Zhuang J, Li XK. Mesenchymal stem cells attenuate rat graft-versus-host disease. *Methods Mol Biol* (2014) 1213:341–53. doi:10.1007/978-1-4939-1453-1_28
77. Clancy J Jr, Mauser L, Chapman AL. Level and temporal pattern of naturally cytolytic cells during acute Graft-versus-Host Disease (GVHD) in the rat. *Cell Immunol* (1983) 79:1–10. doi:10.1016/0008-8749(83)90045-X
78. Goral J, Mathews HL, Clancy J Jr. Antibodies specific for the 70-kDa heat-shock protein parallel the development of acute graft-versus-host disease in (DA x LEW)F1 rats. *Clin Immunol Immunopathol* (1995) 75:147–53. doi:10.1006/clin.1995.1064
79. Kimura T, Enosawa S, Kamada N, Kobayashi E, Toyama N, Doy M, et al. Evidence that the elevation of soluble MHC class I antigens in the serum precedes the onset of graft-versus-host disease and is correlated with the severity of the disease in rats. *Transpl Immunol* (1995) 3:299–304. doi:10.1016/0966-3274(95)80015-8
80. Kobayashi E, Enosawa S, Fujimura A, Kamada N, Suzuki S. Timing of lymphocyte transfusion and portal clamping for the development of lethal graft-versus-host disease in the rat. *Surg Today* (1998) 28:1036–41. doi:10.1007/BF02483957
81. Vaidya S, Wang CC, Roorda C, Billings A, Rajaraman S, Fish JC. Prevention of graft-versus-host disease by intrathymic injection of recipient-type splenocytes into donor. *Transplantation* (1996) 62:1366–8. doi:10.1097/00007890-199611150-00035
82. Nestel FP, Price KS, Seemayer TA, Lapp WS. Macrophage priming and lipopolysaccharide-triggered release of tumor necrosis factor alpha during graft-versus-host disease. *J Exp Med* (1992) 175:405–13. doi:10.1084/jem.175.2.405
83. Hill GR, Crawford JM, Cooke KR, Brinson YS, Pan L, Ferrara JL. Total body irradiation and acute graft-versus-host disease: the role of gastrointestinal damage and inflammatory cytokines. *Blood* (1997) 90:3204–13.
84. Cooke KR, Gerbitz A, Crawford JM, Teshima T, Hill GR, Tesolin A, et al. LPS antagonism reduces graft-versus-host disease and preserves graft-versus-leukemia activity after experimental bone marrow transplantation. *J Clin Invest* (2001) 107:1581–9. doi:10.1172/JCI12156
85. Shlomchik WD, Couzens MS, Tang CB, McNiff J, Robert ME, Liu J, et al. Prevention of graft versus host disease by inactivation of host antigen-presenting cells. *Science* (1999) 285:412–5. doi:10.1126/science.285.5426.412
86. Hill GR. Inflammation and bone marrow transplantation. *Biol Blood Marrow Transplant* (2009) 15:139–41. doi:10.1016/j.bbmt.2008.11.008
87. Yu C, Storb R, Mathey B, Deeg HJ, Schuening FG, Graham TC, et al. DLA-identical bone marrow grafts after low-dose total body irradiation: effects of high-dose corticosteroids and cyclosporine on engraftment. *Blood* (1995) 86:4376–81.
88. Xun CQ, Thompson JS, Jennings CD, Brown SA, Widmer MB. Effect of total body irradiation, busulfan-cyclophosphamide, or cyclophosphamide conditioning on inflammatory cytokine release and development of acute and chronic graft-versus-host disease in H-2-incompatible transplanted SCID mice. *Blood* (1994) 83:2360–7.
89. van Bekkum DW, Roodenburg J, Heidt PJ, van der Waaij D. Mitigation of secondary disease of allogeneic mouse radiation chimeras by modification of the intestinal microflora. *J Natl Cancer Inst* (1974) 52:401–4.
90. Jones JM, Wilson R, Bealmear PM. Mortality and gross pathology of secondary disease in germfree mouse radiation chimeras. *Radiat Res* (1971) 45:577–88. doi:10.2307/3573066
91. Petersen FB, Buckner CD, Clift RA, Nelson N, Counts GW, Meyers JD, et al. Infectious complications in patients undergoing marrow transplantation: a prospective randomized study of the additional effect of decontamination and laminar air flow isolation among patients receiving prophylactic systemic antibiotics. *Scand J Infect Dis* (1987) 19:559–67. doi:10.3109/00365548709032423
92. Russell JA, Chaudhry A, Booth K, Brown C, Woodman RC, Valentine K, et al. Early outcomes after allogeneic stem cell transplantation for leukemia and myelodysplasia without protective isolation: a 10-year experience. *Biol Blood Marrow Transplant* (2000) 6:109–14. doi:10.1016/S1083-8791(00)70073-5
93. Heimesaat MM, Nogai A, Bereswill S, Plickert R, Fischer A, Loddenkemper C, et al. MyD88/TLR9 mediated immunopathology and gut microbiota dynamics in a novel murine model of intestinal graft-versus-host disease. *Gut* (2010) 59:1079–87. doi:10.1136/gut.2009.197434
94. Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, Nishikawa H, et al. Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. *Nature* (2013) 500:232–6. doi:10.1038/nature12331
95. Jenq RR, Ubeda C, Taur Y, Menezes CC, Khanin R, Dudakov JA, et al. Regulation of intestinal inflammation by microbiota following allogeneic bone marrow transplantation. *J Exp Med* (2012) 209:903–11. doi:10.1084/jem.20112408
96. Bollyky PL, Evanko SP, Wu RP, Potter-Perigo S, Long SA, Kinsella B, et al. Th1 cytokines promote T-cell binding to antigen-presenting cells via enhanced hyaluronan production and accumulation at the immune synapse. *Cell Mol Immunol* (2010) 7:211–20. doi:10.1038/cmi.2010.9

97. Murai M, Yoneyama H, Ezaki T, Suematsu M, Terashima Y, Harada A, et al. Peyer's patch is the essential site in initiating murine acute and lethal graft-versus-host reaction. *Nat Immunol* (2003) 4:154–60. doi:10.1038/ni879
98. Anderson BE, McNiff J, Yan J, Doyle H, Mamula M, Shlomchik MJ, et al. Memory CD4+ T cells do not induce graft-versus-host disease. *J Clin Invest* (2003) 112:101–8. doi:10.1172/JCI17601
99. Chen BJ, Cui X, Sempowski GD, Liu C, Chao NJ. Transfer of allogeneic CD62L- memory T cells without graft-versus-host disease. *Blood* (2004) 103:1534–41. doi:10.1182/blood-2003-08-2987
100. Yi T, Chen Y, Wang L, Du G, Huang D, Zhao D, et al. Reciprocal differentiation and tissue-specific pathogenesis of Th1, Th2, and Th17 cells in graft-versus-host disease. *Blood* (2009) 114:3101–12. doi:10.1182/blood-2009-05-219402
101. Burman AC, Banovic T, Kuns RD, Clouston AD, Stanley AC, Morris ES, et al. IFNgamma differentially controls the development of idiopathic pneumonia syndrome and GVHD of the gastrointestinal tract. *Blood* (2007) 110:1064–72. doi:10.1182/blood-2006-12-063982
102. Iclozan C, Yu Y, Liu C, Liang Y, Yi T, Anasetti C, et al. T helper17 cells are sufficient but not necessary to induce acute graft-versus-host disease. *Biol Blood Marrow Transplant* (2010) 16:170–8. doi:10.1016/j.bbmt.2009.09.023
103. Yu Y, Wang D, Liu C, Kaosaard K, Semple K, Anasetti C, et al. Prevention of GVHD while sparing GVL effect by targeting Th1 and Th17 transcription factor T-bet and RORgammat in mice. *Blood* (2011) 118:5011–20. doi:10.1182/blood-2011-03-340315
104. Rowe V, Banovic T, MacDonald KP, Kuns R, Don AL, Morris ES, et al. Host B cells produce IL-10 following TBI and attenuate acute GVHD after allogeneic bone marrow transplantation. *Blood* (2006) 108:2485–92. doi:10.1182/blood-2006-04-016063
105. Renkonen R, Wangel A, Hayry P. Bone marrow transplantation in the rat. B lymphocyte activation in acute graft-versus-host disease. *Transplantation* (1986) 41:290–6. doi:10.1097/00007890-198606000-00007
106. Wysocki CA, Panoskaltsis-Mortari A, Blazar BR, Serody JS. Leukocyte migration and graft-versus-host disease. *Blood* (2005) 105:4191–9. doi:10.1182/blood-2004-12-4726
107. Wysocki CA, Burkett SB, Panoskaltsis-Mortari A, Kirby SL, Luster AD, McKinnon K, et al. Differential roles for CCR5 expression on donor T cells during graft-versus-host disease based on pretransplant conditioning. *J Immunol* (2004) 173:845–54. doi:10.4049/jimmunol.173.2.845
108. Murai M, Yoneyama H, Harada A, Yi Z, Vestergaard C, Guo B, et al. Active participation of CCR5(+)CD8(+) T lymphocytes in the pathogenesis of liver injury in graft-versus-host disease. *J Clin Invest* (1999) 104:49–57. doi:10.1172/JCI6642
109. Duffner U, Lu B, Hildebrandt GC, Teshima T, Williams DL, Reddy P, et al. Role of CXCR3-induced donor T-cell migration in acute GVHD. *Exp Hematol* (2003) 31:897–902. doi:10.1016/S0301-472X(03)00198-X
110. Ueha S, Murai M, Yoneyama H, Kitabatake M, Imai T, Shimaoka T, et al. Intervention of MAdCAM-1 or fractalkine alleviates graft-versus-host reaction associated intestinal injury while preserving graft-versus-tumor effects. *J Leukoc Biol* (2007) 81:176–85. doi:10.1189/jlb.0306231
111. Varona R, Cadenas V, Lozano M, Moreno-Ortiz MC, Kremer L, Martínez-A C, et al. CCR6 regulates the function of alloreactive and regulatory CD4+ T cells during acute graft-versus-host disease. *Leuk Lymphoma* (2006) 47:1469–76. doi:10.1080/10428190500513819
112. Teshima T, Ordemann R, Reddy P, Gagin S, Liu C, Cooke KR, et al. Acute graft-versus-host disease does not require alloantigen expression on host epithelium. *Nat Med* (2002) 8:575–81. doi:10.1038/nm0602-575
113. Theiss-Suennemann J, Jörß K, Messmann JJ, Reichardt SD, Montes-Cobos E, Lühder F, et al. Glucocorticoids attenuate acute graft-versus-host disease by suppressing the cytotoxic capacity of CD8(+) T cells. *J Pathol* (2015) 235:646–55. doi:10.1002/path.4475
114. Braun MY, Lowin B, French L, Acha-Orbea H, Tschopp J. Cytotoxic T cells deficient in both functional fas ligand and perforin show residual cytolytic activity yet lose their capacity to induce lethal acute graft-versus-host disease. *J Exp Med* (1996) 183:657–61. doi:10.1084/jem.183.2.657
115. Graubert TA, DiPersio JF, Russell JH, Ley TJ. Perforin/granzyme-dependent and independent mechanisms are both important for the development of graft-versus-host disease after murine bone marrow transplantation. *J Clin Invest* (1997) 100:904–11. doi:10.1172/JCI119606
116. Maeda Y, Levy RB, Reddy P, Liu C, Clouthier SG, Teshima T, et al. Both perforin and Fas ligand are required for the regulation of alloreactive CD8+ T cells during acute graft-versus-host disease. *Blood* (2005) 105:2023–7. doi:10.1182/blood-2004-08-3036
117. Ghosh A, Dogan Y, Moroz M, Holland AM, Yim NL, Rao UK, et al. Adoptively transferred TRAIL+ T cells suppress GVHD and augment antitumor activity. *J Clin Invest* (2013) 123:2654–62. doi:10.1172/JCI66301
118. Storb R, Epstein RB, Graham TC, Thomas ED. Methotrexate regimens for control of graft-versus-host disease in dogs with allogeneic marrow grafts. *Transplantation* (1970) 9:240–6. doi:10.1097/00007890-197003000-00007
119. Thomas ED, Collins JA, Herman EC Jr, Ferrebee JW. Marrow transplants in lethally irradiated dogs given methotrexate. *Blood* (1962) 19:217–28.
120. Deeg HJ, Storb R, Weiden PL, Raff RF, Sale GE, Atkinson K, et al. Cyclosporin A and methotrexate in canine marrow transplantation: engraftment, graft-versus-host disease, and induction of intolerance. *Transplantation* (1982) 34:30–5. doi:10.1097/00007890-198207000-00006
121. Deeg HJ, Storb R, Appelbaum FR, Kennedy MS, Graham TC, Thomas ED. Combined immunosuppression with cyclosporine and methotrexate in dogs given bone marrow grafts from DLA-haploidentical littermates. *Transplantation* (1984) 37:62–5. doi:10.1097/00007890-198401000-00017
122. Deeg HJ, Storb R, Weiden PL, Graham T, Atkinson K, Thomas ED. Cyclosporin-A: effect on marrow engraftment and graft-versus-host disease in dogs. *Transplant Proc* (1981) 13:402–4.
123. Storb R, Deeg HJ, Pepe M, Appelbaum F, Anasetti C, Beatty P, et al. Methotrexate and cyclosporine versus cyclosporine alone for prophylaxis of graft-versus-host disease in patients given HLA-identical marrow grafts for leukemia: long-term follow-up of a controlled trial. *Blood* (1989) 73:1729–34.
124. Sorror ML, Leisenring W, Deeg HJ, Martin PJ, Storb R. Twenty-year follow-up of a controlled trial comparing a combination of methotrexate plus cyclosporine with cyclosporine alone for prophylaxis of graft-versus-host disease in patients administered HLA-identical marrow grafts for leukemia. *Biol Blood Marrow Transplant* (2005) 11:814–5. doi:10.1016/j.bbmt.2005.05.016
125. Sun J, Dotti G, Huye LE, Foster AE, Savoldo B, Gramatges MM, et al. T cells expressing constitutively active Akt resist multiple tumor-associated inhibitory mechanisms. *Mol Ther* (2010) 18:2006–17. doi:10.1038/mt.2010.185
126. Ruutu T, Gratwohl A, de Witte T, Afanasyev B, Apperley J, Bacigalupo A, et al. Prophylaxis and treatment of GVHD: EBMT-ELN working group recommendations for a standardized practice. *Bone Marrow Transplant* (2014) 49:168–73. doi:10.1038/bmt.2013.107
127. Piguet PF, Grau GE, Allet B, Vassalli P. Tumor necrosis factor/cachectin is an effector of skin and gut lesions of the acute phase of graft-vs.-host disease. *J Exp Med* (1987) 166:1280–9. doi:10.1084/jem.166.5.1280
128. Cooke KR, Hill GR, Crawford JM, Bungard D, Brinson YS, Delmonte J Jr, et al. Tumor necrosis factor-alpha production to lipopolysaccharide stimulation by donor cells predicts the severity of experimental acute graft-versus-host disease. *J Clin Invest* (1998) 102:1882–91. doi:10.1172/JCI4285
129. Tawara I, Koyama M, Liu C, Toubai T, Thomas D, Evers R, et al. Interleukin-6 modulates graft-versus-host responses after experimental allogeneic bone marrow transplantation. *Clin Cancer Res* (2011) 17:77–88. doi:10.1158/1078-0432.CCR-10-1198
130. Givon T, Revel M, Slavin S. Potential use of interleukin-6 in bone marrow transplantation: effects of recombinant human interleukin-6 after syngeneic and semiallogeneic bone marrow transplantation in mice. *Blood* (1994) 83:1690–7.
131. Chen X, Das R, Komorowski R, Beres A, Hessner MJ, Mihara M, et al. Blockade of interleukin-6 signaling augments regulatory T-cell reconstitution and attenuates the severity of graft-versus-host disease. *Blood* (2009) 114:891–900. doi:10.1182/blood-2009-01-197178
132. Drobyski WR, Pasquini M, Kovatovic K, Palmer J, Douglas Rizzo J, Saad A, et al. Tocilizumab for the treatment of steroid refractory graft-versus-host disease. *Biol Blood Marrow Transplant* (2011) 17:1862–8. doi:10.1016/j.bbmt.2011.07.001
133. Sykes M, Abraham VS, Harty MW, Pearson DA. IL-2 reduces graft-versus-host disease and preserves a graft-versus-leukemia effect by selectively inhibiting CD4+ T cell activity. *J Immunol* (1993) 150:197–205.
134. Perol L, Martin GH, Maury S, Cohen JL, Piaggio E. Potential limitations of IL-2 administration for the treatment of experimental acute graft-versus-host disease. *Immunol Lett* (2014) 162:173–84. doi:10.1016/j.imlet.2014.10.027
135. Shin HJ, Baker J, Leveson-Gower DB, Smith AT, Sega EI, Negrin RS. Rapamycin and IL-2 reduce lethal acute graft-versus-host disease associated

with increased expansion of donor type CD4+CD25+Foxp3+ regulatory T cells. *Blood* (2011) 118:2342–50. doi:10.1182/blood-2010-10-313684
136. Zenewicz LA, Flavell RA. Recent advances in IL-22 biology. *Int Immunol* (2011) 23:159–63. doi:10.1093/intimm/dxr001
137. Sanos SL, Vonarbourg C, Mortha A, Diefenbach A. Control of epithelial cell function by interleukin-22-producing RORgammat+ innate lymphoid cells. *Immunology* (2011) 132:453–65. doi:10.1111/j.1365-2567.2011.03410.x
138. Hanash AM, Dudakov JA, Hua G, O'Connor MH, Young LF, Singer NV, et al. Interleukin-22 protects intestinal stem cells from immune-mediated tissue damage and regulates sensitivity to graft versus host disease. *Immunity* (2012) 37:339–50. doi:10.1016/j.immuni.2012.05.028
139. Lamarthee B, Malard F, Gamonet C, Bossard C, Couturier M, et al. Donor interleukin-22 and host type I interferon signaling pathway participate in intestinal graft-versus-host disease via STAT1 activation and CXCL10. *Mucosal Immunol* (2015) 9:309–21. doi:10.1038/mi.2015.61
140. He S, Cao Q, Qiu Y, Mi J, Zhang JZ, Jin M, et al. A new approach to the blocking of alloreactive T cell-mediated graft-versus-host disease by in vivo administration of anti-CXCR3 neutralizing antibody. *J Immunol* (2008) 181:7581–92. doi:10.4049/jimmunol.181.11.7581
141. Wysocki CA, Jiang Q, Panoskaltsis-Mortari A, Taylor PA, McKinnon KP, Su L, et al. Critical role for CCR5 in the function of donor CD4+CD25+ regulatory T cells during acute graft-versus-host disease. *Blood* (2005) 106:3300–7. doi:10.1182/blood-2005-04-1632
142. Hasegawa H, Inoue A, Kohno M, Lei J, Miyazaki T, Yoshie O, et al. Therapeutic effect of CXCR3-expressing regulatory T cells on liver, lung and intestinal damages in a murine acute GVHD model. *Gene Ther* (2008) 15:171–82. doi:10.1038/sj.gt.3303051
143. Miklos S, Mueller G, Chang Y, Bouazzaoui A, Spacenko E, Schubert TE, et al. Preventive usage of broad spectrum chemokine inhibitor NR58-3.14.3 reduces the severity of pulmonary and hepatic graft-versus-host disease. *Int J Hematol* (2009) 89:383–97. doi:10.1007/s12185-009-0272-y
144. Blazar BR, Sharpe AH, Taylor PA, Panoskaltsis-Mortari A, Gray GS, Korngold R, et al. Infusion of anti-B7.1 (CD80) and anti-B7.2 (CD86) monoclonal antibodies inhibits murine graft-versus-host disease lethality in part via direct effects on CD4+ and CD8+ T cells. *J Immunol* (1996) 157:3250–9.
145. Yu XZ, Bidwell SJ, Martin PJ, Anasetti C. CD28-specific antibody prevents graft-versus-host disease in mice. *J Immunol* (2000) 164:4564–8. doi:10.4049/jimmunol.164.9.4564
146. Durie FH, Aruffo A, Ledbetter J, Crassi KM, Green WR, Fast LD, et al. Antibody to the ligand of CD40, gp39, blocks the occurrence of the acute and chronic forms of graft-vs-host disease. *J Clin Invest* (1994) 94:1333–8. doi:10.1172/JCI117453
147. Hargreaves RE, Monk NJ, Jurcevic S. Selective depletion of activated T cells: the CD40L-specific antibody experience. *Trends Mol Med* (2004) 10:130–5. doi:10.1016/j.molmed.2004.01.009
148. Taylor PA, Friedman TM, Korngold R, Noelle RJ, Blazar BR. Tolerance induction of alloreactive T cells via ex vivo blockade of the CD40:CD40L costimulatory pathway results in the generation of a potent immune regulatory cell. *Blood* (2002) 99:4601–9. doi:10.1182/blood.V99.12.4601
149. Tittle TV, Weinberg AD, Steinkeler CN, Maziarz RT. Expression of the T-cell activation antigen, OX-40, identifies alloreactive T cells in acute graft-versus-host disease. *Blood* (1997) 89:4652–8.
150. Blazar BR, Sharpe AH, Chen AI, Panoskaltsis-Mortari A, Lees C, Akiba H, et al. Ligation of OX40 (CD134) regulates graft-versus-host disease (GVHD) and graft rejection in allogeneic bone marrow transplant recipients. *Blood* (2003) 101:3741–8. doi:10.1182/blood-2002-10-3048
151. Nozawa K, Ohata J, Sakurai J, Hashimoto H, Miyajima H, Yagita H, et al. Preferential blockade of CD8(+) T cell responses by administration of anti-CD137 ligand monoclonal antibody results in differential effect on development of murine acute and chronic graft-versus-host diseases. *J Immunol* (2001) 167:4981–6. doi:10.4049/jimmunol.167.9.4981
152. Taylor PA, Panoskaltsis-Mortari A, Freeman GJ, Sharpe AH, Noelle RJ, Rudensky AY, et al. Targeting of inducible costimulator (ICOS) expressed on alloreactive T cells down-regulates graft-versus-host disease (GVHD) and facilitates engraftment of allogeneic bone marrow (BM). *Blood* (2005) 105:3372–80. doi:10.1182/blood-2004-10-3869
153. Xu Y, Flies AS, Flies DB, Zhu G, Anand S, Flies SJ, et al. Selective targeting of the LIGHT-HVEM costimulatory system for the treatment of graft-versus-host disease. *Blood* (2007) 109:4097–104. doi:10.1182/blood-2006-09-047332
154. Karimi MA, Bryson JL, Richman LP, Fesnak AD, Leichner TM, Satake A, et al. NKG2D expression by CD8+ T cells contributes to GVHD and GVT effects in a murine model of allogeneic HSCT. *Blood* (2015) 125:3655–63. doi:10.1182/blood-2015-02-629006
155. Nabekura T, Shibuya K, Takenaka E, Kai H, Shibata K, Yamashita Y, et al. Critical role of DNAX accessory molecule-1 (DNAM-1) in the development of acute graft-versus-host disease in mice. *Proc Natl Acad Sci U S A* (2010) 107:18593–8. doi:10.1073/pnas.1005582107
156. Blazar BR, Levy RB, Mak TW, Panoskaltsis-Mortari A, Muta H, Jones M, et al. CD30/CD30 ligand (CD153) interaction regulates CD4+ T cell-mediated graft-versus-host disease. *J Immunol* (2004) 173:2933–41. doi:10.4049/jimmunol.173.5.2933
157. Ringdén O, Uzunel M, Rasmusson I, Remberger M, Sundberg B, Lönnies H, et al. Mesenchymal stem cells for treatment of therapy-resistant graft-versus-host disease. *Transplantation* (2006) 81:1390–7. doi:10.1097/01.tp.0000214462.63943.14
158. Méndez-Ferrer S, Michurina TV, Ferraro F, Mazloom AR, Macarthur BD, Lira SA, et al. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* (2010) 466:829–34. doi:10.1038/nature09262
159. Li MQ, Hou XF, Shao J, Tang CL, Li DJ. The DSCs-expressed CD82 controls the invasiveness of trophoblast cells via integrinbeta1/MAPK/MAPK3/1 signaling pathway in human first-trimester pregnancy. *Biol Reprod* (2010) 82:968–79. doi:10.1095/biolreprod.109.080739
160. Mattar P, Bieback K. Comparing the immunomodulatory properties of bone marrow, adipose tissue, and birth-associated tissue mesenchymal stromal cells. *Front Immunol* (2015) 6:560. doi:10.3389/fimmu.2015.00560
161. Ball LM, Bernardo ME, Roelofs H, van Tol MJ, Contoli B, Zwaginga JJ, et al. Multiple infusions of mesenchymal stromal cells induce sustained remission in children with steroid-refractory, grade III-IV acute graft-versus-host disease. *Br J Haematol* (2013) 163:501–9. doi:10.1111/bjh.12545
162. Badillo AT, Peranteau WH, Heaton TE, Quinn C, Flake AW. Murine bone marrow derived stromal progenitor cells fail to prevent or treat acute graft-versus-host disease. *Br J Haematol* (2008) 141:224–34. doi:10.1111/j.1365-2141.2008.07040.x
163. Mielcarek M, Storb R, Georges GE, Golubev L, Nikitine A, Hwang B, et al. Mesenchymal stromal cells fail to prevent acute graft-versus-host disease and graft rejection after dog leukocyte antigen-haploidentical bone marrow transplantation. *Biol Blood Marrow Transplant* (2011) 17:214–25. doi:10.1016/j.bbmt.2010.08.015
164. Sudres M, Norol F, Trenado A, Grégoire S, Charlotte F, Levacher B, et al. Bone marrow mesenchymal stem cells suppress lymphocyte proliferation in vitro but fail to prevent graft-versus-host disease in mice. *J Immunol* (2006) 176:7761–7. doi:10.4049/jimmunol.176.12.7761
165. Li H, Guo Z, Jiang X, Zhu H, Li X, Mao N. Mesenchymal stem cells alter migratory property of T and dendritic cells to delay the development of murine lethal acute graft-versus-host disease. *Stem Cells* (2008) 26:2531–41. doi:10.1634/stemcells.2008-0146
166. Tisato V, Naresh K, Girdlestone J, Navarrete C, Dazzi F. Mesenchymal stem cells of cord blood origin are effective at preventing but not treating graft-versus-host disease. *Leukemia* (2007) 21:1992–9. doi:10.1038/sj.leu.2404847
167. Galipeau J. The mesenchymal stromal cells dilemma – does a negative phase III trial of random donor mesenchymal stromal cells in steroid-resistant graft-versus-host disease represent a death knell or a bump in the road? *Cytotherapy* (2013) 15:2–8. doi:10.1016/j.jcyt.2012.10.002
168. Tolar J, Villeneuve P, Keating A. Mesenchymal stromal cells for graft-versus-host disease. *Hum Gene Ther* (2011) 22:257–62. doi:10.1089/hum.2011.1104
169. Waterman RS, Tomchuck SL, Henkle SL, Betancourt AM. A new mesenchymal stem cell (MSC) paradigm: polarization into a pro-inflammatory MSC1 or an immunosuppressive MSC2 phenotype. *PLoS One* (2010) 5:e10088. doi:10.1371/journal.pone.0010088
170. Moll G, Le Blanc K. Engineering more efficient multipotent mesenchymal stromal (stem) cells for systemic delivery as cellular therapy. *ISBT Sci Ser* (2015) 10:357–65. doi:10.1111/voxs.12133
171. Stephens LA, Mason D. CD25 is a marker for CD4+ thymocytes that prevent autoimmune diabetes in rats, but peripheral T cells with this function

are found in both CD25+ and CD25- subpopulations. *J Immunol* (2000) 165:3105–10. doi:10.4049/jimmunol.165.6.3105
172. Stephens LA, Mottet C, Mason D, Powrie F. Human CD4(+)CD25(+) thymocytes and peripheral T cells have immune suppressive activity in vitro. *Eur J Immunol* (2001) 31:1247–54. doi:10.1002/1521-4141(200104)31:4<1247::AID-IMMU1247>3.0.CO;2-M
173. Itoh M, Takahashi T, Sakaguchi N, Kuniyasu Y, Shimizu J, Otsuka F, et al. Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J Immunol* (1999) 162:5317–26.
174. Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* (2005) 22:329–41. doi:10.1016/j.immuni.2005.01.016
175. Cohen JL, Trenado A, Vasey D, Klatzmann D, Salomon BL. CD4(+)CD25(+) immunoregulatory T cells: new therapeutics for graft-versus-host disease. *J Exp Med* (2002) 196:401–6. doi:10.1084/jem.20020090
176. Hoffmann P, Ermann J, Edinger M, Fathman CG, Strober S. Donor-type CD4(+)CD25(+) regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation. *J Exp Med* (2002) 196:389–99. doi:10.1084/jem.20020399
177. Ermann J, Hoffmann P, Edinger M, Dutt S, Blankenberg FG, Higgins JP, et al. Only the CD62L+ subpopulation of CD4+CD25+ regulatory T cells protects from lethal acute GVHD. *Blood* (2005) 105:2220–6. doi:10.1182/blood-2004-05-2044
178. Taylor PA, Panoskaltsis-Mortari A, Swedin JM, Lucas PJ, Gress RE, Levine BL, et al. L-Selectin(hi) but not the L-selectin(lo) CD4+25+ T-regulatory cells are potent inhibitors of GVHD and BM graft rejection. *Blood* (2004) 104:3804–12. doi:10.1182/blood-2004-05-1850
179. Fantini MC, Becker C, Monteleone G, Pallone F, Galle PR, Neurath MF. Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7. *J Immunol* (2004) 172:5149–53. doi:10.4049/jimmunol.172.9.5149
180. Koenecke C, Czeloth N, Bubke A, Schmitz S, Kissenpfennig A, Malissen B, et al. Alloantigen-specific de novo-induced Foxp3+ Treg revert in vivo and do not protect from experimental GVHD. *Eur J Immunol* (2009) 39:3091–6. doi:10.1002/eji.200939432
181. Beres A, Komorowski R, Mihara M, Drobyski WR. Instability of Foxp3 expression limits the ability of induced regulatory T cells to mitigate graft versus host disease. *Clin Cancer Res* (2011) 17:3969–83. doi:10.1158/1078-0432.CCR-10-3347
182. Bucher C, Koch L, Vogtenhuber C, Goren E, Munger M, Panoskaltsis-Mortari A, et al. IL-21 blockade reduces graft-versus-host disease mortality by supporting inducible T regulatory cell generation. *Blood* (2009) 114:5375–84. doi:10.1182/blood-2009-05-221135
183. Laurence A, Amarnath S, Mariotti J, Kim YC, Foley J, Eckhaus M, et al. STAT3 transcription factor promotes instability of nTreg cells and limits generation of iTreg cells during acute murine graft-versus-host disease. *Immunity* (2012) 37:209–22. doi:10.1016/j.immuni.2012.05.027
184. Rolstad B, Ford WL. The rapid elimination of allogeneic lymphocytes: relationship to established mechanisms of immunity and to lymphocyte traffic. *Immunol Rev* (1983) 73:87–113. doi:10.1111/j.1600-065X.1983.tb01080.x
185. Tønnesen B, Rolstad B. In vivo elimination of allogeneic lymphocytes in normal and T-cell-deficient rats. Elimination does not require T cells. *Scand J Immunol* (1983) 17:303–12. doi:10.1111/j.1365-3083.1983.tb00794.x
186. Cudkowicz G, Stimpfling JH. Hybrid resistance to parental marrow grafts: association with the K region of H-2. *Science* (1964) 144:1339–40. doi:10.1126/science.144.3624.1339
187. Engh E, Benestad HB, Strøm-Gundersen I, Vaage JT, Bell EB, Rolstad B. Role of classical (RT1.A) and nonclassical (RT1.C) MHC class I regions in natural killer cell-mediated bone marrow allograft rejection in rats. *Transplantation* (1998) 65:319–24. doi:10.1097/00007890-199802150-00005
188. Engh E, Strøm-Gundersen I, Benestad HB, Rolstad B. Long-term donor chimerism after MHC (RT1) mismatched bone marrow transplantation in the rat: the role of host alloreactive NK cells. *Scand J Immunol* (2001) 54:198–203. doi:10.1046/j.1365-3083.2001.00929.x
189. Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, Tosti A, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science* (2002) 295:2097–100. doi:10.1126/science.1068440
190. Huber CM, Doisne JM, Colucci F. IL-12/15/18-preactivated NK cells suppress GvHD in a mouse model of mismatched hematopoietic cell transplantation. *Eur J Immunol* (2015) 45:1727–35. doi:10.1002/eji.201445200
191. Filipovich AH, Weisdorf D, Pavletic S, Socie G, Wingard JR, Lee SJ, et al. National Institutes of Health consensus development project on criteria for clinical trials in chronic graft-versus-host disease: I. Diagnosis and staging working group report. *Biol Blood Marrow Transplant* (2005) 11:945–56. doi:10.1016/j.bbmt.2005.09.004
192. Flowers ME, Inamoto Y, Carpenter PA, Lee SJ, Kiem HP, Petersdorf EW, et al. Comparative analysis of risk factors for acute graft-versus-host disease and for chronic graft-versus-host disease according to National Institutes of Health consensus criteria. *Blood* (2011) 117:3214–9. doi:10.1182/blood-2010-08-302109
193. Schroeder MA, DiPersio JF. Mouse models of graft-versus-host disease: advances and limitations. *Dis Model Mech* (2011) 4:318–33. doi:10.1242/dmm.006668
194. Shustov A, Nguyen P, Finkelman F, Elkon KB, Via CS. Differential expression of Fas and Fas ligand in acute and chronic graft-versus-host disease: up-regulation of Fas and Fas ligand requires CD8+ T cell activation and IFN-gamma production. *J Immunol* (1998) 161:2848–55.
195. Morris SC, Cheek RL, Cohen PL, Eisenberg RA. Allotype-specific immunoregulation of autoantibody production by host B cells in chronic graft-versus host disease. *J Immunol* (1990) 144:916–22.
196. Wu T, Young JS, Johnston H, Ni X, Deng R, Racine J, et al. Thymic damage, impaired negative selection, and development of chronic graft-versus-host disease caused by donor CD4+ and CD8+ T cells. *J Immunol* (2013) 191:488–99. doi:10.4049/jimmunol.1300657
197. Oliner H, Schwartz R, Dameshek W. Studies in experimental autoimmune disorders. I. Clinical and laboratory features of autoimmunization (runt disease) in the mouse. *Blood* (1961) 17:20–44.
198. Stastny P, Stembridge VA, Ziff M. Homologous disease in the adult rat, a model for autoimmune disease. I. General features and cutaneous lesions. *J Exp Med* (1963) 118:635–48. doi:10.1084/jem.118.4.635
199. Lewis RM, Armstrong MY, André-Schwartz J, Muftuoglu A, Beldotti L, Schwartz RS. Chronic allogeneic disease. I. Development of glomerulonephritis. *J Exp Med* (1968) 128:653–79. doi:10.1084/jem.128.4.653
200. Rappaport H, Khalil A, Halle-Pannenko O, Pritchard L, Dantchev D, Mathé G. Histopathologic sequence of events in adult mice undergoing lethal graft-versus-host reaction developed across H-2 and/or non-H-2 histocompatibility barriers. *Am J Pathol* (1979) 96:121–42.
201. Beschorner WE, Tutschka PJ, Santos GW. Chronic graft-versus-host disease in the rat radiation chimera. I. Clinical features, hematology, histology, and immunopathology in long-term chimeras. *Transplantation* (1982) 33:393–9. doi:10.1097/00007890-198204000-00010
202. Jaffee BD, Claman HN. Chronic graft-versus-host disease (GVHD) as a model for scleroderma. I. Description of model systems. *Cell Immunol* (1983) 77:1–12. doi:10.1016/0008-8749(83)90001-1
203. Claman HN, Jaffee BD, Huff JC, Clark RA. Chronic graft-versus-host disease as a model for scleroderma. II. Mast cell depletion with deposition of immunoglobulins in the skin and fibrosis. *Cell Immunol* (1985) 94:73–84. doi:10.1016/0008-8749(85)90086-3
204. Allison AC, Denman AM, Barnes RD. Cooperating and controlling functions of thymus-derived lymphocytes in relation to autoimmunity. *Lancet* (1971) 2:135–40. doi:10.1016/S0140-6736(71)92306-3
205. Fialkow PJ, Gilchrist C, Allison AC. Autoimmunity in chronic graft-versus-host disease. *Clin Exp Immunol* (1973) 13:479–86.
206. Zhang Y, Hexner E, Frank D, Emerson SG. CD4+ T cells generated de novo from donor hemopoietic stem cells mediate the evolution from acute to chronic graft-versus-host disease. *J Immunol* (2007) 179:3305–14. doi:10.4049/jimmunol.179.5.3305
207. Zhao D, Young JS, Chen YH, Shen E, Yi T, Todorov I, et al. Alloimmune response results in expansion of autoreactive donor CD4+ T cells in transplants that can mediate chronic graft-versus-host disease. *J Immunol* (2011) 186:856–68. doi:10.4049/jimmunol.1002195
208. Hamilton BL. L3T4-positive T cells participate in the induction of graft-vs-host disease in response to minor histocompatibility antigens. *J Immunol* (1987) 139:2511–5.

209. Korngold R, Sprent J. Variable capacity of L3T4+ T cells to cause lethal graft-versus-host disease across minor histocompatibility barriers in mice. *J Exp Med* (1987) 165:1552–64. doi:10.1084/jem.165.6.1552
210. Yamashita K, Choi U, Woltz PC, Foster SF, Sneller MC, Hakim FT, et al. Severe chronic graft-versus-host disease is characterized by a preponderance of CD4(+) effector memory cells relative to central memory cells. *Blood* (2004) 103:3986–8. doi:10.1182/blood-2003-09-3286
211. Xystrakis E, Bernard I, Dejean AS, Alsaati T, Druet P, Saoudi A. Alloreactive CD4 T lymphocytes responsible for acute and chronic graft-versus-host disease are contained within the CD45RChigh but not the CD45RClow subset. *Eur J Immunol* (2004) 34:408–17. doi:10.1002/eji.200324528
212. Tschetter JR, Mozes E, Shearer GM. Progression from acute to chronic disease in a murine parent-into-F1 model of graft-versus-host disease. *J Immunol* (2000) 165:5987–94. doi:10.4049/jimmunol.165.10.5987
213. Kim J, Kim HJ, Choi WS, Nam SH, Cho HR, Kwon B. Maintenance of CD8+ T-cell anergy by CD4+CD25+ regulatory T cells in chronic graft-versus-host disease. *Exp Mol Med* (2006) 38:494–501. doi:10.1038/emm.2006.58
214. Biedermann BC, Sahner S, Gregor M, Tsakiris DA, Jeanneret C, Pober JS, et al. Endothelial injury mediated by cytotoxic T lymphocytes and loss of microvessels in chronic graft versus host disease. *Lancet* (2002) 359:2078–83. doi:10.1016/S0140-6736(02)08907-9
215. Morris SC, Cheek RL, Cohen PL, Eisenberg RA. Autoantibodies in chronic graft versus host result from cognate T-B interactions. *J Exp Med* (1990) 171:503–17. doi:10.1084/jem.171.2.503
216. Via CS, Rus V, Nguyen P, Linsley P, Gause WC. Differential effect of CTLA4Ig on murine graft-versus-host disease (GVHD) development: CTLA4Ig prevents both acute and chronic GVHD development but reverses only chronic GVHD. *J Immunol* (1996) 157:4258–67.
217. Shustov A, Luzina I, Nguyen P, Papadimitriou JC, Handwerger B, Elkon KB, et al. Role of perforin in controlling B-cell hyperactivity and humoral autoimmunity. *J Clin Invest* (2000) 106:R39–47. doi:10.1172/JCI8876
218. Perruche S, Marandin A, Kleinclauss F, Angonin R, Fresnay S, Baron MH, et al. Association of mixed hematopoietic chimerism with elevated circulating autoantibodies and chronic graft-versus-host disease occurrence. *Transplantation* (2006) 81:573–82. doi:10.1097/01.tp.0000183878.53367.77
219. Patriarca F, Skert C, Bonifazi F, Sperotto A, Fili C, Stanzani M, et al. Effect on survival of the development of late-onset non-infectious pulmonary complications after stem cell transplantation. *Haematologica* (2006) 91:1268–72.
220. Sarantopoulos S, Stevenson KE, Kim HT, Bhuiya NS, Cutler CS, Soiffer RJ, et al. High levels of B-cell activating factor in patients with active chronic graft-versus-host disease. *Clin Cancer Res* (2007) 13:6107–14. doi:10.1158/1078-0432.CCR-07-1290
221. Asavaroengchai W, Wang H, Wang S, Wang L, Bronson R, Sykes M, et al. An essential role for IFN-gamma in regulation of alloreactive CD8 T cells following allogeneic hematopoietic cell transplantation. *Biol Blood Marrow Transplant* (2007) 13:46–55. doi:10.1016/j.bbmt.2006.09.014
222. Sarantopoulos S, Stevenson KE, Kim HT, Cutler CS, Bhuiya NS, Schowalter M, et al. Altered B-cell homeostasis and excess BAFF in human chronic graft-versus-host disease. *Blood* (2009) 113:3865–74. doi:10.1182/blood-2008-09-177840
223. Srinivasan M, Flynn R, Price A, Ranger A, Browning JL, Taylor PA, et al. Donor B-cell alloantibody deposition and germinal center formation are required for the development of murine chronic GVHD and bronchiolitis obliterans. *Blood* (2012) 119:1570–80. doi:10.1182/blood-2011-07-364414
224. Schultz KR, Paquet J, Bader S, HayGlass KT. Requirement for B cells in T cell priming to minor histocompatibility antigens and development of graft-versus-host disease. *Bone Marrow Transplant* (1995) 16:289–95.
225. Zorn E, Miklos DB, Floyd BH, Mattes-Ritz A, Guo L, Soiffer RJ, et al. Minor histocompatibility antigen DBY elicits a coordinated B and T cell response after allogeneic stem cell transplantation. *J Exp Med* (2004) 199:1133–42. doi:10.1084/jem.20031560
226. Young JS, Wu T, Chen Y, Zhao D, Liu H, Yi T, et al. Donor B cells in transplants augment clonal expansion and survival of pathogenic CD4+ T cells that mediate autoimmune-like chronic graft-versus-host disease. *J Immunol* (2012) 189:222–33. doi:10.4049/jimmunol.1200677
227. Via CS, Rus V, Gately MK, Finkelman FD. IL-12 stimulates the development of acute graft-versus-host disease in mice that normally would develop chronic, autoimmune graft-versus-host disease. *J Immunol* (1994) 153:4040–7.
228. Okubo T, Hagiwara E, Ohno S, Tsuji T, Ihata A, Ueda A, et al. Administration of an IL-12-encoding DNA plasmid prevents the development of chronic graft-versus-host disease (GVHD). *J Immunol* (1999) 162:4013–7.
229. De Wit D, Van Mechelen M, Zanin C, Doutrelepont JM, Velu T, Gérard C, et al. Preferential activation of Th2 cells in chronic graft-versus-host reaction. *J Immunol* (1993) 150:361–6.
230. Bogunia-Kubik K, Mlynarczewska A, Wysoczanska B, Lange A. Recipient interferon-gamma 3/3 genotype contributes to the development of chronic graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. *Haematologica* (2005) 90:425–6.
231. Watanabe Y, Kawakami H, Kawamoto H, Ikemoto Y, Masuda K, Takezaki E, et al. Effect of neonatal thymectomy on experimental autoimmune hepatitis in mice. *Clin Exp Immunol* (1987) 67:105–13.
232. Tung KS, Smith S, Matzner P, Kasai K, Oliver J, Feuchter F, et al. Murine autoimmune oophoritis, epididymoorchitis, and gastritis induced by day 3 thymectomy. Autoantibodies. *Am J Pathol* (1987) 126:303–14.
233. Sakoda Y, Hashimoto D, Asakura S, Takeuchi K, Harada M, Tanimoto M, et al. Donor-derived thymic-dependent T cells cause chronic graft-versus-host disease. *Blood* (2007) 109:1756–64. doi:10.1182/blood-2006-08-042853
234. Zhang C, Todorov I, Zhang Z, Liu Y, Kandeel F, Forman S, et al. Donor CD4+ T and B cells in transplants induce chronic graft-versus-host disease with autoimmune manifestations. *Blood* (2006) 107:2993–3001. doi:10.1182/blood-2005-09-3623
235. Anderson BE, McNiff JM, Jain D, Blazar BR, Shlomchik WD, Shlomchik MJ. Distinct roles for donor- and host-derived antigen-presenting cells and costimulatory molecules in murine chronic graft-versus-host disease: requirements depend on target organ. *Blood* (2005) 105:2227–34. doi:10.1182/blood-2004-08-3032
236. Na IK, Lu SX, Yim NL, Goldberg GL, Tsai J, Rao U, et al. The cytolytic molecules Fas ligand and TRAIL are required for murine thymic graft-versus-host disease. *J Clin Invest* (2010) 120:343–56. doi:10.1172/JCI39395
237. McCormick LL, Zhang Y, Tootell E, Gilliam AC. Anti-TGF-beta treatment prevents skin and lung fibrosis in murine sclerodermatous graft-versus-host disease: a model for human scleroderma. *J Immunol* (1999) 163:5693–9.
238. Olivieri A, Locatelli F, Zecca M, Sanna A, Cimminiello M, Raimondi R, et al. Imatinib for refractory chronic graft-versus-host disease with fibrotic features. *Blood* (2009) 114:709–18. doi:10.1182/blood-2009-02-204156
239. Magro L, Mohty M, Catteau B, Coiteux V, Chevallier P, Terriou L, et al. Imatinib mesylate as salvage therapy for refractory sclerotic chronic graft-versus-host disease. *Blood* (2009) 114:719–22. doi:10.1182/blood-2009-02-204750
240. Zerr P, Distler A, Palumbo-Zerr K, Tomcik M, Vollath S, Dees C, et al. Combined inhibition of c-Abl and PDGF receptors for prevention and treatment of murine sclerodermatous chronic graft-versus-host disease. *Am J Pathol* (2012) 181:1672–80. doi:10.1016/j.ajpath.2012.07.017
241. Cutler C, Miklos D, Kim HT, Treister N, Woo SB, Bienfang D, et al. Rituximab for steroid-refractory chronic graft-versus-host disease. *Blood* (2006) 108:756–62. doi:10.1182/blood-2006-01-0233
242. Ratanatharathorn V, Ayash L, Reynolds C, Silver S, Reddy P, Becker M, et al. Treatment of chronic graft-versus-host disease with anti-CD20 chimeric monoclonal antibody. *Biol Blood Marrow Transplant* (2003) 9:505–11. doi:10.1016/S1083-8791(03)00216-7
243. Okamoto M, Okano A, Akamatsu S, Ashihara E, Inaba T, Takenaka H, et al. Rituximab is effective for steroid-refractory sclerodermatous chronic graft-versus-host disease. *Leukemia* (2006) 20:172–3. doi:10.1038/sj.leu.2403996
244. Gong Q, Ou Q, Ye S, Lee WP, Cornelius J, Diehl L, et al. Importance of cellular microenvironment and circulatory dynamics in B cell immunotherapy. *J Immunol* (2005) 174:817–26. doi:10.4049/jimmunol.174.2.817
245. Zorn E, Kim HT, Lee SJ, Floyd BH, Litsa D, Arumugarajah S, et al. Reduced frequency of FOXP3+ CD4+CD25+ regulatory T cells in patients with chronic graft-versus-host disease. *Blood* (2005) 106:2903–11. doi:10.1182/blood-2005-03-1257
246. Rieger K, Loddenkemper C, Maul J, Fietz T, Wolff D, Terpe H, et al. Mucosal FOXP3+ regulatory T cells are numerically deficient in acute and chronic GvHD. *Blood* (2006) 107:1717–23. doi:10.1182/blood-2005-06-2529
247. Taylor PA, Lees CJ, Blazar BR. The infusion of ex vivo activated and expanded CD4(+)CD25(+) immune regulatory cells inhibits graft-versus-host disease lethality. *Blood* (2002) 99:3493–9. doi:10.1182/blood.V99.10.3493

248. Koreth J, Matsuoka K, Kim HT, McDonough SM, Bindra B, Alyea EP III, et al. Interleukin-2 and regulatory T cells in graft-versus-host disease. *N Engl J Med* (2011) 365:2055–66. doi:10.1056/NEJMoa1108188
249. Hakim F, Fowler DH, Shearer GM, Gress RE. Animal models of acute and chronic graft-versus-host disease. *Curr Protoc Immunol* (2001) Chapter 4:Unit4.3. doi:10.1002/0471142735.im0403s27
250. Storb R, Epstein RB, Ragde H, Bryant J, Thomas ED. Marrow engraftment by allogeneic leukocytes in lethally irradiated dogs. *Blood* (1967) 30:805–11.
251. Rosinski SL, Stone B, Graves SS, Fuller DH, De Rosa SC, Spies GA, et al. Development of a minor histocompatibility antigen vaccine regimen in the canine model of hematopoietic cell transplantation. *Transplantation* (2015) 99:2083–94. doi:10.1097/TP.0000000000000744
252. Ferrara JL, Reddy P. Pathophysiology of graft-versus-host disease. *Semin Hematol* (2006) 43:3–10. doi:10.1053/j.seminhematol.2005.09.001
253. Hulsdunker J, Zeiser R. Insights into the pathogenesis of GvHD: what mice can teach us about man. *Tissue Antigens* (2015) 85:2–9. doi:10.1111/tan.12497
254. Reddy P, Negrin R, Hill GR. Mouse models of bone marrow transplantation. *Biol Blood Marrow Transplant* (2008) 14:129–35. doi:10.1016/j.bbmt.2007.10.021
255. Yap KK. Modelling human development and disease: the role of animals, stem cells, and future perspectives. *Aust Med Stud J* (2012) 3:8–10.
256. Kihara T, Morita H, Kitano Y. Studies on transient graft-versus-host disease in BALB/c nude mice injected with allogeneic C57BL/6 splenocytes. *J Dermatol Sci* (1996) 11:76–83. doi:10.1016/0923-1811(95)00423-8
257. Sprent J, Schaefer M, Korngold R. Role of T cell subsets in lethal graft-versus-host disease (GVHD) directed to class I versus class II H-2 differences. II. Protective effects of L3T4+ cells in anti-class II GVHD. *J Immunol* (1990) 144:2946–54.
258. Ali N, Flutter B, Sanchez Rodriguez R, Sharif-Paghaleh E, Barber LD, Lombardi G, et al. Xenogeneic graft-versus-host-disease in NOD-scid IL-2Rgammanull mice display a T-effector memory phenotype. *PLoS One* (2012) 7:e44219. doi:10.1371/journal.pone.0044219
259. Becker C, Taube C, Bopp T, Becker C, Michel K, Kubach J, et al. Protection from graft-versus-host disease by HIV-1 envelope protein gp120-mediated activation of human CD4+CD25+ regulatory T cells. *Blood* (2009) 114:1263–9. doi:10.1182/blood-2009-02-206730
260. Bohana-Kashtan O, Morisot S, Hildreth R, Brayton C, Levitsky HI, Civin CI. Selective reduction of graft-versus-host disease-mediating human T cells by ex vivo treatment with soluble Fas ligand. *J Immunol* (2009) 183:696–705. doi:10.4049/jimmunol.0800561
261. King MA, Covassin L, Brehm MA, Racki W, Pearson T, Leif J, et al. Human peripheral blood leucocyte non-obese diabetic-severe combined immunodeficiency interleukin-2 receptor gamma chain gene mouse model of xenogeneic graft-versus-host-like disease and the role of host major histocompatibility complex. *Clin Exp Immunol* (2009) 157:104–18. doi:10.1111/j.1365-2249.2009.03933.x
262. Brehm MA, Shultz LD, Luban J, Greiner DL. Overcoming current limitations in humanized mouse research. *J Infect Dis* (2013) 208(Suppl 2):S125–30. doi:10.1093/infdis/jit319
263. Zinöcker S, Dressel R, Wang XN, Dickinson AM, Rolstad B. Immune reconstitution and graft-versus-host reactions in rat models of allogeneic hematopoietic cell transplantation. *Front Immunol* (2012) 3:355. doi:10.3389/fimmu.2012.00355
264. Konstandin MH, Wabnitz GH, Aksoy H, Kirchgessner H, Dengler TJ, Samstag Y. A sensitive assay for the quantification of integrin-mediated adhesiveness of human stem cells and leukocyte subpopulations in whole blood. *J Immunol Methods* (2007) 327:30–9. doi:10.1016/j.jim.2007.07.005
265. Lin JY, Tsai FC, Wallace CG, Huang WC, Wei FC, Liao SK. Optimizing chimerism level through bone marrow transplantation and irradiation to induce long-term tolerance to composite tissue allotransplantation. *J Surg Res* (2012) 178:487–93. doi:10.1016/j.jss.2012.02.064
266. Vogelsang GB, Santos GW, Colvin OM, Chen T. Thalidomide for graft-versus-host disease. *Lancet* (1988) 1:827. doi:10.1016/S0140-6736(88)91690-X
267. Pakkala I, Taskinen E, Pakkala S, Raisanen-Sokolowski A. MC1288, a vitamin D analog, prevents acute graft-versus-host disease in rat bone marrow transplantation. *Bone Marrow Transplant* (2001) 27:863–7. doi:10.1038/sj.bmt.1702873
268. Gendelman M, Hecht T, Logan B, Vodanovic-Jankovic S, Komorowski R, Drobyski WR. Host conditioning is a primary determinant in modulating the effect of IL-7 on murine graft-versus-host disease. *J Immunol* (2004) 172:3328–36. doi:10.4049/jimmunol.172.5.3328
269. Mabed M, Maroof S, Zalta K, El-Awadee M. Delayed or delayed sequential bone marrow transplantation: relevance for acute graft-versus-host disease prevention after major H2 incompatible transplantation. *Bone Marrow Transplant* (2005) 35:803–6. doi:10.1038/sj.bmt.1704877
270. Schwarte S, Hoffmann MW. Influence of radiation protocols on graft-vs-host disease incidence after bone-marrow transplantation in experimental models. *Methods Mol Med* (2005) 109:445–58.
271. Zeiser R, Nguyen VH, Hou JZ, Beilhack A, Zambricki E, Buess M, et al. Early CD30 signaling is critical for adoptively transferred CD4+CD25+ regulatory T cells in prevention of acute graft-versus-host disease. *Blood* (2007) 109:2225–33. doi:10.1182/blood-2006-07-038455
272. Sadeghi B, Aghdami N, Hassan Z, Forouzanfar M, Rozell B, Abedi-Valugerdi M, et al. GVHD after chemotherapy conditioning in allogeneic transplanted mice. *Bone Marrow Transplant* (2008) 42:807–18. doi:10.1038/bmt.2008.261
273. Riesner K, Kalupa M, Shi Y, Elezkurtaj S, Penack O. A preclinical acute GVHD mouse model based on chemotherapy conditioning and MHC-matched transplantation. *Bone Marrow Transplant* (2015) 51:410–7. doi:10.1038/bmt.2015.279
274. Seok J, Warren HS, Cuenca AG, Mindrinos MN, Baker HV, Xu W, et al. Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc Natl Acad Sci U S A* (2013) 110:3507–12. doi:10.1073/pnas.1222878110
275. Teshima T, Hill GR, Pan L, Brinson YS, van den Brink MR, Cooke KR, et al. IL-11 separates graft-versus-leukemia effects from graft-versus-host disease after bone marrow transplantation. *J Clin Invest* (1999) 104:317–25. doi:10.1172/JCI7111
276. Antin JH, Lee SJ, Neuberg D, Alyea E, Soiffer RJ, Sonis S, et al. A phase I/II double-blind, placebo-controlled study of recombinant human interleukin-11 for mucositis and acute GVHD prevention in allogeneic stem cell transplantation. *Bone Marrow Transplant* (2002) 29:373–7. doi:10.1038/sj.bmt.1703394
277. Ferrara JL, Cooke KR, Pan L, Krenger W. The immunopathophysiology of acute graft-versus-host-disease. *Stem Cells* (1996) 14:473–89. doi:10.1002/stem.140473
278. Anasetti C, Martin PJ, Hansen JA, Appelbaum FR, Beatty PG, Doney K, et al. A phase I-II study evaluating the murine anti-IL-2 receptor antibody 2A3 for treatment of acute graft-versus-host disease. *Transplantation* (1990) 50:49–54. doi:10.1097/00007890-199007000-00010
279. Belanger C. Use of an anti-interleukine 2 receptor monoclonal antibody for GvHD prophylaxis in unrelated bone marrow transplantation. GEGMO Group. *Bone Marrow Transplant* (1993) 11(Suppl 1):112–3.
280. Stevens JC, Banks GT, Festing MF, Fisher EM. Quiet mutations in inbred strains of mice. *Trends Mol Med* (2007) 13:512–9. doi:10.1016/j.molmed.2007.10.001
281. Storb R, Deeg HJ, Whitehead J, Appelbaum F, Beatty P, Bensinger W, et al. Methotrexate and cyclosporine compared with cyclosporine alone for prophylaxis of acute graft versus host disease after marrow transplantation for leukemia. *N Engl J Med* (1986) 314:729–35. doi:10.1056/NEJM198603203141201
282. Mestas J, Hughes CC. Of mice and not men: differences between mouse and human immunology. *J Immunol* (2004) 172:2731–8. doi:10.4049/jimmunol.172.5.2731
283. Ordemann R, Hutchinson R, Friedman J, Burakoff SJ, Reddy P, Duffner U, et al. Enhanced allostimulatory activity of host antigen-presenting cells in old mice intensifies acute graft-versus-host disease. *J Clin Invest* (2002) 109:1249–56. doi:10.1172/JCI0214793
284. Wang XN, Collin M, Sviland L, Marshall S, Jackson G, Schulz U, et al. Skin explant model of human graft-versus-host disease: prediction of clinical outcome and correlation with biological risk factors. *Biol Blood Marrow Transplant* (2006) 12:152–9. doi:10.1016/j.bbmt.2005.11.188
285. Sviland L, Dickinson AM. A human skin explant model for predicting graft-versus-host disease following bone marrow transplantation. *J Clin Pathol* (1999) 52:910–3. doi:10.1136/jcp.52.12.910

286. Vogelsang GB, Hess AD, Berkman AW, Tutschka PJ, Farmer ER, Converse PJ, et al. An in vitro predictive test for graft versus host disease in patients with genotypic HLA-identical bone marrow transplants. *N Engl J Med* (1985) 313:645–50. doi:10.1056/NEJM198509123131101
287. Novota P, Zinöcker S, Norden J, Wang XN, Sviland L, Opitz L, et al. Expression profiling of major histocompatibility and natural killer complex genes reveals candidates for controlling risk of graft versus host disease. *PLoS One* (2011) 6:e16582. doi:10.1371/journal.pone.0016582
288. Jucker M. The benefits and limitations of animal models for translational research in neurodegenerative diseases. *Nat Med* (2010) 16:1210–4. doi:10.1038/nm.2224
289. Chu YW, Gress RE. Murine models of chronic graft-versus-host disease: insights and unresolved issues. *Biol Blood Marrow Transplant* (2008) 14:365–78. doi:10.1016/j.bbmt.2007.12.002
290. Socie G, Blazar BR. Acute graft-versus-host disease: from the bench to the bedside. *Blood* (2009) 114:4327–36. doi:10.1182/blood-2009-06-204669
291. Kilkenny C, Parsons N, Kadyszewski E, Festing MF, Cuthill IC, Fry D, et al. Survey of the quality of experimental design, statistical analysis and reporting of research using animals. *PLoS One* (2009) 4:e7824. doi:10.1371/journal.pone.0007824

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Milestones of Hematopoietic Stem Cell Transplantation – From First Human Studies to Current Developments

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Since the early beginnings, in the 1950s, hematopoietic stem cell transplantation (HSCT) has become an established curative treatment for an increasing number of patients with life-threatening hematological, oncological, hereditary, and immunological diseases. This has become possible due to worldwide efforts of preclinical and clinical research focusing on issues of transplant immunology, reduction of transplant-associated morbidity, and mortality and efficient malignant disease eradication. The latter has been accomplished by potent graft-versus-leukemia (GvL) effector cells contained in the stem cell graft. Exciting insights into the genetics of the human leukocyte antigen (HLA) system allowed improved donor selection, including HLA-identical related and unrelated donors. Besides bone marrow, other stem cell sources like granulocyte-colony stimulating-mobilized peripheral blood stem cells and cord blood stem cells have been established in clinical routine. Use of reduced-intensity or non-myeloablative conditioning regimens has been associated with a marked reduction of non-hematological toxicities and eventually, non-relapse mortality allowing older patients and individuals with comorbidities to undergo allogeneic HSCT and to benefit from GvL or antitumor effects. Whereas in the early years, malignant disease eradication by high-dose chemotherapy or radiotherapy was the ultimate goal; nowadays, allogeneic HSCT has been recognized as cellular immunotherapy relying prominently on immune mechanisms and to a lesser extent on non-specific direct cellular toxicity. This chapter will summarize the key milestones of HSCT and introduce current developments.

Keywords: hematopoietic stem cell transplantation, milestones, conditioning, HLA typing, stem cell source

INTRODUCTION

Seven decades ago, scientists working on the Manhattan Project in the United States discovered that the hematopoietic system was the most radiation-sensitive tissue. In 1945, the plutonium and the atom bomb ended World War II by striking Japan with over 200,000 fatalities. Subsequently, scientists began to explore ways of protecting humans from irradiation. In 1949, Jacobson and

colleagues made the observation that mice were able to survive otherwise lethal irradiation when their spleen was exteriorized and protected from irradiation (1). Furthermore, intraperitoneal injection of spleen cells (1) or infusion of bone marrow (BM) cells (2) achieved the same protective effect resulting in animals' survival. In the late 1950s, engraftment of donor-derived BM cells in lethally irradiated mice and dogs was reported (3, 4). Later on, with the concept of using irradiation for therapeutic elimination of leukemia, the use of conditioning regimens for successful transplantation was introduced into clinic. Thomas performed the first ever BM transplantation (BMT) for acute leukemia patients. He conditioned the patients with total body irradiation (TBI) and high-dose chemotherapy to get rid of the underlying disease and then infused BM, which led to hematological reconstitution (5). Unfortunately, major complications including graft failure, graft rejection, graft-versus-host disease (GvHD), and/or death from opportunistic infections led to poor transplant outcomes, and no patients who were transplanted in the late 1950s and early 1960s survived.

In 1958, van Rood and colleagues recognized that, during pregnancy, about one-third of women formed antibodies against human leukocyte antigens (HLA), which made it possible to unravel the genetics of HLA (6, 7). Thereafter, numerous studies elucidated the role of these antigens in hematopoietic stem cell transplantation (HSCT) leading to an improved understanding of the importance of HLA typing and thus, improved donor selection strategies. In 1968, van Bekkum, Balner, and colleagues (8) had successfully developed a HSCT protocol in monkeys and shared that information not only in the Netherlands but also with Good and coworkers in the United States. That same year, three patients, two in the United States and one in the Netherlands, all suffering from a congenital immune deficiency, were succesfully transplanted with hemopoietic stem cells from a HLA-identical sibling donor (9). In 1972, Thomas and colleagues reported the first experience with allografting for severe aplastic anemia (SAA) (10). In the following years, more centers were able to perform allogeneic HSCT successfully in patients with hematologic malignancies including acute leukemia.

In the 1970s, a major concern was the limitation of allogeneic grafting to HLA-identical sibling pairs. Only about one-fourth of the patients in need had a suitable stem cell donor. In 1979, Hansen and colleagues performed the first successful marrow graft from an unrelated donor (URD) for a patient with leukemia (11). After establishing URD registries in numerous countries and their cooperation under the umbrella of the BM donors worldwide (BMDW), an increasing number of patients have received allogeneic HSCT.

The use of peripheral blood stem cells (PBSC) or cord blood (CB) instead of BM for HSCT has meantime become a routine part of transplantation. Until the early 1919s, only myeloablative (MA) conditioning, including cyclophosphamide (CY), busulfan (BU), and/or TBI, was in clinical use (12, 13). In the mid-1990s, introduction of fludarabine (FLU) (14, 15) and reduction of doses of alkylating agents (16) as well as TBI dose (17), established non-MA (NMA) or reduced-intensity conditioning (RIC).

In the following sections, we will describe the current developments in allogeneic HSCT focusing on conditioning therapies, donor selection, and stem cell sources.

CONDITIONING THERAPY FOR HSCT

For successful HSCT, it is necessary that the incoming donor stem cells have sufficient graft space and support for proliferation and differentiation. Therefore, the existing host stem cells must be eradicated from the host stem cell niche in the BM, or suppressed from growth in order for donor stem cells to engraft adequately. It is also crucial that recipients are immunocompromised to prevent rejection of the incoming donor cells by the host immune system. The pretransplant conditioning regimen suppresses and functionally eradicates the host immune system and thus allows donor stem cells to home in the BM microenvironment without the risk of graft rejection. Finally and most importantly, the conditioning therapy eradicates the underlying malignant disease. This provides long-term disease control by reducing leukemic cells to a minimum, which allows final elimination by graft-versus-leukemia (GvL) effects. An exception to this rule due to a deficiency in their own immune system are infants suffering from severe combined immunodeficiency (SCID) (18) and patients with SAA with an identical twin donor who may be grafted without conditioning therapy (19).

Types of Conditioning Regimens

Many different conditioning treatments exist, but a generally accepted definition is of two types: MA conditioning and NMA/reduced-intensity conditioning (19).

Myeloablative conditioning is of high-dose intensity consisting of a single agent or combination of agents that eradicate the patient's hematopoietic cells in the BM and induce long-lasting trilineage aplasia. This strategy includes TBI and/or alkylating agents at doses that will not allow autologous hematologic recovery resulting in profound pancytopenia within days from the time of administration (19). Pancytopenia is life-threatening and fatal unless patients' hematopoiesis is restored by infusion of hematopoietic stem cells (HSCs). TBI has been the primary therapeutic modality for allogeneic HSCT for patients with hematological malignancies. TBI has retained wide usage during the last decades due to its excellent immunosuppressive properties, activity against a wide variety of malignancies including ones refractory to chemotherapy, penetration of sanctuary sites such as the central nervous system (CNS) and the relative lack of non-hematologic toxicities when given at high doses. Most frequently, fractionated TBI of 12–14 Gy given over 3–4 days has been combined with CY at a dose of 120 mg/kg body weight (BW) administered over 2 days (20) as initially used for successful BMT in the late 1970s (13). Since patients with lymphoma previously given dose-limiting local radiotherapy to the mediastinum experienced a high incidence of fatal interstitial pneumonitis syndrome (IPS) following TBI (21), non-TBI-containing conditioning regimens were explored. Chemotherapy regimens also allowed to avoid the long-term sequelae of TBI including cataracts, sterility, growth, and developmental problems in children and secondary malignancies such as myelodysplasia (MDS) (22).

BU is an alkylating agent with profound MA properties and marked activity against a variety of malignancies. A regimen of BU at a dose of 4 mg/kg/day for 4 days combined with CY at a dose of 120 mg/kg BW has been widely administered for the treatment of malignant and non-malignant diseases followed by allogeneic HSCT (12, 20). Few studies compared chemotherapy regimens with TBI-based conditioning. Two randomized studies demonstrated the equivalency of BU/CY and CY/TBI in patients with chronic myeloid leukemia (CML) in chronic phase receiving HLA-identical allografts (23, 24). One randomized study in patients with acute myeloid leukemia (AML) given HLA-identical transplants showed superiority for CY/TBI conditioning due to a lower relapse rate (25).

Although MA conditioning therapy provides rapid hematopoietic engraftment of donor cells, it also causes myelotoxicity, considerable morbidity, and mortality (20). Tissues containing proliferating cells such as gonads, hair follicles, oral mucosa, and the gastrointestinal (GI) tract are most susceptible, followed by the lung and other organs such as liver and, to a lesser extent, the renal and cardiac system. Besides mucositis, nausea, diarrhea, peripheral neuropathies, alopecia, and skin rash have been reported after MA conditioning. High-dose BU has been associated with interstitial pneumonitis, hepatic sinusoidal obstructive syndrome (26), and increased risk of chronic GvHD (27). The endothelial system has been increasingly recognized as an additional highly sensitive target, and this may explain some of the observed other organ toxicities (28).

Non-myeloablative conditioning can be defined as a regimen that will cause minimal cytopenia, little early toxicity, and does not require hematopoietic stem cell support (17, 19). Nevertheless, NMA conditioning regimens are immunosuppressive to the extent that, when followed by granulocyte-colony stimulating factor (G-CSF) mobilized PBSC or BM infusion, donor lymphohematopoietic cells can engraft with at least mixed donor/recipient chimerism (29). The final elimination of host hematopoiesis is then achieved by graft-versus-hematopoietic and GvL effects of the donor immune cells resulting eventually in full donor chimerism (17). Since Storb and colleagues demonstrated in the dog model that 2 Gy of TBI in combination with systemic immunosuppression allowed establishment of stable mixed hematopoietic chimerism after BM infusion of a DLA-identical littermate (30), low dose TBI at a dose of 2 Gy on the day of graft infusion has become a well-established NMA regimen (17, 20). Furthermore, low dose TBI has been combined with FLU at a dose of 90 mg/m^2 over 3 days (17, 20). The Stanford group combined total lymphoid irradiation of 8–12 Gy delivered over 11 days and antithymocyte globuline (ATG) administered over 5 days in order to facilitate the presence of natural killer/T cells that suppress GvHD, but retain GvL effects (31).

Non-myeloablative conditioning regimens usually exert minor antitumor effects and rely mainly on the subsequent GvL effects of the reconstituted donor immune cells for eradication of the underlying disease.

Reduced-intensity conditioning regimens try to fill the gap between MA and NMA conditioning therapies. The concept of RIC is based on the idea of preventing the high toxicity and mortality associated with MA conditioning regimens in patients with advanced age or relevant comorbidities but providing sufficient immunoablation to prevent graft rejection (20). The goal of RIC is not always complete tumor eradication and thus complete destruction of host hematopoiesis but sufficient control of the underlying disease by cytotoxic therapy followed by the immune-mediated effects of donor graft cells (20). Although intensity of regimens applied vary considerably, all investigators aimed at replacing cytotoxic components of the conditioning regimen with less toxic, but immunosuppressive, agents to enable hematopoietic engraftment. A commonly used RIC regimen consists of FLU at a dose of 125–150 mg/m^2 administered over 5 days in combination with melphalan at a dose of 100–140 mg/m^2 given over 2 days showing efficacy in patients with AML and MDS (32). Slavin and colleagues reported a regimen consisting of FLU, BU, and ATG in patients both with hematologic malignancies as well as genetic disorders resulting in neutropenia and complete or partial donor chimerism in all patients (16). A sequential regimen of cytoreduction with FLU, cytarabine, and amsacrine followed by 3 days of rest and then 4 Gy of TBI, ATG, and CY (FLAMSA regimen) achieved promising results in patients with high-risk AML and MDS including ones with primary refractory disease and adverse risk cytogenetics (33). Subsequent replacement of TBI with BU further improved outcomes (34).

During the last few years, a variety of new agents have been introduced for RIC therapies including other alkylating agents such as high-dose treosulfan, clofarabine, or thiotepa in order to improve patients' outcome by reducing relapse rates in individuals with advanced disease stages prior to HSCT (35).

Table 1 summarizes the currently and most frequently used conditioning regimens for allogeneic HSCT.

Selection of Conditioning Therapy in HSCT

There is, as yet, no standard decision-making criteria for choosing a conditioning regimen for HSCT. Due to the scarcity of available direct comparative data from randomized clinical trials, assessing the efficacy of the various conditioning treatments is difficult. Before making a choice for a given patient, clinicians should consider relevant comorbidities, disease status, patient's age, risk of rejection, and risk of relapse. In many diseases, MA conditioning therapy achieves a higher control of underlying malignancy, but this is at the risk of increased toxicity and higher

TABLE 1 | Frequently used conditioning regimens in various transplant centers worldwide.

Intensity	Regimen	Comments
Myeloablative	CY/TBI BU/CY	Profound pancytopenia, require stem cell support, substantial non-hematological toxicities
Non-myeloablative	FLU/TBI TLI/ATG Low dose TBI	Minimal cytopenia, do not require stem cell support
Reduced intensity	FLU/MEL FLU/BU FLU/CY	Intermittent cytopenia, reduced non-hematological toxicities

CY, cyclophosphamide; TBI, total body irradiation; BU, busulfan; FLU, fludarabine; TLI, total lymphoid irradiation; ATG, antithymocyte globulin; MEL, melphalan.

incidence of transplant-related mortality (TRM). In contrast, RIC regimens have been associated with a higher relapse risk especially in patients with advanced stage of disease (36–38). Dreger and colleagues reported that RIC contributed to 18% of 1-year TRM (39), while MA conditioning generally contributes to over 30% of 1-year TRM, respectively (40).

In a multicenter retrospective study, Martino and colleagues reported the outcome of 836 patients receiving HLA-identical sibling donor transplants with either MA or RIC therapy (41). They observed that the 3-year relapse rate was significantly increased after RIC whereas 3-year NRM was decreased in RIC compared to MA conditioning with a similar rate of overall survival in both groups (41). This suggests that RIC is promising regarding early NRM but at the cost of disease relapse.

New tools for risk assessment before allogeneic HSCT such as the hematopoietic cell transplantation-specific comorbidity index have been used for valid and reliable scoring of pretransplant comorbidities that have predicted non-relapse mortality (NRM) and survival in large patient cohorts (42). These pretransplant assessments aim to improve HSCT outcomes by allowing the selection of conditioning intensity based on the patients' comorbidity index.

Relapse has remained the major cause of mortality after HSCT. Peritransplant and posttransplant strategies to reduce the relapse risk have been discussed by various investigators and research groups (43, 44). So far, available clinical interventions are limited including timely reduction of systemic immunosuppression and prophylactic administration of donor lymphocyte infusions (DLI) (33, 44–46). In patients with high-risk AML and MDS, adjuvant DLI after RIC according to the FLAMSA protocol resulted in significantly improved 7-year survival and lower relapse rates compared to control HSCT patients not given additional DLIs (45). The German Lymphoma group investigated rituximab or no additional therapy in patients with relapsed or refractory lymphoma starting 21 days after allogeneic HSCT (47). Peggs and colleagues administered DLI for mixed chimerism after HSCT with RIC achieving full donor status in 19 of 22 patients (86%) with Hodgkin's lymphoma (46). Of note, 4-year relapse incidence was 5% in these patients. Targeted tyrosine kinase inhibitors including sorafenib, sunitinib, and midostaurin have been used pre- and posttransplant in patients with AML as relapse treatment or maintenance therapy for prevention of relapse (48). Another strategy consists of posttransplant monitoring of $CD34^+$ donor cell chimerism in patients with AML and azacytidine treatment for patients with a decline of $CD34^+$ donor cells below 80% (49).

Conditioning-Mediated Inflammation and GvHD

After administration of any conditioning therapy, but especially prominent after MA and RIC in contrast to NMA regimens, the major finding is epithelial damage caused by chemotherapeutic drugs and TBI leading to release of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin-1 (IL-1) and resulting in the so-called "cytokine storm" (50). Endotoxins such as lipopolysaccharides (LPS) are also translocated across the damaged intestinal mucosa, resulting in a further activation of the host's innate immune system and further cytokine release (51). A whole set of damage-associated molecular patterns (DAMPs) released from damaged cells such as uric acid and ATP and various pathogen-associated molecular patterns (PAMPs) released by the microbiota contribute to this activation (52). The signals generated cause activation of host antigen-presenting cells (APCs) such as dendritic cells (DC) (53) and increased presentation of HLA major and minor antigens. As a result, naive donor T-cells are recruited, activated, and expanded leading to the interaction with host APCs. At this stage, DCs initiate GvHD and prime naive T-cells (53). Recipient's hematopoietic APCs activate donor $CD8^+$ T-cells while, in the gut, non-hematopoietic APCs can activate donor $CD4^+$ T-cells for the induction of GvHD (54). In this way, conditioning therapy can mediate tissue damage leading to donor T-cell expansion and attack on target organs (preferentially gut) leading to acute and/or chronic GvHD.

Couriel and colleagues evaluated the influence of MA and NMA regimens in 137 patients undergoing HLA-identical sibling donor transplantation (55). They observed significantly higher incidences of grades II–IV acute GvHD in patients given MA conditioning therapy. Furthermore, the cumulative incidence of chronic GvHD was 40% higher in patients receiving MA conditioning when compared to NMA. These results suggested that MA conditioning was not only myelotoxic but also accounted for profound higher incidences of both acute and chronic GvHD (55). Similar results were observed by Mielcarek and coworkers (56).

It can be noted that, currently, there is no best conditioning regimen available that can ensure disease-free survival (DFS) of patients after HSCT. Choice of conditioning therapy used prior to transplantation highly depends on recipient age, underlying disease, and disease status prior to HSCT, relevant comorbidities, and type of donor (matched or mismatched; related, or unrelated). MA conditioning is perhaps preferred for younger patients, and RIC may be given to patients whose underlying disease has been well controlled. A choice among various conditioning regimes is largely based upon center experience. However, randomized clinical trials comparing different conditioning therapy intensities are highly warranted to increase the level of evidence for choosing the appropriate pretransplant treatment wisely in order to allow long-term DFS with good quality of life. In addition, a standardized developed therapy worldwide, or even between European centers, would greatly facilitate the evaluation of biomarkers predicting outcome and response to therapy. This would further improve transplant results in the future.

IMPORTANCE OF THE HLA REGION

HLA-Typing Techniques

Improvements in HSCT would not have been possible without the significant progress made in the understanding of the HLA system and the development of HLA typing techniques. The major HLA antigens essential for immune responses are HLA-A, -B, -C, -DR, -DQ, and -DP, which are encoded by polymorphic genes in the human genome, with 1–1543 alleles per locus (for the most up to date number of HLA alleles reported

in IMGT-HLA[1]). The remarkable allelic polymorphism makes HLA typing very challenging (57). The pioneering work of HLA typing was carried out with serological and cellular assays. Serological techniques started with agglutination, but were soon based on complement-dependent cytotoxicity, cell cultures in mixed lymphocyte reactions, and cell-mediated cytolysis. One of the most important drawbacks of those methods is the need for viable cells expressing surface antigens. Over the years, several improvements were made to the serological techniques culminating in the development of the Terasaki microlymphocytotoxicity test (58). After modifications, it is still in use today, especially to clarify the absence of some "null alleles" (variants affecting expression of protein) or to decrease the number of primers or probes in DNA-based tests (59).

In the 1980s, molecular techniques were introduced into HLA typing, namely, restriction fragment length polymorphism (RFLP). Amplified DNA was digested with restriction enzymes to generate specific restriction patterns, thus leading to the identification of alleles according to the pattern. Although RFLP allowed for typing with higher sensitivity and specificity than serological methods, the procedure was still very labor-intensive and did not replace serological typing (57). Further development of PCR technologies and Sanger sequencing provided new options in the field of HLA typing, such as sequence-specific oligonucleotide probes (SSOP), sequence-specific priming (SSP), and sequencing-based typing (SBT). The SSOP system in the most practical format (reverse SSOP) involved PCR amplification of the target sequence labeled with biotinylated primers followed by hybridization with the immobilized sequence-specific probes, incubation with streptavidin conjugated to an enzyme and chromogenic substrate (60). The idea of SSOP typing was also adopted for the flow cytometry technology Luminex by changing immobilization on nylon membrane to microbeads and colorimetric to fluorescence detection technology. This allowed faster, reliable, and automated typing (61). SSP typing was the alternative and the complementary system to SSOP typing, developed based on the extension of the 3′ ends of primers, which were either matched or mismatched with the target sequence. The results of SSOP and SSP typing are considered as "low" and "intermediate" HLA resolution typing (57). Low resolution (on antigen level) and also called "2-digit typing" corresponds to the identification of broad families of alleles that cluster into serotypes (e.g., A*02). It is thus, the equivalent of serological typing (A2) (62). High-resolution (HR) typing is on an allele level and allows identification of the set of alleles encoding the same protein sequence for the region of the antigen-binding site of the HLA molecule. Alleles that are not expressed as cell surface molecules are excluded. Intermediate level is the level of resolution in between high- and low-resolution (63). SBT, which is the combination of DNA amplification and direct sequencing, provided HR HLA typing. However, ambiguity at the allelic level (linked polymorphic sequence can be outside the typed region) or genotype ambiguity (inability to establish whether linked polymorphisms are on the same -cis or different -trans allele coming from the father or the mother) still remains an important problem. In order to deal with that issue, scientists included additional exons for typing or investigated preliminary/additional typing methods to HR typing, e.g., SSP (method, which can distinguish cis/trans ambiguities) (57). Over the last few years, the breakthrough in HLA typing was the development of the next-generation sequencing (NGS) technology, which offers HR and high-throughput typing. However, it requires complex sample preparation including elaborate library preparation and sample enrichment steps and considerable bioinformatics resources for data analysis. Recently, several labs have applied NGS to genotype highly polymorphic HLA genes using different strategies of amplification, library preparation, platforms for sequencing, and sequence analysis approaches to enhance sequencing coverage and resolve ambiguities (64–66). There are still some limitations to overcome, but it is highly probable that NGS will soon become the routine method for HLA typing. Thus, in the near future, centralized typing facilities could offer reasonably priced NGS-based typing when large numbers of samples can be processed in a more automated fashion.

All described methods of HLA typing are shown in **Table 2**.

Choice of Donors for HSCT

Over the last few years, based on the outcome of many studies, identification of 10 alleles in 5 HLA loci, namely, HLA-A, -B, -C, -DRB1, and -DQB1 using HR typing has become the gold standard of URD matching in accordance with the guidelines of the European Society for Blood and Marrow Transplantation (62). In the United States, the National Marrow Donor Program

TABLE 2 | Comparison of HLA Typing Techniques.

Method	Benefits	Drawbacks
Serological	Preliminary or supportive method for molecular assays; fast and cheap	Low resolution; requires viable cells; poor reagent supply in the past, labor intense, not the current standard
Cellular	Used for HLA class II typing until approx. 2000	Low resolution; requires viable cells; labor intense, but informative; rarely used currently
RLFP	Used for HLA class II typing until approx. 2000	Low resolution; labor intense; did not replace serological methods; rarely used currently
SSOP	Involved in preliminary typing used today	Low or intermediate resolution; limited to previously known polymorphisms; restricted to selected exons
SSP	Nowadays used to distinguish cis/trans ambiguities	Low or intermediate resolution; limited to previously known polymorphisms; restricted to selected exons
SBT	High resolution	Does not distinguish cis/trans ambiguities; restricted to selected exons
NGS	High resolution; high-throughput typing; increases rate of resolved ambiguities	Complicated workflow and data analysis, novel technique, could become reasonably priced when used in centralized facilities

HLA, human leukocyte antigen; RLFP, restriction fragment length polymorphism; SSOP, sequence-specific oligonucleotide probes; SSP, sequence-specific priming; SBT, sequencing-based typing; NGS, next-generation sequencing; approx., approximately.

[1]Immunopolymorphism database (IPD) – International ImMunoGeneTics project (IMGT) database. Available from: http://www.ebi.ac.uk/ipd/imgt/hla/stats.html.

Committee has recommended allele-level typing for HLA-A, -B, -C, and -DRB1 to obtain 8/8 or 7/8 allelic identity (67) questioning the importance of HLA-DQB1 matching for outcome (68, 69). Due to the low numbers of mature T-cells in CB, higher levels of HLA-incompatibility between donor and recipient are accepted. Therefore, selection of CB units is primarily based on HLA-A, -B intermediate resolution level, and DRB1 HR level. A recent study by the Center for International Blood and Marrow Transplantation Research (CIBMTR) and Eurocord reported better outcomes in single CB transplants with improved allele-level matching for four HLA loci (-A, -B, -C, and -DRB1) suggesting that CBT with three or more allele level mismatches should be avoided, due to unacceptable levels of NRM and poorer survival (70). HR typing at 4 loci and selecting CB units matched for at least 5/8 alleles also improved TRM after double CBT (71).

In the search algorithm, genotypically identical related donors are considered to be first choice based on rapid availability and the likelihood of not only major but also minor histocompatibility antigen identity. The probability of HLA identity of a sibling is 25%. In a study utilizing birth data and statistical modeling, Besse and colleagues reported considerable variation in the likelihood in families of an HLA-identical sibling donor, ranging from 13 to 51% depending upon patient age and race/ethnicity (72). Furthermore, the present 40-year decline in birth rates is expected to lead to a 1.5-fold decrease in access to an HLA-identical sibling for today's young adults (18–44 years) when they reach the peak age for potential HSCT (61 years) compared to their contemporary counterparts (72). HLA typing of parents and siblings not only allows the identification of a potential-related donor but also reveals the distribution of haplotypes that can provide valid information whether an extended family search may be useful. HLA typing of a family is usually performed at low resolution level unless homozygosity is expected in the family requiring HR typing (59). Typing for HLA-A, -B, and -DR (6/6 matching) at low resolution enables, in most cases, determination of the paternal and maternal haplotypes present in the patient and a potential related donor (62).

For patients lacking an HLA-identical sibling donor, searches for HLA-matched donors among extended family members (grandparents, uncles/aunts, cousins, nieces, and nephews) have proven fruitful in populations where consanguineous or related marriage is common (73, 74). Otherwise, the alternative is an HLA-identical URD or a CB donor. The probability to find a matched unrelated donor (MUD) is around 30–70%, depending on the frequency of the HLA genotype in the donor registries and the patient's ethnicity (67). HR HLA typing is performed when searching for URDs to provide in depth information on the HLA type of the recipient and the potential URD. As a consequence of HR typing and thus, more adequate donor selection, the outcomes of patients transplanted from matched URD have become comparable to patients transplanted from matched sibling donors (75).

In case of a lack of a MRD or MUD, a mismatched donor can be considered (9/10 or 7/8 alleles matched) when patients urgently need a HSCT. This includes haploidentical family donors (5–9/10 or 4–7/8 alleles matched) and mismatched CB donors (<6 alleles matched). Almost all patients have a haplotype-mismatched related donor (MMRD) available. This provides the enormous advantage of immediate access to this donor, a fact that is most important for patients suffering from acute leukemia, who cannot afford a lengthy donor search and are at risk of dying of their malignancy prior to HSCT. It also allows collection of additional donor cells for peritransplant or posttransplant cellular immunotherapy, if needed. In addition, the immediate donor availability has financial implications since costs for additional donor typing and URD search can be reduced.

Until a few years ago, the use of a haplotype MMRD was associated with a significantly higher risk of GvHD and graft rejection unless the graft was T-cell depleted (76). Recently, the post-transplant administration of CY on days +3 and +4 after infusion of unmanipulated BM cells from a haploidentical donor has resulted in improved outcome with low incidence rates of both acute and chronic GvHD (77, 78). Posttransplant CY promotes immune tolerance by selectively depleting rapidly proliferating alloreactive host and donor T-cells while sparing non-alloreactive memory T-cells, regulatory T-cells, and hematopoietic progenitor cells and thus, preventing antitumor and antimicrobial immunity (79). Whereas initial protocols contained BM as graft source, comparable outcomes with BM or PBSC as stem cell sources for HSCT from haploidentical donors have meantime been reported (80). Haploidentical HSCT with posttransplant CY provided survival outcomes comparable to HSCT with an HLA-identical sibling or URD in patients with lymphoma, AML, and ALL (81, 82). In retrospective analyses, results of haploidentical HSCT for patients with AML in remission appear to be comparable to the best results of CB transplantation (83). Prospective clinical trials comparing haploidentical HSCT to CB transplantation and HSCT from other donor sources are currently ongoing.

Interestingly, the superior outcome of the maternal graft over the paternal graft has been described in haploidentical transplants (84, 85). Van Rood and colleagues demonstrated that recipients of non T-cell depleted maternal transplants had a lower incidence of acute and chronic GvHD than recipients of paternal transplants (84). Moreover, Stern and colleagues showed that haploidentical T-cell depleted stem cell transplants from mother to child had a lower relapse rate and improved survival compared to paternal grafts (85). The explanation of the observed effects can be the fact that, during pregnancy, the fetal immune system is exposed to the non-inherited maternal antigens (NIMA), and the mother is sensitized to the fetus inherited paternal antigens (IPA), establishing bidirectional immunity, which is achieved by regulatory T-cells between mother and fetus (86). This concept is supported by the persistence of fetal microchimerism in the mothers after pregnancy (87).

Effect of HLA Incompatibility and Other Clinical Parameters on HSCT Outcome

The effect of HLA mismatches on the outcome of HSCT depends mostly on the number of mismatches, locus of the mismatch, and direction of the mismatch (75, 88, 89). The immune reaction caused by an HLA-mismatch differs when the mismatch is: in the GvH direction – donor homozygous at mismatched loci; in

the host-versus-graft (HvG) direction – recipient homozygous at mismatched loci or is bidirectional – donor and recipient heterozygous at mismatched loci. The mismatched antigen in the GvH direction may be targeted by donor T-cells and cause GvHD, a mismatch in the HvG direction may be recognized by recipient T-cells and promote graft rejection, whereas a bidirectional mismatch may affect both outcomes (88, 90). Whereas patients with hematologic malignancies may benefit from the GvL effect associated with HLA-mismatched donors, this is different for patients with non-malignant diseases requiring allogeneic HSCT where the adverse effect of GvHD is not counterbalanced by a beneficial GvL effect. Mismatched transplants for patients with non-malignant disorders are strongly associated with an increased risk of graft failure, probably also due to the increased use of T-cell depletion prior to HSCT in order to decrease harmful GvHD in those patients. The recommendation for transplantation of patients with non-malignant disorders is to use matched donors whenever possible (91, 92).

Human leukocyte antigen disparity between donor and recipient impacts on the risk of severe GvHD, graft failure, and delayed immune reconstitution (93–96). On the other hand, HLA mismatches can be tolerated in transplant settings using *in vitro* T-cell depleted grafts and permissive HLA mismatches, which do not result in worse outcome (97–99).

During the last few years, the impact of allelic mismatches in specific HLA loci on the risk of GvHD development has been investigated. Several groups have shown an association between allelic mismatches in HLA-A, -B, -C, and -DRB1 and higher rates of acute GvHD (94, 100, 101). However, limited data have been published on the impact of HLA class I and class II disparities on the incidence and severity of chronic GVHD. Interestingly, chronic GvHD was triggered mainly by mismatches in HLA class I (94, 102). Morishima and colleagues found HLA-A and/or HLA-B allele mismatches to be a significant risk factor for the occurrence of chronic GvHD (94).

Since HLA-disparity between recipient and URD is a known risk factor for GvHD, and this complication also increases the incidence of opportunistic infections after HSCT, it is difficult to investigate the impact of HLA-disparity *per se* on immune reconstitution and infectious complications. However, Maury and colleagues identified an independent association of HLA incompatibility between recipient and URD on delayed recovery of $CD4^+$ T-cells and decreased T-cell proliferative responses (103). Few studies explored the impact of HLA mismatches on the rate of infections after HSCT. It has been shown that mismatched donors or URDs are independent risk factors for death due to late infection (later than 6 months after HSCT) (104). Moreover, Ljungman and colleagues reported results from a multivariate analysis indicating that recipients of mismatched family or URD grafts were more prone to develop cytomegalovirus (CMV) disease and die due to CMV-associated complications than recipients of grafts from HLA-matched sibling donors (105). In addition, Poutsiaka and colleagues observed that HLA mismatches between donor and recipient independently increased the risk of blood stream infections (106). Reasons for delayed immune reconstitution after HLA-incompatible donor HSCT may be impaired antigen presentation by APCs or impaired thymic function, since it has been previously shown that HLA mismatches negatively influence thymic-dependent T-cell reconstitution (107). However, further research on long-term immune reconstitution in the context of HLA-mismatched HSCT, especially in the adult population, is warranted.

In addition to HLA disparity, other factors are known to influence the outcome of HSCT including patient and donor age, ethnicity, and gender. The impact of patient age has been investigated by Cornelissen and colleagues in AML patients observing an adverse effect of increasing patient age on outcome due to an age-related rise of treatment-related complications (108). On the other hand, administration of RIC regimens for HSCT in older patients with AML was well tolerated and NRM at 2 years was 15% (109).

Donor age appears to be also an important factor for selecting the best donor. The data from several studies suggest that younger donor age is associated with better outcome after HSCT (110–113). Bastida and colleagues reported that patients with AML and MDS who received a graft from a donor above the age of 50 years had a worse overall survival, higher TRM, and higher relapse rates (113).

The effect of recipients' ethnicity has been reported as additional factor affecting outcome after HSCT. A comparison of results obtained after HSCT of Caucasians, African Americans, Hispanics, and Asians showed a decreased overall survival and higher risk of treatment failure among Hispanics (114–116). These differences in the outcome after HSCT are not well understood. They might be explained by polymorphisms in cytokine genes (117) and differences in minor histocompatibility antigens (mHAs) (118). However, the evaluation of the impact of donor ethnicity and donor-recipient ethnic identity did not support drawing donor ethnicity into consideration in the donor selection algorithm (119).

Various investigators observed a higher risk for transplant-related complications including GvHD after HSCT of male recipients with female donor grafts (120, 121). Of note, risk of relapse was significantly decreased in male recipients experiencing chronic GvHD and having an antibody response to recipient HY antigen (122).

Graft-versus-Leukemia Effect

While both GvL and GvHD are caused by major or minor histocompatibility antigen mismatches, prevention of leukemic relapse by enhancing the GvL effect is frequently limited by GvHD. It has become a major clinical issue to improve outcomes by separating GvL from GvHD effects in the field of HSCT. The role of mHAs in matched donor transplantation has been predominantly investigated in order to overcome this challenge (123).

However, few researchers have addressed the problem in terms of major HLA antigens. Kawase and colleagues identified eight mismatch combinations (two HLA-Cw and six HLA-DPB1), which were associated with decreased risk of relapse and differed from mismatches responsible for severe acute GvHD (124) Moreover, patients given grafts with these combinations of HLA-DPB1 had significantly better overall survival compared to recipients of completely matched donor/recipient pairs (124).

Shaw and colleagues reported comparable data concerning the role of HLA-DPB1 mismatch and a lower risk of relapse, but this effect was accompanied by an increased risk of acute GvHD (125). A model for identification of non-permissive HLA-DPB1 mismatches by the presence of T-cell-epitope mismatching has been proposed in order to provide a clinical strategy for lowering the risk of mortality after URD transplants (98, 126). Recently, Petersdorf and colleagues revealed the mechanism leading to the higher incidence of acute GvHD in recipients of grafts mismatched for HLA-DPB1 (69). They found that the risk of GvHD was influenced by the single nucleotide polymorphism in the HLA-DPB1 region responsible for the genetic control of HLA-DP expression levels. Thus, these data need further investigation but may be helpful in the future for selection of the best donor.

In a retrospective study of single unit CB recipients, van Rood and colleagues demonstrated that patients with AML and ALL who shared one or more HLA-A, -B, or -DRB1 antigens with their CB donor's IPAs had a significant decrease in leukemic relapse after HSCT compared with those who did not, providing indirect evidence that maternal microchimerism in CB mediates a GvL effect in CB transplantation (127).

Role of KIR Ligand Mismatches

Killer immunoglobulin-like receptors (KIRs) are NK receptors binding to the HLA class I molecules and thus, control the activity of NK cells. There are two types of KIRs; one inhibits the ability of NK cells to kill foreign cells and the other activates NK cells (128). Apart from the broad diversity of activating and inhibitory receptors on NK cells, differences in the expression of NK cell ligands on the cell surface of target cells determine the induction or inhibition of NK cell activity. NK cell alloreactivity in patients after HSCT is directed against leukemic cells and mediated by mismatches in the graft-versus-host (GvH) direction in HLA class I molecules, which cause the incompatibility in binding to KIRs (129). There are three known KIR ligand mismatches in the GvH direction, all of which are present in donor/missing in recipient: (1) HLA-C1, (2) HLA-C2, and (3) HLA-BW4 (130). HLA and KIR genes segregate independently on different chromosomes, thus only 25% of HLA identical siblings and less than 1% of MUD are KIR identical (130). It has been demonstrated by *in vitro* studies, murine models, and several clinical studies that KIR ligand mismatches in GvH direction are important for the success of HSCT with a haploidentical donor in patients with AML. GvH NK alloreactivity was associated with significantly improved survival, favored engraftment, eradication of AML, and reduced GvHD (131, 132). These clinical observations are based on the fact that NK cells mediate clearance of (1) residual leukemia cells resulting in lower relapse rate, (2) host T-cells improving hematopoietic engraftment, and (3) host dendritic cells reducing GvHD incidence (133).

On the other hand, conflicting results were presented on the beneficial effect of KIR ligand incompatibilities and outcome after unrelated HSCT. Giebel and colleagues reported that overall survival of patients with ALL, AML, or CML, transplanted with unmanipulated grafts of MUD with KIR ligand incompatibilities, was significantly improved (134), but other studies failed to reproduce these results (135–137). The advantage of KIR ligand mismatches on survival became more pronounced, when analysis was limited to AML patients (138, 139). The discrepancies in the results of the aforementioned studies can be explained by the heterogeneity of treatment protocols and patient cohorts. However, difficulties arise in connection with KIR ligand mismatches and outcome after HSCT. In analyses, it is difficult to show advantages of KIR-ligand mismatches when mismatches in the GvH and HvG direction exist on the same HLA molecules. Strong response from alloreactive T-cells toward the incompatible HLA molecule can override the favorable effect of KIR ligand mismatch (140).

STEM CELL SOURCES

For many years, BM harvested from the posterior iliac crests under general anesthesia had been used as the source of HSC for transplantation. In the 1990s, two new HSC options, namely, G-CSF-mobilized PBSCs and CB became available for clinical use. Although there are many differences between these three HSC sources, clinical results after HSCT seem to be comparable (141–143). The choice of different stem cell sources depends on age of the donor and the recipient, clinical comorbidities, as well as disease stage, and varies depending on the preferences of different centers and donors (**Table 3**).

PBSC – Benefits for Patients and Donors

One of the major changes in HSCT was the replacement of BM by G-CSF-mobilized PBSCs (144, 145). Over the past decade, PBSCs have become the preferable stem cell source in many transplant centers, accounting for around 75% of all HSCTs performed (142, 146, 147).

Use of PBSCs holds several advantages over BM. HSC collection from peripheral blood (PB) is preferred by donors as it spares them general anesthesia and cells can be harvested in the outpatient setting (145, 148). Karlsson and colleagues analyzed

TABLE 3 | Comparison of hematopoietic stem cell sources.

Stem cell source	Benefits		Drawbacks	
	Donor	Recipient	Donor	Recipient
BM		Lower risk of GvHD	More invasive HSC collection	
PBSC	No general anesthesia for collection; less discomfort and pain	Faster hematopoietic engraftment and immune reconstitution; enhanced GvL effect		Higher risk of GvHD
CB	Non-invasive	Lower risks of GvHD and relapse; rapid availability; increased level of HLA-disparity tolerated		Lower number of HSCs; slower immune reconstitution

BM, bone marrow; PBSC, peripheral blood stem cells; CB, cord blood; GvHD, graft-versus-host disease; HSC, hematopoietic stem cell; GvL, graft-versus-leukemia, HLA, human leukocyte antigen.

171 donors and reported significantly more prolonged pain and severe fatigue in BM donors compared to PBSC donors (149). So far, complications of growth factor administration and leukapheresis such as malignancy and stroke are no higher than those of BM collection (141). A large study in more than 9000 PBSC and BM donors demonstrated a lower risk of serious adverse events (SAE) in donors of PBSC (150). Furthermore, PBSC donors treated with G-CSF have shown no increased risk of cancer, autoimmune disease, or stroke compared with BM donors and even a lower incidence of cancer compared with the general population (150).

Besides benefits for the donors, there are advantages for the recipients as well. Faster hematopoietic engraftment and immune reconstitution have been observed in patients receiving PBSC compared to those given BM grafts (145, 151). In a clinical study using MA conditioning therapy with HLA-identical related donors, 5 and 6 days of earlier neutrophil and platelet engraftment, respectively, were observed after PBSC compared to BM grafts (152). Furthermore, after HSCT with MUD, a shorter time to absolute neutrophil count equal to 0.5×10^9/L and a shorter time of platelet engraftment were reported in the PBSC compared to the BM group (153). Several other studies and a meta-analysis including eight different trials in MUD confirm these findings and showed a higher rate of engraftment in recipients of PBSC (146, 154, 155).

Moreover, high numbers of lymphocytes in the PBSC, namely, immunocompetent T-cells may enhance the GvL effect (145, 155). Unmodified PBSC grafts may contain one log more T-lymphocytes than unmodified BM grafts (156). However, these high T-cell numbers could in parallel lead to a higher risk of GvHD (155, 156), which as a consequence may have a higher mortality. An increased incidence of chronic GvHD, but no difference in acute GvHD, was observed between PBSC and BM graft recipients in HLA-matched related settings by Campregher and colleagues (157). Eapen and colleagues reported a significantly higher incidence of chronic GvHD after MA conditioning and URD PBSC infusion (158). Although mortality risks were higher in patients with chronic GvHD, both in PBSC and BM settings, and PBSC recipients had more severe chronic GvHD, there was no difference in mortality between these two graft types (158). A multicentre, randomized trial published by Anasetti and colleagues similarly reported no difference in acute GvHD and a higher incidence of chronic GvHD in PBSC recipients, but no difference in the 2-year-survival rate compared to BM recipients (142).

Progression-free survival seems to be comparable between PBSC and BM recipients, and the risk of relapse appears to be lower in patients given PBSC (142, 151, 155).

Cord Blood – A Life-Saving Alternative

Despite the advances made in HSCT over the last decades, donor availability has remained a major obstacle and introduction of CB provided an alternative for these patients (159, 160). The first CB transplantation was performed successfully in Paris in 1988 in a pediatric patient suffering from Fanconi anemia (FA) (161). Results of CBT in adults were less favorable (160, 162). In a study by Laughlin and colleagues on 68 patients who underwent CBT, 17 died most likely as a result of the preparative regimen and 22 patients died due to an infection after HSCT (162). High death rates were attributed in part to the selection of high-risk patients, but slow myeloid engraftment could have also contributed (162). In the following years, better CB and patient selection substantially improved CBT outcome (154, 163–165). Since then, according to the BMDW database, more than 30,000 CBT have been performed and CB banks have been established around the world storing more than 600,000 CB units (160).

One of the main advantages of CB is the fact that an increased level of HLA disparity can be tolerated (166). The current standard for CB selection is donor–recipient matching at six HLA loci, namely, HLA-A, HLA-B antigen, and HLA-DRB1 allele in comparison to 8–10 loci for BM or PBSC donation (167, 168). Despite increased tolerance, HLA matching still remains, together with the cell dose infused, one of the main factors associated with improved engraftment and better survival (169, 170). The negative impact of HLA-disparity on patient outcome could be partially overcome by higher $CD34^+$ cell doses for each level of HLA disparity. Better survival was demonstrated in recipients of CB grafts with two HLA-mismatches given more than 1.7×10^5 $CD34^+$ cells per kilogram BW than those receiving a lower dose (171). Data suggest that the $CD34^+$ cell content should be the most important criterion when choosing CB grafts, followed by the degree of HLA-disparity (154). The number of total nucleated cells collected or infused should not be less than 2.5×10^7/kg BW (168, 170). Indeed, the main limitation of CB is the low number of HSCs in contrast to the numbers typically present in BM or PBSC allografts (172). Since several studies reported better engraftment in recipients of higher doses of $CD34^+$ cells (162, 169, 170), use of double CB units was introduced some years ago and has been proven safe, showing comparable overall outcomes as matched-related and unrelated HSCT (159, 172). Wagner and colleagues compared HSCT with one CB unit with double CB units used in children and adolescents with hematologic malignancies and observed no differences in survival, neutrophil recovery, and immune reconstitution between the two groups (173). However, recipients of single CB units achieved better platelet recovery and had a lower incidence of more severe acute GvHD and chronic GvHD (173).

Several studies have been performed in order to compare outcome of CB versus BM or PBSC transplantation. In 2004, Laughlin and colleagues compared mismatched CBT and mismatched BMT in adult patients and observed no significant differences in TRM, treatment failure, and overall mortality between these patient cohorts (174). Rocha and colleagues reported no significant differences between mismatched CBT and matched BMT regarding TRM, relapse rate, and leukemia-free survival (175). Takahashi and colleagues observed lower TRM and better DFS after CBT compared to BMT despite a higher HLA-mismatching rate in CBT recipients (176). The same group later reported no differences in TRM, DFS, and relapse rate after CBT when compared with BM and PBSC grafting (136). More recent studies support these results (143, 177), respectively. Terakura and colleagues analyzing HSCT outcomes in patients with ALL and AML reported similar OS and NRM comparing 8/8

allele-matched unrelated BMT with CBT leading to the conclusion that CB could be a preferable alternative (178).

Regarding the incidence of GvHD, Laughlin and colleagues reported higher rates of acute GvHD after MMUD marrow grafts and higher chronic GvHD rates after CBT (174), while Rocha and colleagues observed a lower risk of grades II–IV acute GvHD after CBT and a comparable incidence of chronic GvHD between CB and unrelated BM recipients, respectively (175). Others observed similar rates of severe acute GvHD after CBT and 8/8 matched BMT but higher rates after 7/8 matched BM grafting while the incidence of extensive chronic GvHD was significantly lower after CBT compared with 8/8 and 7/8 BM grafting (178).

In conclusion, CB as an alternative HSC source is comparable to BM and PBSC, and offers several advantages, namely, easier availability, higher tolerable HLA-disparity, lower risks of GvHD, and relapse. Nevertheless, limited cell numbers and slow immune reconstitution contributing to infections and impacting survival remain an obstacle. Novel strategies for improvement of hematopoietic and immune reconstitution after CBT include *ex vivo* expansion of CB cells using different cytokine combinations, intra-bone injection of cells, modification of homing, and the coadministration of mesenchymal stromal cells (159, 172).

CONCLUSION AND OUTLOOK

Allogeneic HSCT has become an established curative treatment of a steadily increasing number of life-threatening hematological, oncological, hereditary, and immunological diseases. During the last decades, combined research efforts including preclinical models and clinical studies on a worldwide scale has resulted in an impressive progress in various areas of HSCT. Improved patient selection, development of improved tissue typing methods, availability of URD and CB units as HSC source, and introduction of RIC and NMA conditioning regimens has resulted in improved patients' survival over the years. However, overall survival rates have remained at 40–50% for over two decades. Further, interdisciplinary research and team efforts are necessary to improve malignant disease eradication and further inspire survival in the future. In addition, a worldwide collective effort is necessary to standardize conditioning protocols, which would aid in improving outcomes.

Currently, cellular-based immunotherapies, which were pioneered by the development of allogeneic HSCT are gaining increasing clinical relevance for treatment of patients with hematologic malignancies. For decades, the contribution of donor's immune cells to elimination of host tumor cells in leukemia, lymphoma, and myeloma after HSCT has been appreciated (179–181). To reduce or avoid the occurrence of GvHD that is associated with significant morbidity and mortality, more precise and effective cell-based therapies have been developed. Immune cell engineering including adoptive transfer of T-cells genetically modified to express chimeric antigen receptors (CARs) specific for a selected tumor antigen such as CD19 in B-cell malignancies have demonstrated impressive antileukemic activity in patients with ALL, lymphoma, and chronic lymphocytic leukemia (182–185). Optimizing T-cell receptor gene therapy for hematologic malignancies aims at improving the efficacy of T-cell therapies by maintaining their effector function and promoting memory. Recent gene-editing tools such as transcription activator-like effector nucleases (TALEN) and clustered regularly interspaced short palindromic repeats (CRISPR) allow deletion of endogenous T cell receptor and HLA genes leading to removal of alloreactivity and decreased immunogenicity of third-party T-cells. Talen-engineered CAR19 T-cells from a third-party donor have recently been administered to a 11-month-old girl with relapsed B-ALL after allogeneic HSCT resulting in complete cytogenetic and molecular remission (186). This represents an important scientific development toward generic off-the-shelf T-cell receptor engineered products for treatment of a larger number of patients with hematologic malignancies.

AUTHOR CONTRIBUTIONS

EW, EH, AD, and HG designed the review and revised it critically for important intellectual content. MJ, SG, and JO provided the draft, summarized available data, and selected the references. JR and MO reviewed the manuscript and provided important suggestions. All the authors approved the final version of the manuscript.

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REFERENCES

1. Jacobson LO, Marks EK, Robson MJ, Gaston EO, Zirkle RE. Effect of spleen protection on mortality following X-irradiation. *J Lab Clin Med* (1949) 34:1538–43.
2. Lorenz E, Uphoff D, Reid TR, Shelton E. Modification of irradiation injury in mice and guinea pigs by bone marrow injections. *J Natl Cancer Inst* (1951) 12:197–201.
3. Ford CE, Hamerton JL, Barnes DWH, Loutit JF. Cytological identification of radiation-chimaeras. *Nature* (1956) 177:452–4. doi:10.1038/177452a0
4. Ferrebee JW, Lochte HL, Jaretzki A, Sahler OD, Thomas ED. Successful marrow homograft in the dog after radiation. *Surgery* (1958) 43:516–20.
5. Thomas ED, Lochte HL, Lu WC, Ferrebee JW. Intravenous infusion of bone marrow in patients receiving radiation and chemotherapy. *N Engl J Med* (1957) 257:491–6. doi:10.1056/NEJM195709122571102
6. Van Rood JJ, Eernisse JG, van Leeuwen A. Leucocyte antibodies in sera from pregnant women. *Nature* (1958) 181:1735–6. doi:10.1038/1811735a0
7. Van Rood JJ, van Leeuwen A. Leukocyte grouping. A method and its application. *J Clin Invest* (1963) 42:1382–90. doi:10.1172/JCI104822
8. Van Putten LM, Balner H, Muller-Berat CN, de Vries MJ, van Bekkum DW. Progress in the treatment and prevention of secondary disease after homologous bone marrow transplantation in monkeys. Effects of chemotherapy and of donor selection by histocompatibility testing. *Bibl Haematol* (1968) 29:574–86.

9. Bortin MM, Bach FH, van Bekkum DW, Good RA, van Rood JJ. 25th anniversary of the first successful allogeneic bone marrow transplants. *Bone Marrow Transplant* (1994) 14:211–2.
10. Thomas ED, Storb R, Fefer A, Slichter SJ, Bryant JI, Buckner CD, et al. Aplastic anemia treated by marrow transplantation. *Lancet* (1972) 1:284–9. doi:10.1016/S0140-6736(72)90292-9
11. Hansen JA, Clift RA, Thomas ED, Buckner CD, Storb R, Giblett ER. Transplantation of marrow from an unrelated donor to a patient with acute leukemia. *N Engl J Med* (1980) 303:565–7. doi:10.1056/NEJM198009043031007
12. Santos GW, Tutschka PJ, Brookmeyer R, Saral R, Beschorner WE, Bias WB, et al. Marrow transplantation for acute nonlymphocytic leukemia after treatment with busulfan and cyclophosphamide. *N Engl J Med* (1983) 309:1347–53. doi:10.1056/NEJM198312013092202
13. Thomas ED, Buckner CD, Banaji M, Clift RA, Fefer A, Flournoy N, et al. One hundred patients with acute leukemia treated by chemotherapy, total body irradiation, and allogeneic marrow transplantation. *Blood* (1977) 49:511–33.
14. Giralt S, Estey E, Albitar M, van Besien K, Rondon G, Anderlini P, et al. Engraftment of allogeneic hematopoietic progenitor cells with purine analog-containing chemotherapy: harnessing graft-versus-leukemia without myeloablative therapy. *Blood* (1997) 89:4531–6.
15. Terenzi A, Aristei C, Aversa F, Perruccio K, Chionne F, Raymondi C, et al. Efficacy of fludarabine as an immunosuppressor for bone marrow transplantation conditioning: preliminary results. *Transplant Proc* (1996) 28:3101.
16. Slavin S, Nagler A, Naparstek E, Kapelushnik Y, Aker M, Cividalli G, et al. Nonmyeloablative stem cell transplantation and cell therapy as an alternative to conventional bone marrow transplantation with lethal cytoreduction for the treatment of malignant and nonmalignant hematologic diseases. *Blood* (1998) 91:756–63.
17. McSweeney PA, Niederwieser D, Shizuru JA, Sandmaier MB, Molina AJ, Maloney DG, et al. Hematopoietic cell transplantation in older patients with hematologic malignancies: replacing high-dose cytotoxic therapy with graft-versus-tumor effects. *Blood* (2001) 97:3390–400. doi:10.1182/blood.V97.11.3390
18. Grunebaum E, Mazzolari E, Porta F, Dallera D, Atkinson A, Reid B, et al. Bone marrow transplantation for severe combined immune deficiency. *JAMA* (2006) 295:508–18. doi:10.1001/jama.295.5.508
19. Bacigalupo A, Ballen K, Rizzo D, Giralt S, Lazarus H, Ho V, et al. Defining the intensity of conditioning regimens: working definitions. *Biol Blood Marrow Transplant* (2009) 15:1628–33. doi:10.1016/j.bbmt.2009.07.004
20. Apperley J, Carreras E, Gluckman E, Gratwohl A, Masszi T. Principles of conditioning. In: Apperley J, Carreras E, Gluckman E, Masszi T, editors. *Haematopoietic Stem Cell Transplantation*. Genoa: Forum Service Editore (2008). p. 128–44.
21. Pecego R, Hill R, Appelbaum FR, Amos D, Buckner CD, Fefer A, et al. Interstitial pneumonitis following autologous bone marrow transplantation. *Transplantation* (1986) 42:515–7. doi:10.1097/00007890-198611000-00015
22. Metayer C, Curtis RE, Vose J, Sobocinski KA, Horowitz MM, Bhatia S, et al. Myelodysplastic syndrome and acute myeloid leukemia after autotransplantation for lymphoma: a multi-center case-controll study. *Blood* (2003) 101:2015–23. doi:10.1182/blood-2002-04-1261
23. Clift RA, Buckner CD, Thomas ED, Bensinger WI, Bowden R, Bryant E, et al. Marrow transplantation for chronic myeloid leukemia: a randomized study comparing cyclophosphamide and total body irradiation with busulfan and cyclophosphamide. *Blood* (1994) 84:2036–43.
24. Devergie A, Blaise D, Attal M, Tigaud JD, Jouet JP, Vernant JP, et al. Allogeneic bone marrow transplantation for chronic myeloid leukemia in first chronic phase: a randomized trial of busulfan-cytoxan versus cytoxan-total body irradiation as preparative regimen. *Blood* (1995) 85:2263–8.
25. Blaise D, Maraninchi D, Archimbaud E, Reiffers J, Devergie A, Jouet JP, et al. Allogeneic bone marrow transplantation for acute myeloid leukemia in first remission: a randomzed trial of a busulfan-cytoxan versus cytoxan-total body irradiation as preparative regimen. *Blood* (1992) 79:2578–82.
26. Dix S, Wingard J, Mullins R, Jerkunica I, Davidson T, Gilmore C, et al. Association of busulfan area under the curve with veno-occlusive disease following BMT. *Bone Marrow Transplant* (1996) 17(2):225–30.
27. Ringden O, Remberger M, Ruutu T, Nikoskelainen J, Volin L, Vindelov L, et al. Increased risk of chronic graft-versus-host disease, obstructive bronchiolitis, and alopecia with busulfan versus total body irradiation: long-term results of a randomized trial in allogeneic marrow recipients with leukemia. *Blood* (1999) 93:2196–201.
28. Palomo M, Diaz-Ricart M, Carbo C, Rovira M, Fernandez-Aviles F, Martine C, et al. Endothelial dysfunction after hematopoietic stem cell transplantation: role of the conditioning regimen and the type of transplantation. *Biol Blood Marrow Transplant* (2010) 16:985–93. doi:10.1016/j.bbmt.2010.02.008
29. Mattsson J, Uzunel M, Remberger M, Ringden O. T cell mixed chimerism is significantly correlated to a decreased risk of acute graft-versus-host disease after allogeneic stem cell transplantation. *Transplantation* (2001) 71:433–9. doi:10.1097/00007890-200102150-00017
30. Storb R, Yu C, Wagner JL, Deeg HJ, Georges G, Kiem HP, et al. Stable mixed hematopoietic chimerism in DLA-identicallittermate dogs given sublethal total body irradiation before and pharmacological immunosuppression after marrow transplantation. *Blood* (1997) 89:3048–54.
31. Lowsky R, Takahashi T, Liu YP, Dejbakhsh-Jones S, Grumet C, Shizuru JA, et al. Protective conditioning for acute graft-versus-host disease. *N Engl J Med* (2005) 353:1321–31. doi:10.1056/NEJMoa050642
32. Popat U, de Lima MJ, Saliba RM, Anderlini P, Andersson BS, Alousi AM, et al. Long-term outcome of reduced-intensity allogeneic hematopoietic SCT in patients with AML in CR. *Bone Marrow Transplant* (2012) 47:212–6. doi:10.1038/bmt.2011.61
33. Schmid C, Schleuning M, Ledderose G, Tischer J, Kolb HJ. Sequential regimen of chemotherapy, reduced-intensity conditioning for allogeneic stem-cell transplantation, and prophylactic donor lymphocyte infusion in high-risk acute myeloid leukemia and myelodysplastic syndrome. *J Clin Oncol* (2005) 23:5675–87. doi:10.1200/JCO.2005.07.061
34. Christopeit M, Badbaran A, Alawi M, Zabelina T, Zeck G, Wolschke C, et al. Correlation of somatic mutations with outcome after FLAMSA-busulfan sequential conditioning and allogeneic stem cell transplantation in patients with myelodysplastic syndromes. *Eur J Haematol* (2016) 97(3):288–96. doi:10.1111/ejh.12724
35. Gyurkocza B, Sandmaier BM. Conditioning regimens for hematopoietic cell transplantation: one size does not fit all. *Blood* (2014) 124:344–53. doi:10.1182/blood-2014-02-514778
36. Aschan J. Risk assessment in haematopoietic stem cell transplantation: conditioning. *Best Pract Res Clin Haematol* (2007) 20:295–310. doi:10.1016/j.beha.2006.09.004
37. Abdul Wahid SF, Nor-Azimah I, Mohd-Razif MI, Wan JF, Nor-Rafeah T, Yap SWE, et al. Comparison of reduced-intensity and myeloablative conditioning regimens for allogeneic hematopoietic stem cell transplantation in patients with acute myeloid leukemia and acute lymphoblastic leukemia: a meta-analysis. *Stem Cells Dev* (2014) 23:2535–52. doi:10.1089/scd.2014.0123
38. Blijlevens N, Donnelly J, De Pauw B. Prospective evaluation of gut mucosal barrier injury following various myeloablative regimens for haematopoietic stem cell transplant. *Bone Marrow Transplant* (2005) 35:707–11. doi:10.1038/sj.bmt.1704863
39. Dreger P, Brand R, Hansz J, Milligan D, Corradini P, Finke J, et al. Treatment-related mortality and graft-versus-leukemia activity after allogeneic stem cell transplantation for chronic lymphocytic leukemia using intensity-reduced conditioning. *Leukemia* (2003) 17:841–8. doi:10.1038/sj.leu.2402905
40. Esteve J, Villamor N, Colomer D, Cervantes F, Campo E, Carreras E, et al. Stem cell transplantation for chronic lymphocytic leukemia: different outcome after autologous and allogeneic transplantation and correlation with minimal residual disease status. *Leukemia* (2001) 15:445–51. doi:10.1038/sj.leu.2402036
41. Martino R, Lacobelli S, Brand R, Jansen T, van Biezen A, Finke J, et al. Retrospective comparison of reduced-intensity conditioning and conventional high-dose conditioning for allogeneic hematopoietic stem cell transplantation using HLA-identical sibling donors in myelodysplastic syndromes. *Blood* (2006) 108:836–46. doi:10.1182/blood-2005-11-4503
42. Sorror ML, Maris MB, Storb R, Baron F, Sandmaier BM, Maloney D, et al. Hematopoietic cell transplantation (HCT)-specific comorbidity index: a new tool for risk assessment before allogeneic HCT. *Blood* (2005) 106:2912–9. doi:10.1182/blood-2005-05-2004
43. Porter DL, Alyea EP, Antin JH, DeLima M, Estey E, Falkenburg JHF, et al. NCI First International Workshop on the biology, prevention and treatment of relapse after allogeneic hematopoietic stem cell transplantation: report

from the Committee on Treatment of Relapse after allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant* (2010) 16:1467–503. doi:10.1016/j.bbmt.2010.08.001

44. Kekre N, Koreth J. Novel strategies to prevent relapse after allogeneic haematopoietic stem cell transplantation for acute myeloid leukaemia and myelodysplastic syndromes. *Curr Opin Hematol* (2015) 22:116–22. doi:10.1097/MOH.0000000000000116
45. Jedlickova Z, Schmid C, Koenecke C, Hertenstein B, Baurmann H, Schwerdtfeger R, et al. Long-term results of adjuvant donor lymphocyte transfusion in AML after allogeneic stem cell transplantation. *Bone Marrow Transplant* (2016) 51:663–7. doi:10.1038/bmt.2015.234
46. Peggs KS, Kayani I, Edwards N, Kottaridis P, Goldstone AH, Linch DC, et al. Donor lymphocyte infusions modulate relapse risk in mixed chimeras and induce durable salvage in relapsed patients after T-cell-depleted allogeneic transplantation for Hodgkin's lymphoma. *J Clin Oncol* (2011) 29:971–8. doi:10.1200/JCO.2010.32.1711
47. Glass B, Hasenkamp J, Wulf G, Dreger P, Pfreundschuh M, Gramatzki M, et al. Rituximab after lymphoma-directed conditioning and allogeneic stem-cell transplantation for relapsed and refractory aggressive non-Hodgkin lymphoma (DSHNHL R3): an open-label, randomised, phase 2 trial. *Lancet Oncol* (2014) 15:757–66. doi:10.1016/S1470-2045(14)70161-5
48. Mawad R, Lionberger JM, Pagel JM. Strategies to reduce relapse after allogeneic hematopoietic cell transplantation in acute myeloid leukemia. *Curr Hematol Malig Rep* (2013) 8:132–40. doi:10.1007/s11899-013-0153-6
49. Platzbecker U, Wermke M, Radke J, Oelschlaegel U, Seltmann F, Kiani A, et al. Azacitidine for treatment of imminent relapse in MDS or AML patients after allogeneic HSCT: results of the RELAZA trial. *Leukemia* (2012) 26:381–9. doi:10.1038/leu.2011.234
50. Xun CQ, Thompson JS, Jennings CD, Brown SA, Widmer MB. Effect of total body irradiation, busulfan-cyclophosphamide, or cyclophosphamide conditioning on inflammatory cytokine release and development of acute and chronic graft-versus-host disease in H-2-incompatible transplanted SCID mice. *Blood* (1994) 83:2360–7.
51. Teshima T, Ordemann R, Reddy P, Gagin S, Liu C, Cooke KR, et al. Acute graft-versus-host disease does not require alloantigen expression on host epithelium. *Nat Med* (2002) 8:575–81. doi:10.1038/nm0602-575
52. Ramadan A, Paczesny S. Various forms of tissue damage and danger signals following hematopoietic stem-cell transplantation. *Front Immunol* (2015) 6:14. doi:10.3389/fimmu.2015.00014
53. Matzinger P. The danger model: a renewed sense of self. *Science* (2002) 296:301–5. doi:10.1126/science.1071059
54. Koyama M, Kuns RD, Olver SD, Raffelt NC, Wilson YA, Don ALJ, et al. Recipient nonhematopoietic antigen-presenting cells are sufficient to induce lethal acute graft-versus-host disease. *Nat Med* (2012) 18:135–42. doi:10.1038/nm.2597
55. Couriel DR, Saliba RM, Giralt S, Khouri I, Andersson B, de Lima M, et al. Acute and chronic graft-versus-host disease after ablative and nonmyeloablative conditioning for allogeneic hematopoietic transplantation. *Biol Blood Marrow Transplant* (2004) 10:178–85. doi:10.1016/j.bbmt.2003.10.006
56. Mielcarek M, Martin PJ, Leisenring W, Flowers MED, Maloney DG, Sandmaier BM, et al. Graft-versus-host disease after nonmyeloablative versus conventional hematopoietic stem cell transplantation. *Blood* (2003) 102:756–62. doi:10.1182/blood-2002-08-2628
57. Erlich H. HLA DNA typing: past, present, and future. *Tissue Antigens* (2012) 80:1–11. doi:10.1111/j.1399-0039.2012.01881.x
58. Terasaki PI, McClelland JD. Microdroplet assay of human serum cytotoxins. *Nature* (1964) 204:998–1000. doi:10.1038/204998b0
59. Nowak J. Role of HLA in hematopoietic SCT. *Bone Marrow Transplant* (2008) 42:S71–6. doi:10.1038/bmt.2008.288
60. Saiki RK, Walsh PS, Levenson CH, Erlich HA. Genetic analysis of amplified DNA with immobilized sequence-specific oligonucleotide probes. *Proc Natl Acad Sci U S A* (1989) 86:6230–4. doi:10.1073/pnas.86.16.6230
61. Dalva K, Beksac M. HLA typing with sequence-specific oligonucleotide primed PCR (PCR-SSO)and use of the Luminex technology. *Methods Mol Med* (2007) 134:61–9. doi:10.1007/978-1-59745-223-6_5
62. Tiercy J. The role of HLA in HSCT. In: Apperley J, Carreras E, Gluckman E, editors. *Haematopoietic Stem Cell Transplantation – EBMT-ESH Handbook*. (2008). p. 46–65.
63. Nunes E, Heslop H, Fernandez-Vina M, Taves C, Wagenknecht DR, Eisenbrey AB, et al. Definitions of histocompatibility typing terms. *Blood* (2011) 118:e180–3. doi:10.1182/blood-2011-05-353490
64. Ehrenberg P, Geretz A, Baldwin K, Apps R, Polonis V, Robb M, et al. High-throughput multiplex HLA genotyping by next-generation sequencing using multi-locus individual tagging. *BMC Genomics* (2014) 15:864. doi:10.1186/1471-2164-15-864
65. Wittig M, Anmarkrud JA, Kässens JC, Koch S, Forster M, Ellinghaus E, et al. Development of a high-resolution NGS-based HLA-typing and analysis pipeline. *Nucleic Acids Res* (2015) 43:e70. doi:10.1093/nar/gkv184
66. Gabriel C, Fürst D, Faé I, Wenda S, Zollikofer C, Mytilineos J, et al. HLA typing by next-generation sequencing – getting closer to reality. *Tissue Antigens* (2014) 83:65–75. doi:10.1111/tan.12298
67. Gragert L, Eapen M, Williams E, Freeman J, Spellman S, Baitty R, et al. HLA match likelihoods for hematopoietic stem-cell grafts in the U.S. registry. *N Engl J Med* (2014) 371:339–48. doi:10.1056/NEJMsa1311707
68. Petersdorf EW, Gooley T, Malkki M, Anasetti C, Martin P, Woolfrey A, et al. The biological significance of HLA-DP gene variation in haematopoietic cell transplantation. *Br J Haematol* (2001) 112:988–94. doi:10.1046/j.1365-2141.2001.02655.x
69. Petersdorf EW, Malkki M, O'hUigin C, Carrington M, Gooley T, Haagenson MD, et al. High HLA-DP expression and graft-versus-host disease. *N Engl J Med* (2015) 373:599–609. doi:10.1056/NEJMoa1500140
70. Eapen M, Klein JP, Ruggeri A, Spellman S, Lee SJ, Anasetti C, et al. Impact of allele-level HLA matching on outcomes after myeloablative single unit umbilical cord blood transplantation for hematologic malignancy. *Blood* (2014) 123:133–40. doi:10.1182/blood-2013-05-506253
71. Oran B, Cao K, Saliba RM, Rezvani K, de Lima M, Ahmed S, et al. Better allele-level matching improves transplant-related mortality after double cord blood transplantation. *Haematologica* (2015) 100:1361–70. doi:10.3324/haematol.2015.127787
72. Besse K, Maiers M, Confer D, Albrecht M. On modeling human leukocyte antigen-identical sibling match probability for allogeneic hematopoietic cell transplantation: estimating the need for an unrelated donor source. *Biol Blood Marrow Transplant* (2016) 22:410–7. doi:10.1016/j.bbmt.2015.09.012
73. Balci YI, Tavil B, Tan CS, Ozgur TT, Bulum B, Cetin M, et al. Increased availibility of family donors for hematopoietic stem cell transplantation in a population with increased incidence of consanguinity: increased availability of family donors for HSCT patients. *Clin Transplant* (2011) 25:475–80. doi:10.1111/j.1399-0012.2010.01310.x
74. Klein T, Yaniv I, Stein J, Narinsky R, Finkelstein Y, Garty BZ. Extended family studies for the identification of allogeneic stem cell transplant donors in Jewish and Arabic patients in Israel. *Pediatr Transplant* (2005) 9:52–5. doi:10.1111/j.1399-3046.2004.00222.x
75. Robin M, Porcher R, Ades L, Boissel N, Raffoux E, Xhaard A, et al. Matched unrelated or matched sibling donors result in comparable outcomes after non-myeloablative HSCT in patients with AML or MDS. *Bone Marrow Transplant* (2013) 48:1296–301. doi:10.1038/bmt.2013.50
76. Reisner Y, Hagin D, Martelli MF. Haploidentical hematopoietic transplantation: current status and future perspectives. *Blood* (2011) 118:6006–17. doi:10.1182/blood-2011-07-338822
77. Bashey A, Solomon SR. T-cell replete haploidentical donor transplantation using post-transplant CY: an emerging standard-of-care option for patients who lack an HLA-identical sibling donor. *Bone Marrow Transplant* (2014) 49:999–1008. doi:10.1038/bmt.2014.62
78. Luznik L, O'Donnell PV, Symons HJ, Chen AR, Leffell MS, Zahurak M, et al. HLA-haploidentical bone marrow transplantation for hematologic malignancies using nonmyeloablative conditioning and high-dose, post-transplantation cyclophosphamide. *Biol Blood Marrow Transplant* (2008) 14:641–50. doi:10.1016/j.bbmt.2008.03.005
79. Luznik L, O'Donnell PV, Fuchs EJ. Post-transplantation cyclophosphamide for tolerance induction in HLA-haploidentical bone marrow transplantation. *Semin Oncol* (2012) 39:683–93. doi:10.1053/j.seminoncol.2012.09.005
80. O'Donnell PV, Eapen M, Horowitz MM, Logan BR, DiGilio A, Brunstein C, et al. Comparable outcomes with marrow or peripheral blood as stem cell sources for hematopoietic cell transplantation from haploidentical donors after non-ablative conditioning: a matched pair analysis. *Bone Marrow Transplant* (2016) 1–3. doi:10.1038/bmt.2016.215

81. Ghosh N, Karmali R, Rocha V, Ahn KW, DiGilio A, Hari PN, et al. Reduced-intensity transplantation for lymphomas using haploidentical related donors versus HLA-matched sibling donors: a Center for International Blood and Marrow Transplant research analysis. *J Clin Oncol* (2016) 34:3141–9. doi:10.1200/JCO.2015.66.3476
82. Ballen KK, Spitzer TR. The great debate: haploidentical or cord blood transplant. *Bone Marrow Transplant* (2011) 46:323–9. doi:10.1038/bmt.2010.260
83. Ciceri F, Labopin M, Aversa F, Rowe JM, Bunies D, Lewalle P, et al. A survey of fully haploidentical hematopoietic stem cell transplantation in adults with high-risk aucte leukemia: a risk factor analysis of outcomes for patients in remission at transplantation. *Blood* (2008) 112:3574–81. doi:10.1182/blood-2008-02-140095
84. van Rood JJ, Loberiza FR, Zhang M-J, Oudshoorn M, Claas F, Cairo MS, et al. Effect of tolerance to noninherited maternal antigens on the occurrence of graft-versus-host disease after bone marrow transplantation from a parent or an HLA-haploidentical sibling. *Blood* (2002) 99:1572–7. doi:10.1182/blood.V99.5.1572
85. Stern M, Ruggeri L, Mancusi A, Bernardo ME, de Angelis C, Bucher C, et al. Survival after T cell–depleted haploidentical stem cell transplantation is improved using the mother as donor. *Blood* (2008) 112:2990–5. doi:10.1182/blood-2008-01-135285
86. Tilburgs T, Scherjon SA, van der Mast BJ, Haasnoot GW, Versteeg-v.d.Voort-Maarschalk M, Roelen DL, et al. Fetal–maternal HLA-C mismatch is associated with decidual T cell activation and induction of functional T regulatory cells. *J Reprod Immunol* (2009) 82:148–57. doi:10.1016/j.jri.2009.05.003
87. Kruchen A, Stahl T, Gieseke F, Binder TM, Oezcan Z, Meisel R, et al. Fetomaternal microchimerism is associated with better outcome in haploidentical hematopoietic stem cell transplantation. *Blood* (2014) 124(21):1242.
88. Hurley CK, Woolfrey A, Wang T, Haagenson M, Umejiego J, Aljurf M, et al. The impact of HLA unidirectional mismatches on the outcome of myeloablative hematopoietic stem cell transplantation with unrelated donors. *Blood* (2013) 121:4800–6. doi:10.1182/blood-2013-01-480343
89. Kanda J, Ichinohe T, Fuji S, Maeda Y, Ohashi K, Fukuda T, et al. Impact of HLA mismatch direction on the outcome of unrelated bone marrow transplantation: a retrospective analysis from the Japan Society for Hematopoietic Cell Transplantation. *Biol Blood Marrow Transplant* (2015) 21:305–11. doi:10.1016/j.bbmt.2014.10.015
90. Petersdorf EW, Hansen JA, Martin PJ, Woolfrey A, Malkki M, Gooley T, et al. Major-histocompatibility-complex class I alleles and antigens in hematopoietic-cell transplantation. *N Engl J Med* (2001) 345:1794–800. doi:10.1056/NEJMoa011826
91. Horan J, Wang T, Haagenson M, Spellman SR, Dehn J, Eapen M, et al. Evaluation of HLA matching in unrelated hematopoietic stem cell transplantation for nonmalignant disorders. *Blood* (2012) 120:2918–24. doi:10.1182/blood-2012-03-417758
92. Kojima S, Matsuyama T, Kato S, Kigasawa H, Kobayashi R, Kikuta A, et al. Outcome of 154 patients with severe aplastic anemia who received transplants from unrelated donors: the Japan Marrow Donor Program. *Blood* (2002) 100:799–803. doi:10.1182/blood.V100.3.799
93. Mattsson J, Ringdén O, Storb R. Graft failure after allogeneic hematopoietic cell transplantation. *Biol Blood Marrow Transplant* (2008) 14(Suppl 1):165–70. doi:10.1016/j.bbmt.2007.10.025
94. Morishima Y, Sasazuki T, Inoko H, Juji T, Akaza T, Yamamoto K, et al. The clinical significance of human leukocyte antigen (HLA) allele compatibility in patients receiving a marrow transplant from serologically HLA-A, HLA-B, and HLA-DR matched unrelated donors. *Blood* (2002) 99:4200–6. doi:10.1182/blood.V99.11.4200
95. Chang YJ, Zhao XY, Huo MR, Xu LP, Liu DH, Liu KY, et al. Immune reconstitution following unmanipulated HLA-mismatched/haploidentical transplantation compared with HLA-identical sibling transplantation. *J Clin Immunol* (2012) 32:268–80. doi:10.1007/s10875-011-9630-7
96. Teshima T, Matsuo K, Matsue K, Kawano F, Taniguchi S, Hara M, et al. Impact of human leucocyte antigen mismatch on graft-versus-host disease and graft failure after reduced intensity conditioning allogeneic haematopoietic stem cell transplantation from related donors. *Br J Haematol* (2005) 130:575–87. doi:10.1111/j.1365-2141.2005.05632.x
97. Shaw BE, Arguello R, Garcia-Sepulveda CA, Madrigal JA. The impact of HLA genotyping on survival following unrelated donor haematopoietic stem cell transplantation. *Br J Haematol* (2010) 150:251–8. doi:10.1111/j.1365-2141.2010.08224.x
98. Fleischhauer K, Shaw BE, Gooley T, Malkki M, Bardy P, Bignon J-D, et al. Effect of T-cell-epitope matching at HLA-DPB1 in recipients of unrelated-donor haemopoietic-cell transplantation: a retrospective study. *Lancet Oncol* (2012) 13:366–74. doi:10.1016/S1470-2045(12)70004-9
99. Mead AJ, Thomson KJ, Morris EC, Mohamedbhai S, Denovan S, Orti G, et al. HLA-mismatched unrelated donors are a viable alternate graft source for allogeneic transplantation following alemtuzumab-based reduced-intensity conditioning. *Blood* (2010) 115:5147–53. doi:10.1182/blood-2010-01-265413
100. Petersdorf EW, Gooley TA, Anasetti C, Martin PJ, Smith AG, Mickelson EM, et al. Optimizing outcome after unrelated marrow transplantation by comprehensive matching of HLA class I and II alleles in the donor and recipient. *Blood* (1998) 92:3515–20.
101. Sasazuki T, Juji T, Morishima Y, Kinukawa N, Kashiwabara H, Inoko H, et al. Effect of matching of class I HLA alleles on clinical outcome after transplantation of hematopoietic stem cells from an unrelated donor. *N Engl J Med* (1998) 339:1177–85. doi:10.1056/NEJM199810223391701
102. Greinix HT, Fae I, Schneider B, Rosenmayr A, Mitterschiffthaler A, Pelzmann B, et al. Impact of HLA class I high-resolution mismatches on chronic graft-versus-host disease and survival of patients given hematopoietic stem cell grafts from unrelated donors. *Bone Marrow Transplant* (2004) 35:57–62. doi:10.1038/sj.bmt.1704741
103. Maury S, Mary J-Y, Rabian C, Schwarzinger M, Toubert A, Scieux C, et al. Prolonged immune deficiency following allogeneic stem cell transplantation: risk factors and complications in adult patients. *Br J Haematol* (2001) 115:630–41. doi:10.1046/j.1365-2141.2001.03135.x
104. Bjorklund A, Aschan J, Labopin M, Remberger M, Ringden O, Winiarski J, et al. Risk factors for fatal infectious complications developing late after allogeneic stem cell transplantation. *Bone Marrow Transplant* (2007) 40:1055–62. doi:10.1038/sj.bmt.1705856
105. Ljungman P, Aschan J, Lewensohn-Fuchs I, Carlens S, Larsson K, Lonnqvist B, et al. Results of different strategies for reducing cytomegalovirus-associated mortality in allogeneic stem cell transplant recipients. *Transplantation* (1998) 66:1330–4. doi:10.1097/00007890-199811270-00012
106. Poutsiaka DD, Price LL, Ucuzian A, Chan GW, Miller KB, Snydman DR. Blood stream infection after hematopoietic stem cell transplantation is associated with increased mortality. *Bone Marrow Transplant* (2007) 40:63–70. doi:10.1038/sj.bmt.1705690
107. Toubert A, Glauzy S, Douay C, Clave E. Thymus and immune reconstitution after allogeneic hematopoietic stem cell transplantation in humans: never say never again. *Tissue Antigens* (2012) 79:83–9. doi:10.1111/j.1399-0039.2011.01820.x
108. Cornelissen JJ, van Putten WLJ, Verdonck LF, Theobald M, Jacky E, Daenen SMG, et al. Results of a HOVON/SAKK donor versus no-donor analysis of myeloablative HLA-identical sibling stem cell transplantation in first remission acute myeloid leukemia in young and middle-aged adults: benefits for whom? *Blood* (2007) 109:3658–66. doi:10.1182/blood-2006-06-025627
109. Devine SM, Owzar K, Blum W, Mulkey F, Stone RM, Hsu JW, et al. Phase II study of allogeneic transplantation for older patients with acute myeloid leukemia in first complete remission using a reduced-intensity conditioning regimen: results from cancer and leukemia Group B 100103 (Alliance for clinical trials in oncology)/Blood and Marrow Transplant Clinical Trial Network 0502. *J Clin Oncol* (2015) 33:4167–75. doi:10.1200/JCO.2015.62.7273
110. Mehta J, Gordon LI, Tallman MS, Winter JN, Evens AO, Frankfurt O, et al. Does younger donor age affect the outcome of reduced-intensity allogeneic hematopoietic stem cell transplantation for hematologic malignancies beneficially? *Bone Marrow Transplant* (2006) 38:95–100. doi:10.1038/sj.bmt.1705388
111. Ayuk F, Zabelina T, Wortmann F, Alchalby H, Wolschke C, Lellek H, et al. Donor choice according to age for allo-SCT for AML in complete remission. *Bone Marrow Transplant* (2013) 48:1028–32. doi:10.1038/bmt.2013.14
112. Kollman C, Howe CWS, Anasetti C, Antin JH, Davies SM, Filipovich AH, et al. Donor characteristics as risk factors in recipients after transplantation of bone marrow from unrelated donors: the effect of donor age. *Blood* (2001) 98:2043–51. doi:10.1182/blood.V98.7.2043

113. Bastida JM, Cabrero M, Lopez-Godino O, Lopez-Parra M, Sanchez-Guijo F, Lopez-Corral L, et al. Influence of donor age in allogeneic stem cell transplant outcome in acute myeloid leukemia and myelodisplastic syndrome. *Leuk Res* (2015) 39:828–34. doi:10.1016/j.leukres.2015.05.003
114. Schwake CJ, Eapen M, Lee SJ, Freytes CO, Giralt SA, Navarro WH, et al. Differences in characteristics of US hematopoietic stem cell transplantation centers by proportion of racial or ethnic minorities. *Biol Blood Marrow Transplant* (2005) 11:988–98. doi:10.1016/j.bbmt.2005.07.013
115. Baker KS, Loberiza FR Jr, Yu H, Cairo MS, Bolwell BJ, Bujan-Boza WA, et al. Outcome of ethnic minorities with acute or chronic leukemia treated with hematopoietic stem-cell transplantation in the United States. *J Clin Oncol* (2005) 23:7032–42. doi:10.1200/JCO.2005.01.7269
116. Serna DS, Lee SJ, Zhang M-J, Baker KS, Eapen M, Horowitz MM, et al. Trends in survival rates after allogeneic hematopoietic stem-cell transplantation for acute and chronic leukemia by ethnicity in the United States and Canada. *J Clin Oncol* (2003) 21:3754–60. doi:10.1200/JCO.2003.03.133
117. Delaney NL, Esquenazi V, Lucas DP, Zachary AA, Leffell MS. TNF-α, TGF-β, IL-10, IL-6, and INF-γ alleles among African Americans and Cuban Americans. Report of the ASHI minority workshops: part IV. *Hum Immunol* (2004) 65:1413–9. doi:10.1016/j.humimm.2004.07.240
118. Spierings E, Hendriks M, Absi L, Canossi A, Chhaya S, Crowley J, et al. Phenotype frequencies of autosomal minor histocompatibility antigens display significant differences among populations. *PLoS Genet* (2007) 3:e103. doi:10.1371/journal.pgen.0030103
119. Ustun C, Bachanova V, Shanley R, MacMillan ML, Majhail NS, Arora M, et al. Importance of donor ethnicity/race matching in unrelated adult and cord blood allogeneic hematopoietic cell transplantation. *Leuk Lymphoma* (2014) 55:358–64. doi:10.3109/10428194.2013.800200
120. Gratwohl A, Hermans J, Niederwieser D, van Biezen A, van Houwelingen HC, Apperley J, et al. Female donors influence transplant-related mortality and relapse incidence in male recipients of sibling blood and marrow transplants. *Hematol J* (2001) 2:363–70. doi:10.1038/sj.thj.6200117
121. Randolph SSB, Gooley TA, Warren EH, Appelbaum FR, Riddell SR. Female donors contribute to a selective graft-versus-leukemia effect in male recipients of HLA-matched, related hematopoietic stem cell transplants. *Blood* (2004) 103:347–52. doi:10.1182/blood-2003-07-2603
122. Miklos DB, Kim HT, Miller KH, Guo L, Zorn E, Lee SJ, et al. Antibody responses to H-Y minor histocompatibility antigens correlate with chronic graft-versus-host disease and disease remission. *Blood* (2005) 105:2973–8. doi:10.1182/blood-2004-09-3660
123. Feng X, Hui KM, Younes HM, Brickner AG. Targeting minor histocompatibility antigens in graft versus tumor or graft versus leukemia responses. *Trends Immunol* (2008) 29:624–32. doi:10.1016/j.it.2008.09.004
124. Kawase T, Matsuo K, Kashiwase K, Inoko H, Saji H, Ogawa S, et al. HLA mismatch combinations associated with decreased risk of relapse: implications for the molecular mechanism. *Blood* (2009) 113:2851–8. doi:10.1182/blood-2008-08-171934
125. Shaw BE, Gooley TA, Malkki M, Madrigal JA, Begovich AB, Horowitz MM, et al. The importance of HLA-DPB1 in unrelated donor hematopoietic cell transplantation. *Blood* (2007) 110:4560–6. doi:10.1182/blood-2007-06-095265
126. Zino E, Frumento G, Marktel S, Sormani MP, Ficara F, Terlizzi SD, et al. A T-cell epitope encoded by a subset of HLA-DPB1 alleles determines nonpermissive mismatches for hematologic stem cell transplantation. *Blood* (2004) 103:1417–24. doi:10.1182/blood-2003-04-1279
127. Van Rood JJ, Scaradavou A, Stevens CE. Indirect evidence that maternal microchimerism in cord blood mediates a graft-versus-leukemia effect in cord blood transplantation. *Proc Natl Acad Sci U S A* (2012) 109:2509–14. doi:10.1073/pnas.1119541109
128. Pegram HJ, Andrews DM, Smyth MJ, Darcy PK, Kershaw MH. Activating and inhibitory receptors of natural killer cells. *Immunol Cell Biol* (2011) 89:216–24. doi:10.1038/icb.2010.78
129. Velardi A, Ruggeri L, Mancusi A, Aversa F, Christiansen FT. Natural killer cell allorecognition of missing self in allogeneic hematopoietic transplantation: a tool for immunotherapy of leukemia. *Curr Opin Immunol* (2009) 21:525–30. doi:10.1016/j.coi.2009.07.015
130. Shilling HG, Young N, Guethlein LA, Cheng NW, Gardiner CM, Tyan D et al. Genetic control of human NK cell repertoire. *J Immunol* (2002) 169: 239–247. doi:10.4049/jimmunol.169.1.239
131. Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, Tosti A, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science* (2002) 295:2097–100. doi:10.1126/science.1068440
132. Ruggeri L, Mancusi A, Capanni M, Urbani E, Carotti A, Aloisi T, et al. Donor natural killer cell allorecognition of missing self in haploidentical hematopoietic transplantation for acute myeloid leukemia: challenging its predictive value. *Blood* (2007) 110:433–40. doi:10.1182/blood-2006-07-038687
133. Malmberg K-J, Schaffer M, Ringdén O, Remberger M, Ljunggren H-G. KIR-ligand mismatch in allogeneic hematopoietic stem cell transplantation. *Mol Immunol* (2005) 42:531–4. doi:10.1016/j.molimm.2004.07.037
134. Giebel S, Locatelli F, Lamparelli T, Velardi A, Davies S, Frumento G, et al. Survival advantage with KIR ligand incompatibility in hematopoietic stem cell transplantation from unrelated donors. *Blood* (2003) 102:814–9. doi:10.1182/blood-2003-01-0091
135. Bornhäuser M, Schwerdtfeger R, Martin H, Frank K-H, Theuser C, Ehninger G. Role of KIR ligand incompatibility in hematopoietic stem cell transplantation using unrelated donors. *Blood* (2004) 103:2860–1. doi:10.1182/blood-2003-11-3893
136. Farag SS, Bacigalupo A, Eapen M, Hurley C, Dupont B, Caligiuri MA, et al. The effect of KIR ligand incompatibility on the outcome of unrelated donor transplantation: a report from the Center for International Blood and Marrow Transplant Research, the European Blood and Marrow Transplant Registry, and the Dutch Registry. *Biol Blood Marrow Transplant* (2006) 12:876–84. doi:10.1016/j.bbmt.2006.05.007
137. Davies SM, Ruggieri L, DeFor T, Wagner JE, Weisdorf DJ, Miller JS, et al. Evaluation of KIR ligand incompatibility in mismatched unrelated donor hematopoietic transplants. *Blood* (2002) 100:3825–7. doi:10.1182/blood-2002-04-1197
138. Cooley S, Weisdorf DJ, Guethlein LA, Klein JP, Wang T, Le CT, et al. Donor selection for natural killer cell receptor genes leads to superior survival after unrelated transplantation for acute myelogenous leukemia. *Blood* (2010) 116:2411–9. doi:10.1182/blood-2010-05-283051
139. Cooley S, Trachtenberg E, Bergemann TL, Saeteurn K, Klein J, Le CT, et al. Donors with group B KIR haplotypes improve relapse-free survival after unrelated hematopoietic cell transplantation for acute myelogenous leukemia. *Blood* (2009) 113:726–32. doi:10.1182/blood-2008-07-171926
140. Lowe EJ, Turner V, Handgretinger R, Horwitz EM, Benaim E, Hale GA, et al. T-cell alloreactivity dominates natural killer cell alloreactivity in minimally T-cell-depleted HLA-non-identical paediatric bone marrow transplantation. *Br J Haematol* (2003) 123:323–6. doi:10.1046/j.1365-2141.2003.04604.x
141. Holtick U, Albrecht M, Chemnitz JM, Theurich S, Shimabukuro-Vornhagen A, Skoetz N, et al. Comparison of bone marrow versus peripheral blood allogeneic hematopoietic stem cell transplantation for hematological malignancies in adults-a systematic review and meta-analysis. *Crit Rev Oncol Hematol* (2015) 94:179–88. doi:10.1016/j.critrevonc.2014.12.007
142. Anasetti C, Logan BR, Lee SJ, Waller EK, Weisdorf DJ, Wingard JR, et al. Peripheral-blood stem cells versus bone marrow from unrelated donors. *N Engl J Med* (2012) 367:1487–96. doi:10.1056/NEJMoa1203517
143. Chen YH, Xu L, Liu D, Chen H, Zhang X, Han W, et al. Comparative outcomes between cord blood transplantation and bone marrow or peripheral blood stem cell transplantation from unrelated donors in patients with hematologic malignancies: a single-institute analysis. *Chin Med J* (2013) 126:2499–503.
144. Schmitz N, Bacigalupo A, Labopin M, Majolino I, Laporte JP, Brinch L, et al. Transplantation of peripheral blood progenitor cells from HLA-identical sibling donors. *Br J Haematol* (1996) 95:715–23. doi:10.1046/j.1365-2141.1996.d01-1958.x
145. Dreger P, Glass B, Uharek L, Schmitz N. Allogeneic peripheral blood progenitor cells: current status and future directions. *J Hematother* (1996) 5:331–7. doi:10.1089/scd.1.1996.5.331
146. Eapen M, Le Rademacher J, Antin JH, Champlin RE, Carreras J, Fay J, et al. Effect of stem cell source on outcomes after unrelated donor transplantation in severe aplastic anemia. *Blood* (2011) 118:2618–21. doi:10.1182/blood-2011-05-354001
147. Korbling M, Freireich EJ. Twenty-five years of peripheral blood stem cell transplantation. *Blood* (2011) 117:6411–6. doi:10.1182/blood-2010-12-322214

148. Kolb HJ. As time goes by. *Biol Blood Marrow Transplant* (2015) 21:1–2. doi:10.1016/j.bbmt.2014.11.011
149. Karlsson L, Quinlan D, Guo D, Brown C, Selinger S, Klassen J, et al. Mobilized blood cells vs bone marrow harvest: experience compared in 171 donors with particular reference to pain and fatigue. *Bone Marrow Transplant* (2004) 33(7):709–13. doi:10.1038/sj.bmt.1704418
150. Pulsipher MA, Chitphakdithai P, Logan BR, Navarro WH, Levine JE, Miller JP, et al. Lower risk for serious adverse events and no increased risk for cancer after PBSC vs BM donation. *Blood* (2014) 123:3655–63. doi:10.1182/blood-2013-12-542464
151. Powles R, Mehta J, Kulkarni S, Treleaven J, Millar B, Marsden J, et al. Allogeneic blood and bone-marrow stem-cell transplantation in haematological malignant diseases: a randomised trial. *Lancet* (2000) 355(9211):1231–7. doi:10.1016/S0140-6736(00)02090-0
152. Bensinger WI, Martin PJ, Storer B, Clift R, Forman SJ, Negrin R, et al. Transplantation of bone marrow as compared with peripheral-blood cells from HLA-identical relatives in patients with hematologic cancers. *N Engl J Med* (2001) 344:175–81. doi:10.1056/NEJM200101183440303
153. Ringden O, Remberger M, Runde V, Bornhäuser M, Blau IW, Basara N, et al. Peripheral blood stem cell transplantation from unrelated donors: a comparison with marrow transplantation. *Blood* (1999) 94:455–64.
154. Eapen M, Rocha V, Sanz G, Scaradavou A, Zhang MJ, Arcese W, et al. Effect of graft source on unrelated donor haemopoietic stem-cell transplantation in adults with acute leukaemia: a retrospective analysis. *Lancet Oncol* (2010) 11(7):653–60. doi:10.1016/S1470-2045(10)70127-3
155. Wu S, Zhang C, Zhang X, Xu YQ, Deng TX. Is peripheral blood or bone marrow a better source of stem cells for transplantation in cases of HLA-matched unrelated donors? A meta-analysis. *Crit Rev Oncol Hematol* (2015) 96(1):20–33. doi:10.1016/j.critrevonc.2015.04.009
156. Storek J, Gooley T, Siadak M, Bensinger WI, Maloney DG, Chauncey TR, et al. Allogeneic peripheral blood stem cell transplantation may be associated with a high risk of chronic graft-versus-host disease. *Blood* (1997) 90:4705–9.
157. Campregher PV, Hamerschlak N, Colturato VA, Mauad MA, de Souza MP, Bouzas LF, et al. Survival and graft-versus-host disease in patients receiving peripheral stem cell compared to bone marrow transplantation from HLA-matched related donor: retrospective analysis of 334 consecutive patients. *Eur J Haematol* (2015) 95(5):421–5. doi:10.1111/ejh.12508
158. Eapen M, Logan BR, Appelbaum FR, Antin JH, Anasetti C, Couriel DR, et al. Long-term survival after transplantation of unrelated donor peripheral blood or bone marrow hematopoietic cells for hematologic malignancy. *Biol Blood Marrow Transplant* (2015) 21:55–9. doi:10.1016/j.bbmt.2014.09.006
159. Copelan EA. Hematopoietic stem-cell transplantation. *N Engl J Med* (2006) 354:1813–26. doi:10.1056/NEJMra052638
160. Ballen KK, Gluckman E, Broxmeyer HE. Umbilical cord blood transplantation: the first 25 years and beyond. *Blood* (2013) 122:491–8. doi:10.1182/blood-2013-02-453175
161. Gluckman E, Broxmeyer HA, Auerbach AD, Friedman HS, Douglas GW, Devergie A, et al. Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical-cord blood from an HLA-identical sibling. *N Engl J Med* (1989) 321:1174–8. doi:10.1056/NEJM198910263211707
162. Laughlin MJ, Barker J, Bambach B, Koc ON, Rizzieri DA, Wagner JE, et al. Hematopoietic engraftment and survival in adult recipients of umbilical-cord blood from unrelated donors. *N Engl J Med* (2001) 344:1815–22. doi:10.1056/NEJM200106143442402
163. Baron F, Labopin M, Ruggeri A, Mohty M, Sanz G, Milpied N, et al. Unrelated cord blood transplantation for adult patients with acute myeloid leukemia: higher incidence of acute graft-versus-host disease and lower survival in male patients transplanted with female unrelated cord blood-a report from Eurocord, the Acute Leukemia Working Party, and the Cord Blood Committee of the Cellular Therapy and Immunobiology Working Party of the European Group for Blood and Marrow Transplantation. *J Hematol Oncol* (2015) 8:107. doi:10.1186/s13045-015-0207-4
164. Takahashi S, Ooi J, Tomonari A, Konuma T, Tsukada N, Oiwa-Monna M, et al. Comparative single-institute analysis of cord blood transplantation from unrelated donors with bone marrow or peripheral blood stem-cell transplants from related donors in adult patients with hematologic malignancies after myeloablative conditioning regimen. *Blood* (2007) 109(3):1322–30. doi:10.1182/blood-2006-04-020172
165. Atsuta Y, Suzuki R, Nagamura-Inoue T, Taniguchi S, Takahashi S, Kai S, et al. Japan Cord Blood Bank Network. Disease-specific analyses of unrelated cord blood transplantation compared with unrelated bone marrow transplantation in adult patients with acute leukemia. *Blood* (2009) 113:1631–8. doi:10.1182/blood-2008-03-147041
166. Milano F, Boelens JJ. Stem cell comparison: what can we learn clinically from unrelated cord blood transplantation as an alternative stem cell source? *Cytotherapy* (2015) 17:695–701. doi:10.1016/j.jcyt.2015.03.003
167. Avery S, Shi W, Lubin M, Gonzales AM, Heller G, Castro-Malaspina H, et al. Influence of infused cell dose and HLA match on engraftment after double-unit cord blood allografts. *Blood* (2011) 117:3277–85. doi:10.1182/blood-2010-08-300491
168. Rocha V, Gluckman E. Improving outcomes of cord blood transplantation: HLA matching, cell dose and other graft- and transplantation-related factors. *Br J Haematol* (2009) 147:262–74. doi:10.1111/j.1365-2141.2009.07883.x
169. Gluckman E, Rocha V, Boyer-Chammard A, Locatelli F, Arcese W, Pasquini R, et al. Outcome of cord-blood transplantation from related and unrelated donors. *N Engl J Med* (1997) 337:373–81. doi:10.1056/NEJM199708073370602
170. Rubinstein P, Carrier C, Scaradavou A, Kurtzberg J, Adamson J, Migliaccio AR, et al. Outcomes among 562 recipients of placental-blood transplants from unrelated donors. *N Engl J Med* (1998) 339:1565–77. doi:10.1056/NEJM199811263392201
171. Wagner JE, Barker JN, DeFor TE, Baker KS, Blazar BR, Eide C, et al. Transplantation of unrelated donor umbilical cord blood in 102 patients with malignant and nonmalignant diseases: influence of CD34 cell dose and HLA disparity on treatment-related mortality and survival. *Blood* (2002) 100(5):1611–8. doi:10.1182/blood-2002-01-0294
172. Lund TC, Boitano AE, Delaney CS, Shpall EJ, Wagner JE. Advances in umbilical cord blood manipulation-from niche to bedside. *Nat Rev Clin Oncol* (2015) 12:163–74. doi:10.1038/nrclinonc.2014.215
173. Wagner JE Jr, Eapen M, Carter S, Wang Y, Schultz KR, Wall DA, et al. One-unit versus two-unit cord-blood transplantation for hematologic cancers. *N Engl J Med* (2014) 371:1685–94. doi:10.1056/NEJMoa1405584
174. Laughlin MJ, Eapen M, Rubinstein P, Wagner JE, Zhang MJ, Champlin RE, et al. Outcomes after transplantation of cord blood or bone marrow from unrelated donors in adults with leukemia. *N Engl J Med* (2004) 351:2265–75. doi:10.1056/NEJMoa041276
175. Rocha V, Labopin M, Sanz G, Arcese W, Schwerdtfeger R, Bosi A, et al. Transplants of umbilical-cord blood or bone marrow from unrelated donors in adults with acute leukemia. *N Engl J Med* (2004) 351:2276–85. doi:10.1056/NEJMoa041469
176. Takahashi S, Iseki T, Ooi J, Tomonari A, Takasugi K, Shimohakamada Y, et al. Single-institute comparative analysis of unrelated bone marrow transplantation and cord blood transplantation for adult patients with hematologic malignancies. *Blood* (2004) 104:3813–20. doi:10.1182/blood-2004-03-1001
177. Rodrigues CA, Rocha V, Dreger P, Brunstein CG, Sengeloev H, Finke J, et al. Alternative donor hematopoietic stem cell transplantation for mature lymphoid malignancies after reduced-intensity conditioning regimen: similar outcomes with umbilical cord blood and unrelated donor peripheral blood. *Haematologica* (2014) 99(2):370–7. doi:10.3324/haematol.2013.088997
178. Terakura S, Atsuta Y, Tsukada N, Kobayashi T, Tanaka M, Kanda J, et al. Comparison of outcomes of 8/8 and 7/8 allele-matched unrelated bone marrow transplantation and single-unit cord blood transplantation in adults with acute leukemia. *Biol Blood Marrow Transplant* (2015) 22:330–8. doi:10.1016/j.bbmt.2015.10.006
179. Rondon G, Giralt S, Huh Y, Khouri I, Andersson B, Andreeff M, et al. Graft-versus-leukemia effect after allogeneic bone marrow transplantation for chronic lymphocytic leukemia. *Bone Marrow Transplant* (1996) 18:669–72.
180. Mandigers CM, Verdonck LF, Meijerink JP, Dekker AW, Schattenberg B, Raemaekers JMM. Graft-versus-lymphoma effect of donor lymphocyte infusion in indolent lymphomas relapsed after allogeneic stem cell transplantation. *Bone Marrow Transplant* (2003) 32:1159–63. doi:10.1038/sj.bmt.1704290
181. Tricot G, Vesole DH, Jagannath S, Hilton J, Munshi N, Barlogie B. Graft-versus-myeloma effect: proof of principle. *Blood* (1996) 87:1196–8.
182. Maude SL, Frey N, Shaw PA, Aplenc R, Barrett DM, Bunin NJ, et al. Chimeric antigen receptor T cells for sustained remissions in leukemia. *N Engl J Med* (2014) 371:1507–17. doi:10.1056/NEJMoa1407222

183. Brentjens RJ, Davila ML, Riviere I, Park J, Wang X, Cowell LG, et al. CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia. *Sci Transl Med* (2013) 5:177ra38. doi:10.1126/scitranslmed.3005930
184. Porter DL, Levine BL, Kalos M, Bagg A, June CH. Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. *N Engl J Med* (2011) 365:725–33. doi:10.1056/NEJMoa1103849
185. Kalos M, Levine BL, Porter DL, Katz S, Grupp SA, Bagg A, et al. T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. *Sci Transl Med* (2011) 3:95ra73. doi:10.1126/scitranslmed.3002842
186. Qasim W, Amrolia PJ, Smarasinghe S, Ghorashian S, Zhan H, Stafford S, et al. First application of Talen engineered universal CAR19 T cells in B-ALL. *Blood* (2015) 126(23):2046.

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REVIEW
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Pathophysiology of GvHD and Other HSCT-Related Major Complications

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For over 60 years, hematopoietic stem cell transplantation has been the major curative therapy for several hematological and genetic disorders, but its efficacy is limited by the secondary disease called graft versus host disease (GvHD). Huge advances have been made in successful transplantation in order to improve patient quality of life, and yet, complete success is hard to achieve. This review assimilates recent updates on pathophysiology of GvHD, prophylaxis and treatment of GvHD-related complications, and advances in the potential treatment of GvHD.

Keywords: pathophysiology, graft versus host disease, T cells, haematopoietic stem cell transplantation, prophylaxis

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INTRODUCTION TO GRAFT VERSUS HOST DISEASE (GvHD)

Graft versus host disease is the most recognized complication post-hematopoietic stem cell transplantation (HSCT) and was first observed in 1956 in a murine model. Barnes and Loutit demonstrated that when irradiated mice were infused with allogenic marrow and spleen cells, mice recovered from radiation injury and aplasia but they developed diarrhea, weight loss, skin changes, and liver abnormalities, and subsequently died due to "secondary disease" (1). This phenomenon was recognized as GvHD. A decade later, in 1966, Billingham postulated three crucial requirements for the development of GvHD:

(i) the transplanted graft must contain immunologically competent cells,
(ii) the recipient must be incapable of rejecting or eliminating transplanted cells,
(iii) the recipient must express tissue antigens that are not present in the transplant donor, thus the recipient antigens are recognized as foreign by donor cells (2).

Today, we know that the immunocompetent cells are T lymphocytes that are present in the stem cell inoculum and are required to mount an effective immune response (3). A normal immune system is able to reject T cells from a foreign donor. However, when recipient's immune system is compromised through the use of various immune-ablative agents (chemotherapy and/or radiotherapy), the recipient is incapable of rejecting the transplanted cells. We now know that the tissue antigens that differ in donor and recipient are major and minor human leukocyte antigens (HLA), and their expression on cell surfaces is crucial for the activation of allogenic T cells and initiation of GvHD (4). Previously, it was believed that acute GvHD occurs within day 100 after transplantation and chronic GvHD (cGvHD) occurs beyond day 100 and that the most affected organs at the onset of GvHD are skin (81%), gastrointestinal tract (54%), and liver (50%) (4). Now, it is clear that acute GvHD can occur after day 100 as late acute GvHD (e.g., after cessation of immunosuppression or after donor lymphocyte infusion) or cause overlap syndrome of both acute GvHD and cGvHD (5).

PATHOPHYSIOLOGY OF ACUTE GvHD: A THREE-STEP MODEL EXPLAINING THE CURRENT STRATEGIES OF PROPHYLAXIS AND TREATMENT

Acute GvHD has been attributed to three stages. Initially, there is tissue damage due to conditioning that in turn activates the host antigen-presenting cells (APCs). Secondly, APCs activate donor T cells, also known as an afferent phase. Finally, in efferent phase, cellular and inflammatory factors work together to damage the target organs.

Conditioning-Mediated Tissue Damage

Conditioning is crucial to eradicate underlying disease and to support engraftment of donor cells without rejection by recipient (6). Prior to donor cell infusion, patient's tissues have been profoundly damaged due to underlying disease itself, treatment for the disease, infections, and the conditioning regimen (7, 8). As a consequence, damaged host tissue releases danger signals, which include pro-inflammatory cytokines such as tumor necrosis factor (TNF) and interleukin-1 (IL-1) (9), that activate host APCs, ultimately activating donor T cells present in the stem cell inoculum (10, 11). Conditioning-mediated damage to the gastrointestinal (GI) tract remains the main concern as GI tract allows systemic translocation of microbial products like lipopolysaccharide (LPS) and other pathogen associated molecular patterns that greatly amplify host APC activation (8), leading to amplified T-cell activation. Conditioning-related damage also explains why the concept of reduced intensity or even non-myeloablative conditioning has contributed to less toxicity, less severe GvHD, and reduced treatment-related mortality. Some studies showed that delaying the transfer of donor cells after conditioning decreased the risk of GvHD (9, 12).

Donor T Cell Activation (the Afferent Phase)

Graft versus host disease occurs when donor T cells activate and respond to HLA differences on recipient's tissue (13). Experimental models have proved that the host APCs are necessary and sufficient to activate donor T cells and initiate GvHD (11, 14). Donor T cells can recognize alloantigen either on host APC, known as direct antigen presentation (15), or on donor APCs, known as indirect presentation (16). T-cell responses depend on the disparity between the donor and the recipient with regard to HLA (13). CD4+ T cells respond to the variations in MHC class II molecule (HLA-DR, -DQ, and -DP), and CD8+ T cells respond to the variations in MHC class I molecule (HLA-A, -B, and -C) (17). Transplants carried out in the HLA-matched sibling or identical twin setting can still give rise to GvHD due to differences in minor HLA (18). The first to be described were HA-1 (19) and HA2 (20), and the subsequent clinical impact of minor histocompatibility antigens including H-Y antigens (21, 22) of female-to-male transplants has recently been reviewed (23, 24). Minor HLAs are T-cell epitopes, which are originally derived from polymorphic or normal tissue proteins. These antigenic peptides can be presented on HLA Class I or Class II molecules, and to date over 50 minor HLA antigens have been identified (24). Minor HLA antigens have been associated with GvHD and graft versus leukemia (GvL) effects due to their tissue distribution. Minor HLA antigens restricted to the hematopoietic system may be able to enhance GvL responses while more broadly expressed minor HLA antigens contribute to both GvHD and GvL (25). As well as cytotoxic T-cell responses of allogeneic H-Y antibodies have shown to predict cGvHD and non-relapse mortality (26, 27).

T-cell activation is in the focus of current immunosuppressive strategies used for prophylaxis and treatment. Calcineurin inhibitors, mycophenolate, and mToR inhibitors interfere with different signals of T-cell activation (28, 29). The broader strategy is T-cell depletion, which is currently applied by *in vivo* approaches such as the use of antithymocyte globulin pretransplant (30). Cytotoxic approaches more or less selectively eliminate activated T cells if applied posttransplant; the old approach of methotrexate prophylaxis but also the more recent approach of using posttransplant cyclophosphamide engages this principle (31).

Target Cell Apoptosis (the Efferent Phase)

In this phase, both innate and adaptive immune cells work synergistically to exacerbate the T cell-induced inflammation. Cellular mediators, such as cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells, utilize the Fas/Fas ligand (FasL) pathway and perforin/granzyme pathway to lyse the target cells (32, 33). Furthermore, inflammatory cytokines synergize with CTLs, resulting in further tissue injury and possible target organ dysfunction (13). In addition, microbial products like LPS, released during conditioning, leak through a damaged intestinal mucosa and skin and stimulate mononuclear cells (monocytes/macrophages) to secret inflammatory cytokines leading to amplification and propagation of a cytokine storm (13). This leads to destruction of epithelial cells, mostly in the GI tract.

The broad activity of corticosteroids including induction of T-cell apoptosis, suppression of macrophage activation, and cytokine release explains why these old drugs are still the treatment of choice for first-line treatment of both acute GvHD and cGvHD. Cytokine inhibitors like TNF blocking agents were thought to be more specific but did not result in increased response rates (34). For almost all second-line strategies in steroid-refractory acute GvHD low response rates associated with high treatment-related mortality have been reported that the urgent need for further improvement (35).

In the last 10 years, the concept of GvHD pathophysiology has been largely extended, and a more differentiated view has been adapted.

Firstly, the mechanism of conditioning-related damage has further been specified. It is now clear that the tissue damage results in release of several danger signals such as uric acid and the metabolites of adenosine triphosphate pathway and its receptor has been shown to be involved in activation of GvHD (36).

Secondly, the concept of LPS-triggered inflammation has been substituted by multiple microbiota derived signals and differential activation of toll-like receptors (TLRs) and NOD-like receptors (NLRs). NOD2/CARD15 has been shown to be

involved in triggering the inflammation both in mice (37, 38) and men (39). More recently, it became clear that the microbiota of epithelial tissues is the major player influencing epithelial integrity and local immune tolerance by commensal bacteria and millions of metabolites are produced to maintain epithelial homeostasis (40, 41).

Finally, and in context with the concept of microbiota as important players, the importance of regulatory immune cells that balance immune reactions is recognized. Regulatory T cells (Tregs) expressing the transcription factor Foxp3 occur as natural, thymus derived T cells and are able to prevent alloreaction (42). On epithelial surfaces, induced peripheral Tregs try to dampen acute inflammation (43). Foxp3 positive T cells act in cooperation with numerous newly identified regulatory populations, such as invariant natural killer T cells (44), myeloid-derived suppressor cells (MDSCs), and a whole new set of innate immune cells such as innate lymphoid cells (45). **Figure 1** represents the GvHD initiation phase. **Figure 2** summarizes the complete pathophysiology of aGvHD.

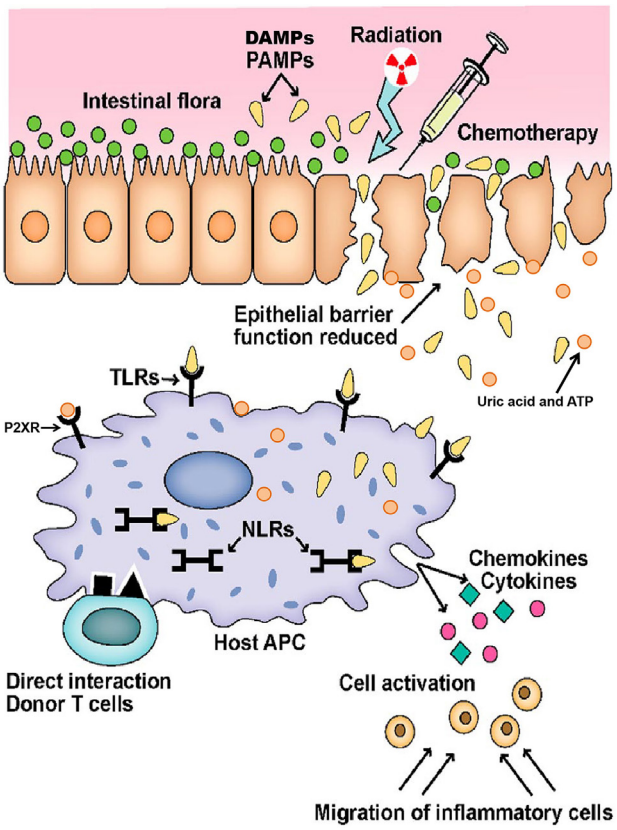

FIGURE 1 | Initiation of graft versus host disease. Conditioning regimen leads to destruction of epithelial cells and their integrity. Damaged epithelia secrete uric acid and adenosine triphosphate (ATP) that result in production of pro-inflammatory cytokines. Pathogen recognition receptors, such as toll-like receptors (TLRs), NOD-like receptors (NLRs), and P2XRs, are activated by pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs). These signals ultimately activate antigen-presenting cells (APCs) that lead to donor T-cell activation. Adopted and modified from Ref. (37).

IN VITRO MODELING OF GvHD TO GIVE INSIGHT INTO THE PATHOPHYSIOLOGY

The skin explant model has long been established as a tool for studying the immunobiology of GvHD (**Figure 3**) and more recently has been used to investigate the specificity of antiviral T cells in graft versus host (GvH) reactions (34–36), the role of Tregs, and mechanisms of apoptosis (46, 47).

The skin explant model has also been used to assess the safety of *ex vivo* expanded Treg cells as well as their capacity to prevent GvH reactions (48). Activated and expanded polyclonal Treg, at any cell concentration, did not induce any significant GvH reactions.

Over recent years, significant advances in the understanding of the benefits of Tregs in hematopoietic stem cell transplantation have resulted in the completion of early stage clinical trials as well as the initiation of trials in solid organ transplantation (49–52). These early stage HSCT trials have provided promising results showing a reduction in the incidence of GvHD without adversely effecting relapse, transplant-related mortality, and engraftment. Using the skin explant model, it has been possible to investigate the cellular and molecular mechanisms by which Tregs are likely to be preventing GvHD following HSCT.

We have shown that for Treg to suppress GvH reactions they need to be present during the priming of alloreactive T cells (48). Polyclonal Treg cells were expanded *ex vivo* and added into the skin explant model at either the priming or the effector stage. The later addition of Treg, during the effector phase, impaired their suppressive capacity. This suggests that Treg may be more effective when given early, as prophylaxis, rather than as a treatment. This study also demonstrated that in humans an effector to Treg ratio of 4:1 was sufficient to modulate GvH reactions, whereas previous studies in mice had suggested a 1:1 ratio was necessary. This study has therefore provided preclinical evidence to support the safety and feasibility of *ex vivo* expanded Treg as a novel therapeutic and provides information on the optimal timing and dose of Treg to prevent GvH reactions.

Further work using the skin explant model has been able to elucidate some of the mechanisms by which Tregs are able to prevent GvHR. The presence of Treg during the priming of alloreactive T cells reduced their cytotoxic capacity (48). Further investigations showed that Treg also impaired the ability of alloreactive T cells to migrate into the target tissues (53). The presence of Treg during priming resulted in a reduction in IFNγ production by CD8+ cytotoxic T cells, as well as a reducing expression of skin homing molecules CXCR3 and CLA. This paired with a reduction in levels of the chemokines CXCL10 and CXCL11 in the skin resulted in a significant reduction in the number of cytotoxic T cells present in the skin and decreased the GvH severity. We have since demonstrated that Tregs are able to modulate GvH reactions through impairment of dendritic cells at a transcriptional level, arresting them in a semi-mature status and leaving them functionally impaired (54).

The skin explant model has also been used to investigate the involvement of epithelial Fas in the pathophysiology of GvHD (55). Animal models have previously shown the critical role for Fas/FasL in GvHD (56). Ruffin et al. showed that there was

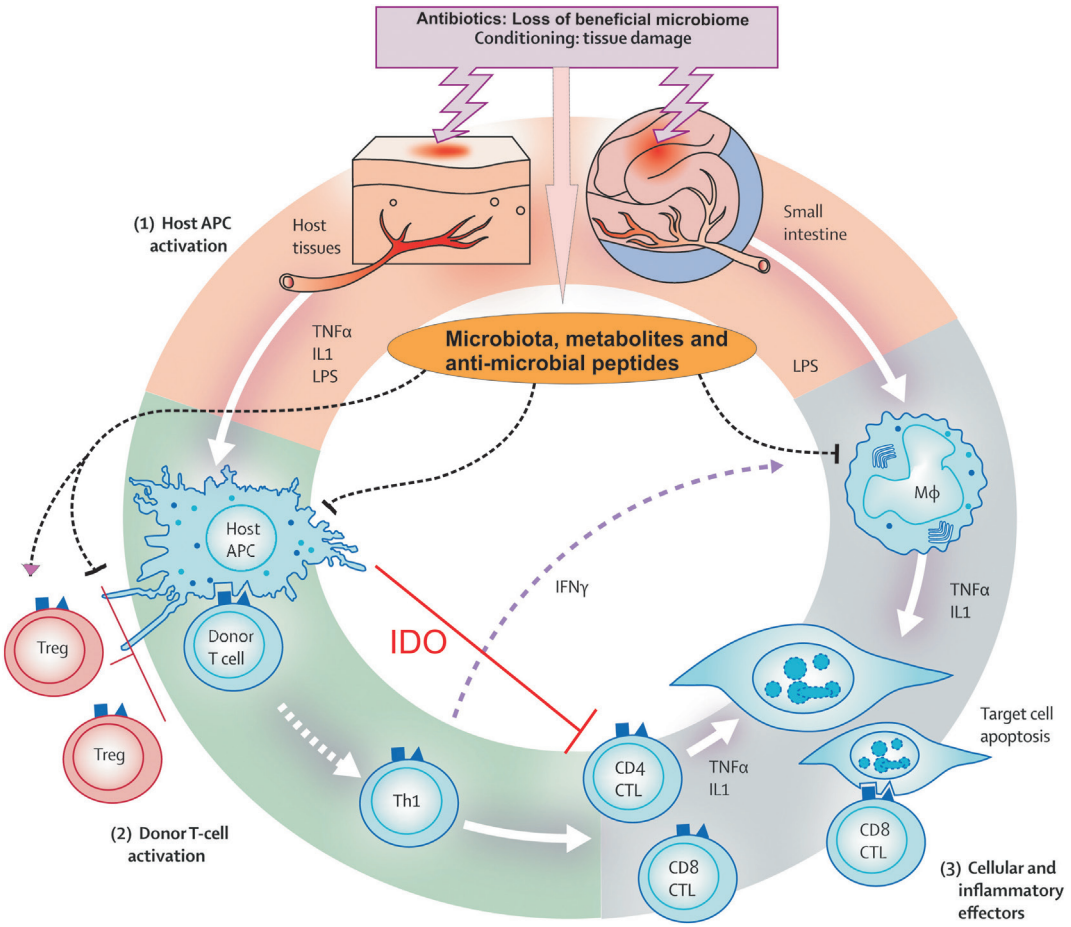

FIGURE 2 | **Pathophysiology of acute graft versus host disease**. Conditioning regimen cause profound damage to the host tissues leading to release of inflammatory cytokines like tumor necrosis factor and interleukin-1. These cytokines activate host antigen-presenting cells (APCs) in phase I. In addition, loss of microbial diversity and metabolites thereof leads to loss of epithelial and immune homeostasis. Host APCs activate mature donor T cells present in stem cell inoculum in phase II. T cells subsequently proliferate and differentiate into Th1 and Th17 type, which are involved in activation of CD4 cytotoxic T lymphocyte (CTL), CD8 CTL, and natural killer cells that mediate tissue damage. In phase III, effector T cells together with pro-inflammatory cytokines attack the epithelial cells of skin, liver, lung, and gastrointestinal tract. This damage is further supported by the lipopolysaccharide (LPS) that has leaked through damaged intestinal mucosa, which then recruits myeloid cells to further produce pro-inflammatory cytokines and thus enhance the cytokine storm. Adopted and modified from Ref. (13).

a significant increase in Fas expressing cells in GvHR positive experiments and that Fas-mediated apoptosis was involved in the induction of GvHR, as blocking Fas-mediated apoptosis reduced the severity of GvHR. They also showed that levels of Fas in the serum of patients who received myeloablative conditioning were increased, possibly due to the higher toxicity. This supports the potential use of Fas as a therapeutic target.

IDENTIFICATION OF BIOMARKERS

As well as investigating the safety of cellular therapies and immunology of GvHD, the skin explant model has been used in recent years to identify biomarkers. Within our group, we used the skin explant model to validate a number of biomarkers, which had been identified in the serum of HSCT patients (57). BAFF and IL-33 levels were elevated pretransplant in patients who then went on to develop aGvHD and therefore could have the potential to act as predictive biomarkers. We also found that CXCL10 and CXCL11 were suitable as diagnostic markers of GvHD. Training and validation cohorts were used to highlight the association of these potential biomarkers to GvHD. Then the skin explant model was used to confirm their association with GvH reactions. Immunohistochemistry was carried out on sections from the skin explant, and increased staining for BAFF, IL-33, CXCL10, and CXCL11 was seen in skin explants with a higher grade GVHR. This was further confirmed in clinical biopsies demonstrating increased levels of protein, measured with immunohistochemistry and gene expression for BAFF, CXCL10, and CXCL11. In this study, the skin explant proved to be a useful tool in validating a panel of biomarkers, which had been identified in patient samples. The skin explant is not exclusive to the human setting. Recently, Zinöcker et al. have

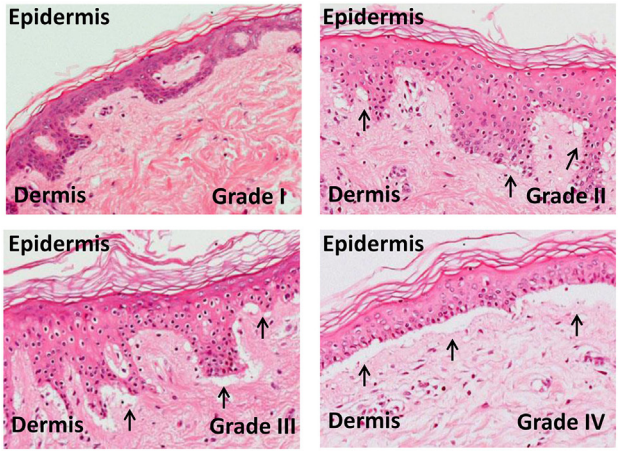

FIGURE 3 | **Skin explant grades I–IV**. The outcome of the skin explant assay is histopathological damage ranging from grade I GvHR (with minimal vacuolization in the epidermis) to grade II GvHR (with vacuolization and dyskeratotic bodies) to grade III GvHR (with sub epidermal cleft formation) and finally to grade IV GvHR (with separation of the dermis from the epidermis).

described the use of a rat skin explant model for investigating the pathophysiology of GvHD (58) as well as gene expression profiling (59).

Harris et al. (60) have recently reviewed the use of biomarkers in predicting acute GvHD, which include genomic factors as well as plasma proteins. One of the first studies demonstrated that a panel of tumor necrosis factor receptor type 1 (TNFR1) interleukin-2 receptor alpha, IL-8, and hepatocyte growth factor (HGF) had prognostic as well as diagnostic value in predicting acute GvHD (61). Other markers in the skin such as elafin (61) and plasma biomarkers of the lower GI tract and liver acute GvHD have been validated in subsequent studies, and the most significant of these was regenerating islet-derived 3 alpha (Reg3a) (62, 63). These studies led to the use of the biomarkers TNFR1, ILRα, IL-8, HGF, Reg3α, and elafin for measuring responsiveness to GvHD therapy. The panel was able to predict 28-day post-therapy non-response and 180-day mortality in a cohort of 112 patients (64).

In addition an algorithm using concentrations of three biomarkers TNFR1, soluble IL-33 receptor (ST2), and Reg3α, Levine and colleagues (65) were able to calculate the probability of non-relapse mortality caused by non-responsive GvHD and divide the patients into distinct groups to predict response to GvHD therapy. The researchers subsequently developed the Mount Sinai Acute GvHD International Consortium, which consists of a group of 10 transplant centers in the United States and Europe who collaborate on the use of this scoring system to test new treatments for acute GvHD.

PATHOPHYSIOLOGY OF cGvHD

Although the pathophysiology of cGvHD is poorly understood, it remains the major cause of late non-relapse death after HSCT (66). cGvHD may manifest simultaneously from aGvHD, develop after the treatment of aGvHD, or may occur *de novo* (67). Classical cGvHD occurs 100 days after transplantation but may also overlap with aGvHD (5, 68).

Acute GvHD is a major risk factor of cGvHD and strategies aiming at T-cell depletion at the time of transplantation to prevent cGvHD demonstrate that early events impact on the development of cGvHD. As immune cells and immune organs, such as thymus, bone marrow niche, and spleen, are the primary targets of acute GvHD, thymus destruction and deficient selection of donor T cells by the thymus are the major factors resulting in allo- and autoimmunity associated with cGvHD (69). Due to early damage of the B cell niche in the bone marrow, B cell development is strongly disturbed resulting in elevated BAFF levels as a predictor of cGvHD and insufficient elimination in B cells producing auto- and alloantibodies (70). A hallmark of cGvHD is development of sclerotic lesions, which can occur in almost every organ (68). While previous data favor a concept of defective wound healing with increase production of sclerotic cytokines, such as TGFβ and PDGF, recent evidence supports a role of specific TH17 subsets in this sclerotic process (71).

TARGET ORGAN DAMAGE DURING GvHD

Skin is the principal target organ of GvHD, and the initial manifestation in the skin is maculopapular rash, which has the potential to spread throughout the body (13). The rash may resemble folliculitis or may resemble sunburn. In extreme cases, skin may blister and ulcerate (13, 72). Acute cutaneous GvHD usually begins with erythematous, rashes on the ears, palms, and soles. Martin and coworkers reported results of 740 allogenic transplantations and 81% of patients with aGvHD had skin involvement (4). Damage to the skin could be defined by vacuolar degeneration of the basal cell layer, dyskeratotic keratinocytes, and mononuclear cell infiltrates (73). Epithelial damage occurs at the tips of rete ridges and hair follicles, regions where selective targeted apoptotic rete cells are located (74). A recent study by Paczesny et al. reported that elafin could be a potential biomarker for diagnosis and prognosis of skin GvHD (75).

Liver is another target organ of GvHD. Hepatic GvHD is manifested by abnormal liver function tests and a rise in the serum level of bilirubin and alkaline phosphatase. Donor lymphocytes attack the bile duct epithelial cells causing endothelialitis, pericholangitis, and apoptotic bile duct destruction (76). While liver GvHD affecting bile ducts and resulting in severe hyperbilirubinemia occurs less frequently, there is an increasing rate of hepatitis like cGvHD as another, but less harmful liver lesion (77).

Gastrointestinal tract represents the most severely affected organ after conditioning. GI GvHD is characterized by secretory and voluminous diarrhea, severe abdominal pain, vomiting, and anorexia (13). Snover and colleagues used immunohistochemistry to explain histologic features of the GI tract during GvHD (78). Single cell apoptosis was observed along with patchy ulcerations and apoptotic bodies in the base of crypts with loss of the surface epithelium (13, 78). The base of the intestinal crypts, where epithelial stem cells are located, is the most sensitive

target for GvHD as it is the site of epithelium regeneration and Paneth cells. Recently, Levine and colleagues observed loss of the Paneth cells at the onset of GI GvHD (79) suggesting these cells as sensitive targets of GvHD. In addition, as stated earlier, it was proposed that regenerating islet-derived 3-a (reg3a), released from Paneth cells, was a potential plasma biomarker for lower GI GvHD (63), Paneth cell damage contributes to loss of antimicrobial peptides and accelerates the loss of microbial diversity in GvHD, a major risk factor of treatment-related mortality (80, 81).

FURTHER HSCT-RELATED COMPLICATIONS

Overview

Although GvHD is the main complication of allogeneic SCT, non-relapse-related mortality (NRM) can occur independently from the occurrence of GvHD or in patients with minor GvHD. Overall, NRM has decreased in the last 10 years as a result of several improvements such as reduced intensity conditioning; resulting in reduced organ toxicity, improved donor selection and matching, and progress in supportive treatment (82).

Major complications include viral and fungal infections, which can occur independently from GvHD due to the immunodeficiencies induced by HSCT. GvHD and its treatment aggravate and prolong the risk of infectious complications, and many patients suffering from severe GvHD die from infectious complications. Beyond the period of acute GvHD, cGvHD and long-term complications are major causes of NRM and morbidity. Long-term complications include organ toxicities, endocrine deficiencies, and most important secondary cancers. HSCT patients' survivors therefore need a long-term follow-up in order to allow early detection of complications, and several guidelines summarize the current recommendations (83, 84).

A detailed presentation of infectious complications, organ toxicities, and long-term complications is beyond the focus of this review; we therefore focus on the most relevant targets of complications: endothelial cells and pulmonary complications.

Endothelial Complications

Endothelial complications occur clinically throughout the different phases of HSCT. In the early weeks after transplantation, sinusoidal obstruction syndrome (SOS) formally known as veno-occlusive disease (VOD) can result in severe liver damage and eventually multi-organ failure (85, 86). SOS results from conditioning-related toxicity in the sinusoids of the liver with subsequent occlusions by thrombosis and fibrosis. In the period of engraftment, cytokine storm-mediated capillary leakage syndrome can occur. With the introduction of calcineurin inhibitors (CNI) for prophylaxis of GvHD, which also give rise to some endothelial toxicity, transplant associated microangiopathy (TAM) has been increasingly observed during acute GvHD (87). Manifestations of intestinal TAM can mimic severe GvHD and provoke intestinal bleeding but require a different treatment regimen. Besides CNI-associated TAM, it can also occur as atypical hemolytic uremic syndrome, which results from a failure of cleaving von Willebrand Factor (88, 89). In long-term patients, cerebro- and cardiovascular complications are increased.

While clinical endothelial complications have been well known for many years, more recently the pathophysiology of endothelial cells in GvHD has been studied. *In vitro* models of endothelial cell cultures reveal that conditioning can induce endothelial apoptosis, which is aggravated by LPS-mediated inflammation and followed allogeneic cytotoxic T-cell damage (90). Murine models have demonstrated the role of endothelial neovascularization induced by conditioning leading to GvHD (91–93) and infiltrating donor T cells. Recently, Schmid et al. showed for the first time in a murine system that not only endothelial venules but also arterial vessels suffer direct endothelial damage during GvHD (94). Detailed studies in patients have shown an association of loss of dermal vessels, with CD8+ T cell infiltrates, demonstrating allogeneic reactions against endothelial cells (95, 96). More recently, endothelial damage has been shown to contribute to steroid resistance and failure to recover from GvHD. Loss of protective thrombomodulin was observed in biopsies from GvHD patients (97) together with increased serum thrombomodulin (98). In addition, genetic SNPs within the thrombomodulin gene have been identified as risk factors for GvHD (99). Finally, circulating endothelial factors such as angiopoietin levels pretransplant and VEGF levels posttransplant have been identified as risk factors of GvHD (100), which paves the way for infiltrating donor T cells.

Pulmonary Complications

A further central target organ of HSCT-related complications is the lung. Early after transplantation, bacterial and fungal pneumonia are common, mainly due to *Aspergillus* predomination. In the posttransplant period of GvHD and immune reconstitution, viral pneumonia caused by CMV, respiratory viruses (influenza, parainfluenza, RSV, and metapneumovirus), and adenoviruses predominate as well as fungal pneumonia, especially in patients with severe immunosuppression (101). In addition, further infectious agents such as *Toxoplasma gondii* and *Pneumocystis jirovecii* causing toxoplasma and pneumocystis pneumonia, respectively, can cause pneumonia during the period of B cell reconstitution while B cell numbers are absent or low. Pneumonias caused by encapsulated bacteria such as pneumococci are also observed (102, 103). Early after HSCT, peri-engraftment respiratory distress syndrome causes rapid deterioration of respiratory functions during leukocyte recovery but responds rapidly to high dose corticosteroid treatment. In the initial stages of aGvHD, idiopathic pneumonia syndrome (IPS) is a serious complication resulting from conditioning-related toxicity and LPS-triggered allogeneic reactions. IPS may or may not be exacerbated by occult or unknown infections (89) and in either case, TNF blocking agents have been shown to be effective in both experimental models and in patients (86–88). The most frequent complication is bronchiolitis obliterans syndrome (BOS) characterized by inflammation of the small bronchiole with subsequent obstruction and lung destruction (104, 105). Early monitoring and intervention with topical corticosteroids, azithromycin and possibly systemic immunosuppression is

needed to prevent progression to irreversible lung damage, which may lead a requirement for lung transplantation (106, 107). Besides BOS, restrictive changes can be observed such as pulmonary fibrosis, bronchiolitis obliterans organizing pneumonia, and pulmonary VOD (108).

Cellular therapy is one approach increasingly used as a second-line treatment. Tregs (109) and MSCs (110–112) are promising cellular products, but phase 3 trials are yet to be conducted.

RECENT ADVANCES AND PERSPECTIVES IN GvHD

For over 30 years, immunosuppressive drugs have served as a central strategy to reduce GvHD. Drugs such as sirolimus, tacrolimus, and methotrexate are the mainstay in the treatment of GvHD (113). Complete *ex vivo* T-cell depletion is no longer routinely used in HLA-matched transplantation as it also largely abolishes GvL effects. A more recent report from Finke and colleagues suggested ATG as an *in vivo* T-cell depletion may be more efficacious in lowering the incidence of severe acute GvHD in matched and mismatched HSCT from unrelated donors while GvL effects seemed less affected (114). Hundred patients were enrolled in the study. Comparable outcomes were obtained for GvHD patients receiving bone marrow or peripheral blood stem cells from matched or one antigen mismatched-unrelated donors when ATG was added to the standard prophylaxis (cyclosporine + methotrexate) (114). The use of ATG may therefore contribute to balance GvH versus GvL effect and enable HLA mismatch donors to be used as well as fully match-unrelated donors, with no difference in outcome. As an alternative, elimination of alloreactive T cells by posttransplant cyclophosphamide may become an option, which is already widely used for GvHD prophylaxis following haploidentical transplantation (115). Whether this approach can be integrated in the HLA-identical setting as a potential alternative to calcineurin inhibitors is under current investigation.

Pathogen recognition receptors like NLRs and TLRs are known to control adaptive immune responses in inflammatory disorders (37), and the research on the role of these receptors has resulted in the description of the interaction of the microbiota and the immune system in the setting of GvHD. Loss of microbiome diversity early after HSCT has been recognized as a new risk factor for GvHD and HSCT-related complications (116). This observation suggests that restoration of a diverse microbiome could be a new approach to induce intestinal and systemic tolerance, and pre-/pro- and post-biotic strategies, as well as several approaches of fecal microbiota transplantation, that are currently being tested in both experimental and clinical settings of HSCT (41).

Regulatory T cells have been expanded *in vitro* and used for prophylaxis and treatment of GvHD in experimental and small clinical trials (117, 118). Another option is induction of Tregs in patients, e.g., by interleukin-2 (IL-2) (119). Induction of Tregs has also been postulated as one mechanism explaining the beneficial action of extracorporeal photopheresis for treatment of acute GvHD and cGvHD (120, 121). Besides Tregs, numerous alternative candidates for cellular therapy of GvHD exist such as MDSCs (122). MSCs are indirect immunoregulatory cells that induce tissue repair and show some promising activity in steroid-refractory GvHD (123, 124).

Among pharmacological agents, drugs with anti-inflammatory effects of corticosteroids but without numerous side effects are urgently needed. Recently, anti-inflammatory JAK2 inhibitors have shown promising effects both in GvHD and in rheumatology. Proteasome inhibitors and histone deacetylase inhibitors originally developed as anticancer drugs now show some promising activity in dampening T-cell responses (125). In cGvHD, the role of aberrant B cells is increasingly recognized, which paves the way for anti-B cell strategies like rituximab or new B cell development inhibitors like the Bruton's tyrosine kinase-inhibitor ibrutinib (70).

A major issue in the treatment of aGvHD is that most approaches are initiated too late, when major changes have already severely damaged the target tissue. Therefore, biomarkers allowing early identification of patients at high risk are needed. A handful of biomarkers have been discovered, which might be used to guide treatment in the future (65).

Finally, the practice of stem cell transplantation differs between countries, within the same countries and between transplantation institutes. Approaches aimed at standardization of diagnosis and treatment are urgently needed, some of which have been addressed by several consensus projects (68, 126).

AUTHOR CONTRIBUTIONS

EH and AD designed and revised the review. SG provided the draft, summarized available data, selected the references, and wrote the review. DW, EM and XNW contributed to the review. All authors approved the final version of the manuscript.

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REFERENCES

1. Barnes D, Loutit J. Treatment of murine leukaemia with x-rays and homologous bone marrow: II. *Br J Haematol* (1957) 3(3):241–52. doi:10.1111/j.1365-2141.1957.tb05793.x
2. Billingham RE. The biology of graft-versus-host reactions. *Harvey Lect* (1966) 62:21–78.
3. Kernan NA, Collins NH, Juliano L, Cartagena T, Dupont B, O'Reilly RJ. Clonable T lymphocytes in T cell-depleted bone marrow transplants correlate with development of graft-v-host disease. *Blood* (1986) 68(3):770–3.

4. Martin PJ, Schoch G, Fisher L, Byers V, Anasetti C, Appelbaum FR, et al. A retrospective analysis of therapy for acute graft-versus-host disease: initial treatment. *Blood* (1990) 76(8):1464–72.
5. Filipovich AH, Weisdorf D, Pavletic S, Socie G, Wingard JR, Lee SJ, et al. National Institutes of Health consensus development project on criteria for clinical trials in chronic graft-versus-host disease: I. Diagnosis and staging working group report. *Biol Blood Marrow Transplant* (2005) 11(12):945–56. doi:10.1016/j.bbmt.2005.09.004
6. Gratwohl A, Carreras E. Principles of conditioning. In: Apperley J, Carreras AE, Gluckman E, Gratwohl A, Masszi T, editors. *Haematopoietic Stem Cell Transplantation. The EBMT Handbook*. 5th ed. Genoa, Italy: Forum Service Editore (2008). p. 128–44.
7. Kaitin KI. Graft-versus-host disease. *N Engl J Med* (1991) 325(5):357–8. doi:10.1056/NEJM199108013250513
8. Hill GR, Crawford JM, Cooke KR, Brinson YS, Pan L, Ferrara JL. Total body irradiation and acute graft-versus-host disease: the role of gastrointestinal damage and inflammatory cytokines. *Blood* (1997) 90(8):3204–13.
9. Xun CQ, Thompson JS, Jennings CD, Brown SA, Widmer MB. Effect of total body irradiation, busulfan-cyclophosphamide, or cyclophosphamide conditioning on inflammatory cytokine release and development of acute and chronic graft-versus-host disease in H-2-incompatible transplanted SCID mice. *Blood* (1994) 83(8):2360–7.
10. Matzinger P. The danger model: a renewed sense of self. *Science* (2002) 296(5566):301–5. doi:10.1126/science.1071059
11. Shlomchik WD, Couzens MS, Tang CB, McNiff J, Robert ME, Liu J, et al. Prevention of graft versus host disease by inactivation of host antigen-presenting cells. *Science* (1999) 285(5426):412–5. doi:10.1126/science.285.5426.412
12. Johnson BD, Truitt R. Delayed infusion of immunocompetent donor cells after bone marrow transplantation breaks graft-host tolerance allows for persistent antileukemic reactivity without severe graft-versus-host disease. *Blood* (1995) 85(11):3302–12.
13. Ferrara JL, Levine JE, Reddy P, Holler E. Graft-versus-host disease. *Lancet* (2009) 373(9674):1550–61. doi:10.1016/S0140-6736(09)60237-3
14. Teshima T, Ordemann R, Reddy P, Gagin S, Liu C, Cooke KR, et al. Acute graft-versus-host disease does not require alloantigen expression on host epithelium. *Nat Med* (2002) 8(6):575–81. doi:10.1038/nm0602-575
15. Newton-Nash DK. The molecular basis of allorecognition assessment of the involvement of peptide. *Hum Immunol* (1994) 41(2):105–11. doi:10.1016/0198-8859(94)90002-7
16. Markey KA, Banovic T, Kuns RD, Olver SD, Don AL, Raffelt NC, et al. Conventional dendritic cells are the critical donor APC presenting alloantigen after experimental bone marrow transplantation. *Blood* (2009) 113(22):5644–9. doi:10.1182/blood-2008-12-191833
17. Sprent J, Schaefer M, Gao EK, Korngold R. Role of T cell subsets in lethal graft-versus-host disease (GVHD) directed to class I versus class II H-2 differences. I. L3T4+ cells can either augment or retard GVHD elicited by Lyt-2+ cells in class I different hosts. *J Exp Med* (1988) 167(2):556–69. doi:10.1084/jem.167.2.556
18. Goulmy E, Schipper R, Pool J, Blokland E, Falkenburg JH, Vossen J, et al. Mismatches of minor histocompatibility antigens between HLA-identical donors and recipients and the development of graft-versus-host disease after bone marrow transplantation. *N Engl J Med* (1996) 334(5):281–5. doi:10.1056/NEJM199602013340501
19. den Haan JM, Meadows LM, Wang W, Pool J, Blokland E, Bishop TL, et al. The minor histocompatibility antigen HA-1: a diallelic gene with a single amino acid polymorphism. *Science* (1998) 279(5353):1054–7. doi:10.1126/science.279.5353.1054
20. den Haan JM, Sherman NE, Blokland E, Huczko E, Koning F, Drijfhout JW, et al. Identification of a graft versus host disease-associated human minor histocompatibility antigen. *Science* (1995) 268(5216):1476–80. doi:10.1126/science.7539551
21. Goulmy E, Gratama JW, Blokland E, Zwaan FE, van Rood JJ. A minor transplantation antigen detected by MHC-restricted cytotoxic T lymphocytes during graft-versus-host disease. *Nature* (1983) 302(5904):159–61. doi:10.1038/302159a0
22. Goulmy E, Termijtelen A, Bradley BA, van Rood JJ. Y-antigen killing by T cells of women is restricted by HLA. *Nature* (1977) 266(5602):544–5. doi:10.1038/266544a0
23. Popli R, Sahaf B, Nakasone H, Lee JY, Miklos DB. Clinical impact of H-Y alloimmunity. *Immunol Res* (2014) 58(2–3):249–58. doi:10.1007/s12026-014-8514-3
24. Spierings E. Minor histocompatibility antigens: past, present, and future. *Tissue Antigens* (2014) 84(4):347–60. doi:10.1111/tan.12445
25. de Bueger M, Bakker A, Van Rood JJ, Van der Woude F, Goulmy E. Tissue distribution of human minor histocompatibility antigens. Ubiquitous versus restricted tissue distribution indicates heterogeneity among human cytotoxic T lymphocyte-defined non-MHC antigens. *J Immunol* (1992) 149(5):1788–94.
26. Nakasone H, Tian L, Sahaf B, Kawase T, Schoenrock K, Perloff S, et al. Allogeneic HY antibodies detected 3 months after female-to-male HCT predict chronic GVHD and nonrelapse mortality in humans. *Blood* (2015) 125(20):3193–201. doi:10.1182/blood-2014-11-613323
27. Miklos DB, Kim HT, Miller KH, Guo L, Zorn E, Lee SJ, et al. Antibody responses to H-Y minor histocompatibility antigens correlate with chronic graft-versus-host disease and disease remission. *Blood* (2005) 105(7):2973–8. doi:10.1182/blood-2004-09-3660
28. Nash RA, Antin JH, Karanes C, Fay JW, Avalos BR, Yeager AM, et al. Phase 3 study comparing methotrexate and tacrolimus with methotrexate and cyclosporine for prophylaxis of acute graft-versus-host disease after marrow transplantation from unrelated donors. *Blood* (2000) 96(6):2062–8.
29. Piñana JL, Valcárcel D, Fernández-Avilés F, Martino R, Rovira M, Barba P, et al. MTX or mycophenolate mofetil with CsA as GVHD prophylaxis after reduced-intensity conditioning PBSCT from HLA-identical siblings. *Bone Marrow Transplant* (2010) 45(9):1449–56. doi:10.1038/bmt.2009.362
30. Bacigalupo A, Lamparelli T, Bruzzi P, Guidi S, Alessandrino PE, di Bartolomeo P, et al. Antithymocyte globulin for graft-versus-host disease prophylaxis in transplants from unrelated donors: 2 randomized studies from Gruppo Italiano Trapianti Midollo Osseo (GITMO). *Blood* (2001) 98(10):2942–7. doi:10.1182/blood.V98.10.2942
31. Storb R, Yu C, Wagner JL, Deeg HJ, Nash RA, Kiem HP, et al. Stable mixed hematopoietic chimerism in DLA-identical littermate dogs given sublethal total body irradiation before and pharmacological immunosuppression after marrow transplantation. *Blood* (1997) 89(8):3048–54.
32. Kägi D, Vignaux F, Ledermann B, Bürki K, Depraetere V, Nagata S, et al. Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science* (1994) 265(5171):528–30. doi:10.1126/science.7518614
33. Lowin B, Hahne M, Mattmann C, Tschopp J. Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways. *Nature* (1994) 370(6491):650–2. doi:10.1038/370650a0
34. Couriel D, Saliba R, Hicks K, Ippoliti C, de Lima M, Hosing C, et al. Tumor necrosis factor-alpha blockade for the treatment of acute GVHD. *Blood* (2004) 104(3):649–54. doi:10.1182/blood-2003-12-4241
35. Westin JR, Saliba RM, De Lima M, Alousi A, Hosing C, Qazilbash MH, et al. Steroid-refractory acute GVHD: predictors and outcomes. *Adv Hematol* (2011) 2011:601953. doi:10.1155/2011/601953
36. Apostolova P, Zeiser R. The role of danger signals and ectonucleotidases in acute graft-versus-host disease. *Hum Immunol* (2016) 77(11):1037–47. doi:10.1016/j.humimm.2016.02.005
37. Penack O, Holler E, van den Brink MR. Graft-versus-host disease: regulation by microbe-associated molecules and innate immune receptors. *Blood* (2010) 115(10):1865–72. doi:10.1182/blood-2009-09-242784
38. Penack O, Smith OM, Cunningham-Bussel A, Liu X, Rao U, Yim N, et al. NOD2 regulates hematopoietic cell function during graft-versus-host disease. *J Exp Med* (2009) 206(10):2101–10. doi:10.1084/jem.20090623
39. Wehkamp J, Harder J, Weichenthal M, Schwab M, Schäffeler E, Schlee M, et al. NOD2 (CARD15) mutations in Crohn's disease are associated with diminished mucosal α-defensin expression. *Gut* (2004) 53(11):1658–64. doi:10.1136/gut.2003.032805
40. Mathewson ND, Jenq R, Mathew AV, Koenigsknecht M, Hanash A, Toubai T, et al. Gut microbiome-derived metabolites modulate intestinal epithelial cell damage and mitigate graft-versus-host disease. *Nat Immunol* (2016) 17(5):505–13. doi:10.1038/ni.3400
41. Peled JU, Jenq RR, Holler E, van den Brink MR. Role of gut flora after bone marrow transplantation. *Nat Microbiol* (2016) 1:16036. doi:10.1038/nmicrobiol.2016.36
42. Edinger M, Hoffmann P, Ermann J, Drago K, Fathman CG, Strober S, et al. CD4+ CD25+ regulatory T cells preserve graft-versus-tumor activity while

inhibiting graft-versus-host disease after bone marrow transplantation. *Nat Med* (2003) 9(9):1144–50. doi:10.1038/nm915
43. Bollrath J, Powrie FM. Controlling the frontier: regulatory T-cells and intestinal homeostasis. *Semin Immunol* (2013) 25(5):352–7. doi:10.1016/j.smim.2013.09.002
44. Schneidawind D, Baker J, Pierini A, Buechele C, Luong RH, Meyer EH, et al. Third-party CD4+ invariant natural killer T cells protect from murine GVHD lethality. *Blood* (2015) 125(22):3491–500. doi:10.1182/blood-2014-11-612762
45. Hanash AM, Dudakov JA, Hua G, O'Connor MH, Young LF, Singer NV, et al. Interleukin-22 protects intestinal stem cells from immune-mediated tissue damage and regulates sensitivity to graft versus host disease. *Immunity* (2012) 37(2):339–50. doi:10.1016/j.immuni.2012.05.028
46. Einsele H, Roosnek E, Rufer N, Sinzger C, Riegler S, Löffler J, et al. Infusion of cytomegalovirus (CMV)-specific T cells for the treatment of CMV infection not responding to antiviral chemotherapy. *Blood* (2002) 99(11):3916–22. doi:10.1182/blood.V99.11.3916
47. Feuchtinger T, Opherk K, Bethge WA, Topp MS, Schuster FR, Weissinger EM, et al. Adoptive transfer of pp65-specific T cells for the treatment of chemorefractory cytomegalovirus disease or reactivation after haploidentical and matched unrelated stem cell transplantation. *Blood* (2010) 116(20):4360–7. doi:10.1182/blood-2010-01-262089
48. Wang XN, Haniffa MA, Holtick U, Collin MP, Jackson G, Hilkens CM, et al. Regulatory T-cell suppression of CD8+ T-cell-mediated graft-versus-host reaction requires their presence during priming. *Transplantation* (2009) 88(2):188–97. doi:10.1097/TP.0b013e3181ac14ce
49. Trzonkowski P, Bieniaszewska M, Juścińska J, Dobyszuk A, Krzystyniak A, Marek N, et al. First-in-man clinical results of the treatment of patients with graft versus host disease with human ex vivo expanded CD4+CD25+CD127- T regulatory cells. *Clin Immunol* (2009) 133(1):22–6. doi:10.1016/j.clim.2009.06.001
50. Brunstein CG, Miller JS, Cao Q, McKenna DH, Hippen KL, Curtsinger J, et al. Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. *Blood* (2011) 117(3):1061–70. doi:10.1182/blood-2010-07-293795
51. Di Ianni M, Falzetti F, Carotti A, Terenzi A, Castellino F, Bonifacio E, et al. Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. *Blood* (2011) 117(14):3921–8. doi:10.1182/blood-2010-10-311894
52. Geissler EK. The ONE Study compares cell therapy products in organ transplantation: introduction to a review series on suppressive monocyte-derived cells. *Transplant Res* (2012) 1:11–11. doi:10.1186/2047-1440-1-10
53. Mavin E, Ahmed SS, O'Boyle G, Turner B, Douglass S, Norden J, et al. Regulatory T cells inhibit CD8(+) T-cell tissue invasion in human skin graft-versus-host reactions. *Transplantation* (2012) 94(5):456–64. doi:10.1097/TP.0b013e31826205d6
54. Mavin E, Nicholson L, Rafez Ahmed S, Gao F, Dickinson A, Wang XN. Human regulatory T cells mediate transcriptional modulation of dendritic cell function. *J Immunol* (2017) 198(1):138–46. doi:10.4049/jimmunol.1502487
55. Ruffin N, Ahmed SS, Osorio LM, Wang XN, Jackson GH, Collin MP, et al. The involvement of epithelial Fas in a human model of graft versus host disease. *Transplantation* (2011) 91(9):946–51. doi:10.1097/TP.0b013e318212c833
56. Mori T, Nishimura T, Ikeda Y, Hotta T, Yagita H, Ando K. Involvement of Fas-mediated apoptosis in the hematopoietic progenitor cells of graft-versus-host reaction-associated myelosuppression. *Blood* (1998) 92(1):101–7.
57. Ahmed SS, Wang XN, Norden J, Pearce K, El-Gezawy E, Atarod S, et al. Identification and validation of biomarkers associated with acute and chronic graft versus host disease. *Bone Marrow Transplant* (2015) 50(12):1563–71. doi:10.1038/bmt.2015.191
58. Zinöcker S, Dressel R, Wang XN, Dickinson AM, Rolstad B. Immune reconstitution and graft-versus-host reactions in rat models of allogeneic hematopoietic cell transplantation. *Front Immunol* (2012) 3:355. doi:10.3389/fimmu.2012.00355
59. Novota P, Zinöcker S, Norden J, Wang XN, Sviland L, Opitz L, et al. Expression profiling of major histocompatibility and natural killer complex genes reveals candidates for controlling risk of graft versus host disease. *PLoS One* (2011) 6(1):e16582. doi:10.1371/journal.pone.0016582
60. Harris AC, Ferrara JL, Levine JE. Advances in predicting acute GVHD. *Br J Haematol* (2013) 160(3):288–302. doi:10.1111/bjh.12142
61. Paczesny S, Krijanovski OI, Braun TM, Choi SW, Clouthier SG, Kuick R, et al. A biomarker panel for acute graft-versus-host disease. *Blood* (2009) 113(2):273–8. doi:10.1182/blood-2008-07-167098
62. Ferrara JL, Harris AC, Greenson JK, Braun TM, Holler E, Teshima T, et al. Regenerating islet-derived 3-alpha is a biomarker of gastrointestinal graft-versus-host disease. *Blood* (2011) 118(25):6702–8. doi:10.1182/blood-2011-08-375006
63. Harris AC, Ferrara JL, Braun TM, Holler E, Teshima T, Levine JE, et al. Plasma biomarkers of lower gastrointestinal and liver acute GVHD. *Blood* (2012) 119(12):2960–3. doi:10.1182/blood-2011-10-387357
64. Levine JE, Logan BR, Wu J, Alousi AM, Bolaños-Meade J, Ferrara JL, et al. Acute graft-versus-host disease biomarkers measured during therapy can predict treatment outcomes: a Blood and Marrow Transplant Clinical Trials Network study. *Blood* (2012) 119(16):3854–60. doi:10.1182/blood-2012-01-403063
65. Levine JE, Braun TM, Harris AC, Holler E, Taylor A, Miller H, et al. A prognostic score for acute graft-versus-host disease based on biomarkers: a multicentre study. *Lancet Haematol* (2015) 2(1):e21–9. doi:10.1016/S2352-3026(14)00035-0
66. Lee SJ, Klein JP, Barrett AJ, Ringden O, Antin JH, Cahn JY, et al. Severity of chronic graft-versus-host disease: association with treatment-related mortality and relapse. *Blood* (2002) 100(2):406–14. doi:10.1182/blood.V100.2.406
67. Shimabukuro-Vornhagen A, Hallek MJ, Storb RF, von Bergwelt-Baildon MS. The role of B cells in the pathogenesis of graft-versus-host disease. *Blood* (2009) 114(24):4919–27. doi:10.1182/blood-2008-10-161638
68. Jagasia MH, Greinix HT, Arora M, Williams KM, Wolff D, Cowen EW, et al. National Institutes of Health Consensus Development Project on Criteria for Clinical Trials in Chronic Graft-versus-Host Disease: I. The 2014 Diagnosis and Staging Working Group Report. *Biol Blood Marrow Transplant* (2015) 21(3):389.e–401.e. doi:10.1016/j.bbmt.2014.12.001
69. Socié G, Ritz J. Current issues in chronic graft-versus-host disease. *Blood* (2014) 124(3):374–84. doi:10.1182/blood-2014-01-514752
70. Sarantopoulos S, Ritz J. Aberrant B-cell homeostasis in chronic GVHD. *Blood* (2015) 125(11):1703–7. doi:10.1182/blood-2014-12-567834
71. Varelias A, Gartlan KH, Kreijveld E, Olver SD, Lor M, Kuns RD, et al. Lung parenchyma-derived IL-6 promotes IL-17A–dependent acute lung injury after allogeneic stem cell transplantation. *Blood* (2015) 125(15):2435–44. doi:10.1182/blood-2014-07-590232
72. Griffith LM, Pavletic SZ, Lee SJ, Martin PJ, Schultz KR, Vogelsang GB, et al. Chronic graft-versus-host disease-implementation of the National Institutes of Health Consensus Criteria for Clinical Trials. *Biol Blood Marrow Transplant* (2008) 14(4):379. doi:10.1016/j.bbmt.2008.01.005
73. Peñas PF, Zaman S. Many faces of graft-versus-host disease. *Australas J Dermatol* (2010) 51(1):1–10. doi:10.1111/j.1440-0960.2009.00577.x
74. Johnson ML, Farmer ER. Graft-versus-host reactions in dermatology. *J Am Acad Dermatol* (1998) 38(3):369–92. doi:10.1016/S0190-9622(98)70495-5
75. Paczesny S, Braun TM, Levine JE, Hogan J, Crawford J, Coffing B, et al. Elafin is a biomarker of graft-versus-host disease of the skin. *Sci Transl Med* (2010) 2(13):ra2–13. doi:10.1126/scitranslmed.3000406
76. Snover DC, Weisdorf SA, Ramsay NK, McGlave P, Kersey JH. Hepatic graft versus host disease: a study of the predictive value of liver biopsy in diagnosis. *Hepatology* (1984) 4(1):123–30. doi:10.1002/hep.1840040122
77. McDonald GB. Hepatobiliary complications of hematopoietic cell transplantation, 40 years on. *Hepatology* (2010) 51(4):1450–60. doi:10.1002/hep.23533
78. Snover DC, Weisdorf SA, Vercellotti GM, Rank B, Hutton S, McGlave P. A histopathologic study of gastric and small intestinal graft-versus-host disease following allogeneic bone marrow transplantation. *Hum Pathol* (1985) 16(4):387–92. doi:10.1016/S0046-8177(85)80232-X
79. Levine JE, Huber E, Hammer ST, Harris AC, Greenson JK, Braun TM, et al. Low Paneth cell numbers at onset of gastrointestinal graft-versus-host

disease identify patients at high risk for nonrelapse mortality. *Blood* (2013) 122(8):1505–9. doi:10.1182/blood-2013-02-485813
80. Weber D, Oefner PJ, Hiergeist A, Koestler J, Gessner A, Weber M, et al. Low urinary indoxyl sulfate levels early after transplantation reflect a disrupted microbiome and are associated with poor outcome. *Blood* (2015) 126(14):1723–8. doi:10.1182/blood-2015-04-638858
81. Eriguchi Y, Takashima S, Oka H, Shimoji S, Nakamura K, Uryu H, et al. Graft-versus-host disease disrupts intestinal microbial ecology by inhibiting Paneth cell production of α-defensins. *Blood* (2012) 120(1):223–31. doi:10.1182/blood-2011-12-401166
82. Gooley TA, Chien JW, Pergam SA, Hingorani S, Sorror ML, Boeckh M, et al. Reduced mortality after allogeneic hematopoietic-cell transplantation. *N Engl J Med* (2010) 363(22):2091–101. doi:10.1056/NEJMoa1004383
83. Bhatia S. Caring for the long-term survivor after allogeneic stem cell transplantation. *Hematology Am Soc Hematol Educ Program* (2014) 2014(1):495–503. doi:10.1182/asheducation-2014.1.495
84. Konopacki J, Porcher R, Robin M, Bieri S, Cayuela JM, Larghero J, et al. Long-term follow up after allogeneic stem cell transplantation in patients with severe aplastic anemia after cyclophosphamide plus antithymocyte globulin conditioning. *Haematologica* (2012) 97(5):710–6. doi:10.3324/haematol.2011.050096
85. Mohty M, Malard F, Abecassis M, Aerts E, Alaskar AS, Aljurf M, et al. Sinusoidal obstruction syndrome/veno-occlusive disease: current situation and perspectives—a position statement from the European Society for Blood and Marrow Transplantation (EBMT). *Bone Marrow Transplant* (2015) 50(6):781–9. doi:10.1038/bmt.2015.52
86. Carreras E. How I manage sinusoidal obstruction syndrome after haematopoietic cell transplantation. *Br J Haematol* (2015) 168(4):481–91. doi:10.1111/bjh.13215
87. Stavrou E, Lazarus HM. Thrombotic microangiopathy in haematopoietic cell transplantation: an update. *Mediterr J Hematol Infect Dis* (2010) 2(3):2010033. doi:10.4084/MJHID.2010.033
88. Jodele S, Davies SM, Lane A, Khoury J, Dandoy C, Goebel J, et al. Diagnostic and risk criteria for HSCT-associated thrombotic microangiopathy: a study in children and young adults. *Blood* (2014) 124(4):645–53. doi:10.1182/blood-2014-03-564997
89. Jodele S, Laskin BL, Dandoy CE, Myers KC, El-Bietar J, Davies SM, et al. A new paradigm: diagnosis and management of HSCT-associated thrombotic microangiopathy as multi-system endothelial injury. *Blood Rev* (2015) 29(3):191–204. doi:10.1016/j.blre.2014.11.001
90. Palomo M, Diaz-Ricart M, Carbo C, Rovira M, Fernandez-Aviles F, Escolar G, et al. The release of soluble factors contributing to endothelial activation and damage after hematopoietic stem cell transplantation is not limited to the allogeneic setting and involves several pathogenic mechanisms. *Biol Blood Marrow Transplant* (2009) 15(5):537–46. doi:10.1016/j.bbmt.2009.01.013
91. Leonhardt F, Grundmann S, Behe M, Bluhm F, Dumont RA, Braun F, et al. Inflammatory neovascularization during graft-versus-host disease is regulated by αv integrin and miR-100. *Blood* (2013) 121(17):3307–18. doi:10.1182/blood-2012-07-442665
92. Penack O, Henke E, Suh D, King CG, Smith OM, Na IK, et al. Inhibition of neovascularization to simultaneously ameliorate graft-vs-host disease and decrease tumor growth. *J Natl Cancer Inst* (2010) 102(12):894–908. doi:10.1093/jnci/djq172
93. Penack O, Socié G, van den Brink MR. The importance of neovascularization and its inhibition for allogeneic hematopoietic stem cell transplantation. *Blood* (2011) 117(16):4181–9. doi:10.1182/blood-2010-10-312934
94. Schmid PM, Bouazzaoui A, Doser K, Schmid K, Hoffmann P, Schroeder JA, et al. Endothelial dysfunction and altered mechanical and structural properties of resistance arteries in a murine model of graft-versus-host disease. *Biol Blood Marrow Transplant* (2014) 20(10):1493–500. doi:10.1016/j.bbmt.2014.05.002
95. Biedermann BC. Vascular endothelium and graft-versus-host disease. *Best Pract Res Clin Haematol* (2008) 21(2):129–38. doi:10.1016/j.beha.2008.02.003
96. Biedermann BC, Sahner S, Gregor M, Tsakiris DA, Jeanneret C, Pober JS, et al. Endothelial injury mediated by cytotoxic T lymphocytes and loss of microvessels in chronic graft versus host disease. *Lancet* (2002) 359(9323):2078–83. doi:10.1016/S0140-6736(02)08907-9
97. Andrulis M, Dietrich S, Longerich T, Koschny R, Burian M, Schmitt-Gräf A, et al. Loss of endothelial thrombomodulin predicts response to steroid therapy and survival in acute intestinal graft-versus-host disease. *Haematologica* (2012) 97(11):1674–7. doi:10.3324/haematol.2011.061051
98. Luft T, Dietrich S, Falk C, Conzelmann M, Hess M, Benner A, et al. Steroid-refractory GVHD: T-cell attack within a vulnerable endothelial system. *Blood* (2011) 118(6):1685–92. doi:10.1182/blood-2011-02-334821
99. Rachakonda SP, Penack O, Dietrich S, Blau O, Blau IW, Radujkovic A, et al. Single-nucleotide polymorphisms within the thrombomodulin gene (THBD) predict mortality in patients with graft-versus-host disease. *J Clin Oncol* (2014) 32(30):3421–7. doi:10.1200/JCO.2013.54.4056
100. Holtan SG, Verneris MR, Schultz KR, Newell LF, Meyers G, He F, et al. Circulating angiogenic factors associated with response and survival in patients with acute graft-versus-host disease: results from Blood and Marrow Transplant Clinical Trials Network 0302 and 0802. *Biol Blood Marrow Transplant* (2015) 21(6):1029–36. doi:10.1016/j.bbmt.2015.02.018
101. Tomblyn M, Chiller T, Einsele H, Gress R, Sepkowitz K, Storek J, et al. Guidelines for preventing infectious complications among hematopoietic cell transplantation recipients: a global perspective. *Biol Blood Marrow Transplant* (2009) 15(10):1143–238. doi:10.1016/j.bbmt.2009.06.019
102. Maschmeyer G, Donnelly JP. How to manage lung infiltrates in adults suffering from haematological malignancies outside allogeneic haematopoietic stem cell transplantation. *Br J Haematol* (2016) 173(2):179–89. doi:10.1111/bjh.13934
103. Rieger CT, Rieger H, Kolb HJ, Peterson L, Huppmann S, Fiegl M, et al. Infectious complications after allogeneic stem cell transplantation: incidence in matched-related and matched-unrelated transplant settings. *Transpl Infect Dis* (2009) 11(3):220–6. doi:10.1111/j.1399-3062.2009.00379.x
104. Hildebrandt GC, Fazekas T, Lawitschka A, Bertz H, Greinix H, Halter J, et al. Diagnosis and treatment of pulmonary chronic GVHD: report from the consensus conference on clinical practice in chronic GVHD. *Bone Marrow Transplant* (2011) 46(10):1283–95. doi:10.1038/bmt.2011.35
105. Wolff D, Hildebrandt G. Bronchiolitis obliterans–pleading for a pragmatic approach. *Biol Blood Marrow Transplant* (2016) 22(4):591–3. doi:10.1016/j.bbmt.2016.01.022
106. Cheng GS, Edelman JD, Madtes DK, Martin PJ, Flowers ME. Outcomes of lung transplantation after allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant* (2014) 20(8):1169–75. doi:10.1016/j.bbmt.2014.04.008
107. Sengsayadeth SM, Srivastava S, Jagasia M, Savani BN. Time to explore preventive and novel therapies for bronchiolitis obliterans syndrome after allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant* (2012) 18(10):1479–87. doi:10.1016/j.bbmt.2012.03.008
108. Bacigalupo A, Chien J, Barisione G, Pavletic S. Late pulmonary complications after allogeneic hematopoietic stem cell transplantation: diagnosis, monitoring, prevention, and treatment. *Semin Hematol* (2012) 49(1):15–24. doi:10.1053/j.seminhematol.2011.10.005
109. Edinger M, Hoffmann P. Regulatory T cells in stem cell transplantation: strategies and first clinical experiences. *Curr Opin Immunol* (2011) 23(5):679–84. doi:10.1016/j.coi.2011.06.006
110. Le Blanc K, Frassoni F, Ball L, Locatelli F, Roelofs H, Lewis I, et al. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. *Lancet* (2008) 371(9624):1579–86. doi:10.1016/S0140-6736(08)60690-X
111. Le Blanc K, Rasmusson I, Sundberg B, Götherström C, Hassan M, Uzunel M, et al. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet* (2004) 363(9419):1439–41. doi:10.1016/S0140-6736(04)16104-7
112. Moll G, Le Blanc K. Engineering more efficient multipotent mesenchymal stromal (stem) cells for systemic delivery as cellular therapy. *ISBT Sci Ser* (2015) 10(S1):357–65. doi:10.1111/voxs.12133
113. Antin JH, Kim HT, Cutler C, Ho VT, Lee SJ, Miklos DB, et al. Sirolimus, tacrolimus, and low-dose methotrexate for graft-versus-host disease prophylaxis in mismatched related donor or unrelated donor transplantation. *Blood* (2003) 102(5):1601–5. doi:10.1182/blood-2003-02-0489
114. Finke J, Schmoor C, Lang H, Potthoff K, Bertz H. Matched and mismatched allogeneic stem-cell transplantation from unrelated donors using combined graft-versus-host disease prophylaxis including rabbit anti-T lymphocyte

globulin. *J Clin Oncol* (2003) 21(3):506–13. doi:10.1200/JCO.2003.03.129
115. Luznik L, O'Donnell PV, Symons HJ, Chen AR, Leffell MS, Zahurak M, et al. HLA-haploidentical bone marrow transplantation for hematologic malignancies using nonmyeloablative conditioning and high-dose, post-transplantation cyclophosphamide. *Biol Blood Marrow Transplant* (2008) 14(6):641–50. doi:10.1016/j.bbmt.2008.03.005
116. Taur Y, Jenq RR, Ubeda C, van den Brink M, Pamer EG. Role of intestinal microbiota in transplantation outcomes. *Best Pract Res Clin Haematol* (2015) 28(2):155–61. doi:10.1016/j.beha.2015.10.013
117. Pierini A, Alvarez M, Negrin RS. NK cell and CD4+ FoxP3+ regulatory T cell based therapies for hematopoietic stem cell engraftment. *Stem Cells Int* (2016) 2016:9025835. doi:10.1155/2016/9025835
118. Theil A, Tuve S, Oelschlägel U, Maiwald A, Döhler D, Oßmann D, et al. Adoptive transfer of allogeneic regulatory T cells into patients with chronic graft-versus-host disease. *Cytotherapy* (2015) 17(4):473–86. doi:10.1016/j.jcyt.2014.11.005
119. Koreth J, Matsuoka K, Kim HT, McDonough SM, Bindra B, Alyea EP III, et al. Interleukin-2 and regulatory T cells in graft-versus-host disease. *N Engl J Med* (2011) 365(22):2055–66. doi:10.1056/NEJMoa1108188
120. Dall'Amico R, Messina C. Extracorporeal photochemotherapy for the treatment of graft-versus-host disease. *Ther Apher* (2002) 6(4):296–304. doi:10.1046/j.1526-0968.2002.00448.x
121. Gatza E, Rogers CE, Clouthier SG, Lowler KP, Tawara I, Liu C, et al. Extracorporeal photopheresis reverses experimental graft-versus-host disease through regulatory T cells. *Blood* (2008) 112(4):1515–21. doi:10.1182/blood-2007-11-125542
122. Koehn BH, Apostolova P, Haverkamp JM, Miller JS, McCullar V, Tolar J, et al. GVHD-associated, inflammasome-mediated loss of function in adoptively transferred myeloid-derived suppressor cells. *Blood* (2015) 126(13):1621–8. doi:10.1182/blood-2015-03-634691
123. Ball LM, Bernardo ME, Roelofs H, van Tol MJ, Contoli B, Zwaginga JJ, et al. Multiple infusions of mesenchymal stromal cells induce sustained remission in children with steroid-refractory, grade III–IV acute graft-versus-host disease. *Br J Haematol* (2013) 163(4):501–9. doi:10.1111/bjh.12545
124. Ringdén O, Uzunel M, Rasmusson I, Remberger M, Sundberg B, Lönnies H, et al. Mesenchymal stem cells for treatment of therapy-resistant graft-versus-host disease. *Transplantation* (2006) 81(10):1390–7. doi:10.1097/01.tp.0000214462.63943.14
125. Teshima T, Reddy P, Zeiser R. Acute graft-versus-host disease: novel biological insights. *Biol Blood Marrow Transplant* (2016) 22(1):11–6. doi:10.1016/j.bbmt.2015.10.001
126. Wolff D, Bertz H, Greinix H, Lawitschka A, Halter J, Holler E. The treatment of chronic graft-versus-host disease: consensus recommendations of experts from Germany, Austria, and Switzerland. *Dtsch Arztebl Int* (2011) 108(43):732–40. doi:10.3238/arztebl.2011

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Graft-versus-Leukemia Effect Following Hematopoietic Stem Cell Transplantation for Leukemia

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The success of hematopoietic stem cell transplantation (HSCT) lies with the ability of the engrafting immune system to remove residual leukemia cells *via* a graft-versus-leukemia effect (GvL), caused either spontaneously post-HSCT or *via* donor lymphocyte infusion. GvL effects can also be initiated by allogenic mismatched natural killer cells, antigen-specific T cells, and activated dendritic cells of leukemic origin. The history and further application of this GvL effect and the main mechanisms will be discussed and reviewed in this chapter.

Keywords: graft-versus-leukemia effect, animal models, donor lymphocyte infusion, allogenic natural killer cells, leukaemia associated antigens (LAA), LAA specific T cells, leukaemia derived dendritic cells, invariant natural killer T cells (i)NKT, leukaemia specific antigens

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INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) transplantation has a major role in the treatment of leukemia and hematological disease, often the only treatment providing a chance of cure in otherwise refractory diseases. The primary approaches involved total body irradiation (TBI) (1) or cyclophosphamide (CY) (2). However, TBI alone was not immunosuppressive enough, and only one patient survived as a chimera (3). CY was readily immunosuppressive, but did not affect leukemic stem cells (2). Recovery from aplastic anemia was even observed after CY and graft failure, indicating the weak effect of CY on hematopoietic stem cells (4). Sustained success was reported with the combination of TBI and CY and other chemotherapy (5), as well as with the combination of CY and busulfan (6). In the 1970s and 1980s, interest focused on the conditioning treatment and the prevention of graft-versus-host disease (GvHD).

The first suggestion of a graft-versus-leukemia (GvL) effect was in 1956, using a mouse transplantation model, where rejected leukemia cells appeared to be eliminated by incoming bone marrow when irradiation was delayed (7). This led to the concept that the donor marrow cells may be responsible for the eradication of the leukemia.

This observation was applied to the clinic nearly 10 years later by Mathé and team in 1965. Mathé coined the term "adoptive immunotherapy" for the treatment of leukemia with allogeneic bone marrow transplantation (8) and showed that leukemia was eliminated by the GvL. In this paper, they also describe the "secondary syndrome," later to be described as GvHD. Interestingly, bone marrow from six family donors were used for the transplant and in order to decide which donor may be used posttransplant to enhance GvL, the patient received skin grafts from all six donors.

Histocompatibility tests at that time (9, 10) showed that the only skin graft, which was not rejected was the one which was closest genetically to the donor. This donor was subsequently used to give incremental doses of leukocyte treatment posttransplant and gave rise to GvL, but also GvHD. The latter was controlled by the use of hydrocortisone. The patient remained in remission for 1-year posttransplant but died from a viral infection with no sign of relapsing disease.

A major step toward successful transplantation was identifying HLA-identical siblings as best donors and syngeneic twins for prevention of rejection and GvHD. These findings were derived from dog experiments where the correct littermate was chosen by first ensuring that the donor leukocytes were negative, both against the donor in mixed lymphocyte reactions and with cytotoxic antisera (11, 12).

In 1990, over 2,000 transplants had been performed and reported by the International Bone Marrow Transplant Registry (IBMTR). An analysis of the transplants by Horowitz et al. showed conclusively that a GvL effect was important to reduce relapse. An increased relapse rate was observed when the graft was T cell depleted to prevent GvHD. In addition, the data showed that grafts with or without T cells had a high incidence of relapse, indicating that the antileukemia effect could occur independent of GvHD (13).

Relapse of residual disease is a common cause of reduced survival following HSCT. This occurs in 20–70% of patients and is dependent on several factors including pretransplant disease status, cytogenetic subtypes [in acute myeloid leukemia (AML) and in acute lymphoid leukemia (ALL)], stem cell source, age of the patient and donor, and type of conditioning regimen (14, 15). In addition, relapse contributes to 40–45% of deaths following HLA-matched identical HSCT and 34% in unrelated donor HSCT (16). The use of reduced intensity conditioning regimens has also led to GvL effects, which have been most marked in chronic myeloid leukemia (CML) and are also detectable in myelodysplastic syndrome (MDS), AML, and ALL.

This review describes the history and advances made in treating and or preventing relapse following HSCT using donor lymphocyte infusion (DLI), allogenic mismatched natural killer (NK) cells, antigen-specific T cells, and activated dendritic cells (DCs) of leukemic origin.

CLINICAL RESULTS USING DLI FOR RELAPSE AFTER HEMATOPOIETIC CELL TRANSPLANTATION

Although adoptive transfer of lymphocytes immediately after transplantation was attempted to induce remission, severe GvHD ensued and was unsuccessful for reducing relapse in high risk acute leukemia (17).

Careful studies in dogs given T cell-depleted marrow from dog lymphocyte antigen-identical littermate donors had shown that donor lymphocytes could be transfused after more than 2 months posttransplant without the risk of GvHD (18); this effect was subsequently shown in both canine and murine transplant models (18–21). Moreover, donor DLI converted mixed chimerism into complete chimerism, transferred immunity, and improved immune reactivity to leukemic antigens. Further work demonstrated the potential role of tolerance in the lack of GvHD development.

It took pioneering work in the 1990s (22, 23) to show that, for CML transplant patients, subsequent separation of the transplant by 2 months from the DLI enabled remissions and prevented GvHD. These first studies in man for CML were carried out by Kolb and colleagues with subsequent remission in all three patients for over a decade (22). Two of the three patients, treated in this first report of DLI, are still in complete remission (CR1) of CML (22). One patient had a cytogenetic relapse 20 years after DLI. She was retreated with DLI from her brother and responded again. The GvL effect has been confirmed for CML in numerous studies worldwide (24, 25) and are frequently durable offering potential cure for the majority of CML patients (26–28). These results have been collected by the European group for Blood and Marrow Transplantation (EBMT) (29), the US (26), and Japanese transplant centers (30). The best results with 70–80% cytogenetic complete remissions were reported for CML in cytogenetic and hematological relapse, other important factors being the presence of chronic GvHD prior to DLI and the time of relapse post-HSCT (31). Donor chimerism was also necessary for a successful GvL in CML. For patients with AML or MDS, the response rate to DLI is much lower (20–40%) and is lower still in ALL (10–13%) with intermediate results (40–52%), compared with CML, being obtained in multiple myeloma (MM) (32). In AML, DLI efficacy is thought to be limited to a small sub group of patients, those with favorable cytogenetics, with a low-tumor burden at relapse or in hematological remission prior to DLI (33). In general, complete remissions were durable in CML and only in a minority of patients with acute leukemia and MM (34).

THE HISTORY OF THE ROLE OF T CELLS AND NK CELLS IN THE GvL EFFECT

As stated previously, the role of T cells was further identified by studies using data from the IBMTR (35). In addition, differences in relapse rates with higher doses of TBI and fractionated TBI versus single-dose TBI were only seen in patients given T-cell-depleted marrow, while in patients given T-replete marrow, no differences were observed (35). In 2004, Ballester et al. described a graft versus myeloma effect after DLI and an autologous stem cell transplant rescue (36). Daguindau et al. described an antitumor effect of HSCT in 14 patients with either acute leukemia or MDS who sustained a long-lasting CR1, despite only transient or absent engraftment with donor cells (37). This effect, therefore, being caused by transient exposure to allogenic T cells and autologous reconstitution.

Natural killer cells were identified in the 1970s by Kiessling and Wigzell (38) and were shown to kill tumor cell lines in the absence of MHC class I molecules (39). This gave rise to Ljunggren and Karre in 1990 introducing the "missing self" hypothesis (40) where NK cells kill targets because they do not express high levels of "self" MHC class I gene products. They predicted the presence of receptors for self-MHC which,

when engaged, would inhibit cytotoxicity. It was subsequently shown that NK cells can distinguish between normal and malignantly transformed cells by the presence of killer-cell immunoglobulin-like receptors (KIRs); these are receptors for MHC class I and are the main inhibitory receptors. The KIR inhibitory receptor family recognizes HLA-A, B, and C molecules (41, 42). Another inhibitory receptor C type lectin NKG2A recognizes the MHC molecule HLA-E (43–45). The inhibitory receptors give rise to a repertoire of NK cells with overlapping specificities. The function of NK cells is regulated by the balance of activatory and inhibitory signals transmitted by different cell surface receptors, such as KIRs, NK Group 2 member D (NKG2D), NKG2A/CD94, NKp46, and others (46, 47) (**Figures 1** and **2**). NK cells recognize both foreign and self-antigens expressed by NK-susceptible targets. NK cells attack cells lacking MHC class I molecules specific to the inhibitory receptors KIRs on the NK cells (47).

The biological and clinical effects of NK cells in allogeneic transplant have recently been reviewed by Benjamin et al. (50).

The first study utilizing NK cell alloreactivity was shown in 2002 by the group of Velardi (48). HLA haploidentical—mismatched family donors were used to transplant 57 AML patients and 35 ALL patients. Donor versus recipient NK cell reactivity was analyzed in groups with and without KIR ligand incompatibility in the graft-versus-host (GvH) direction. Protection from GvHD and AML relapse was observed and showed that KIR ligand incompatibility in the GvH direction predicted survival in AML patients. In contrast, in the ALL patients, KIR ligand incompatibility in the GvH direction had no effect on ALL survival rates. In this study, the transplant regimen was myeloablative and involved T cell-depleted grafts and very large doses of CD34 positive cells/kg, contributing to lack of GvHD and successful engraftment.

In a later study using non-myeloablative conditioning (2 Gy of TBI with or without fludarabine) and HLA-matched grafts, the risk of relapse was less in patients with ligands for all donor KIR, but this did not reach significance (51).

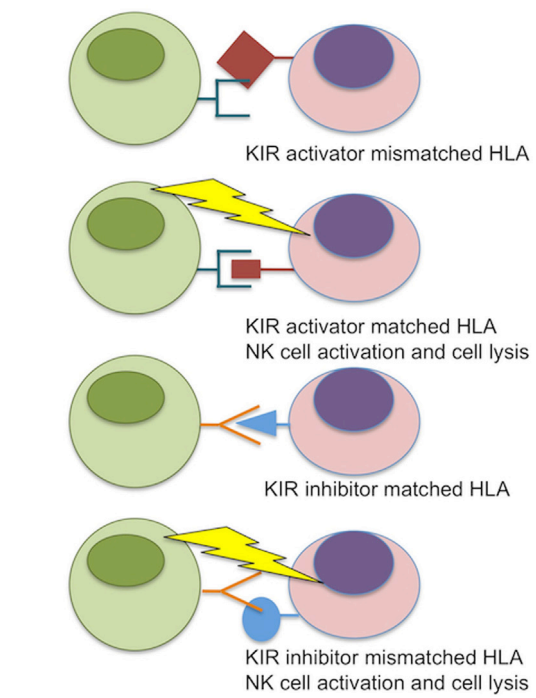

FIGURE 1 | Donor-versus-recipient natural killer (NK) alloreactivity. NK cell function is regulated by KIR interactions with matched HLA class I alleles. If HLA is mismatched in transplant recipient leukemic cells, NK cells are relieved from inhibition and induce cell lysis. In the case for inhibitor KIRs, binding with matching HLA prevents donor NK cell activation to self. For activating KIRs, donor NK cells that bind the matched HLA are activated and induce cell lysis of transplant recipient acute myeloid leukemia (AML) cells. Image adapted from Ref. (48, 49).

PROPHYLACTIC AND PREEMPTIVE DLI

Animal experiments were designed to demonstrate repletion of T cells by DLI after T-cell-depleted transplantation. In dogs, donor lymphocytes eliminated residual host hematopoiesis and converted mixed chimerism into complete chimerism (52). Following these results, prophylactic DLI became part of the FLAMSA regimen (fludarabine, cytarabine, amsocrine) for high risk AML (53). This regimen was designed for sequential therapy of high risk AML with FLAMSA followed by reduced intensity conditioning consisting of reduced TBI (4 Gy) or busulphan, antithymocyte globulin, and CY. DLI after immunosuppressive therapy was stopped for 30 days without development of GvHD. A matched pair analysis of patients receiving or not receiving DLI showed a significant advantage of patients given DLI; matching criteria were CR1 at day 120 from transplantation, absence of GvHD, and infection (54). Prophylactic DLI produced 80% long-term survival in several studies (55–58), involving around 340 AML and ALL patients. GvHD was seen in 28% of patients given DLI, but it was fatal only in 9% of all patients treated (58). However, in a small study of 12 patients in 2001, given DLI prophylactically as early as days 30, 60, and 90 days posttransplant, three patients developed GvHD (59). In a study of 15 patients treated with alemtuzumab in the conditioning treatment, seven patients developed GvHD and, in three patients, it was fatal (60). In lymphoma patients, conditioned with a regimen containing alemtuzumab for *in vivo* T-cell depletion followed by DLI for mixed chimerism, non-fatal GvHD occurred in 4 out of 17 patients (61). In our own unpublished evaluation, using landmark analysis on day 180, remissions were sustained in patients transplanted in CR. Relapse-free survival was improved in patients transplanted in an active phase of the disease. The median time of DLI post-transplantation was 160 days. Patients with active GvHD or relapse prior to 180 days were excluded from the evaluation. Fatal GvHD was observed in a patient treated for increasing mixed chimerism following an infection with Noro virus and another patient with upper respiratory tract infection. Viral infections can induce HLA class II antigens on non-hematopoietic cells leading to GvHD (62). Prophylactic antibiotic and virostatic treatment has been used to improve outcome.

Besides DLI for mixed chimerism, preemptive DLI can be given to patients with minimal residual disease (MRD) after

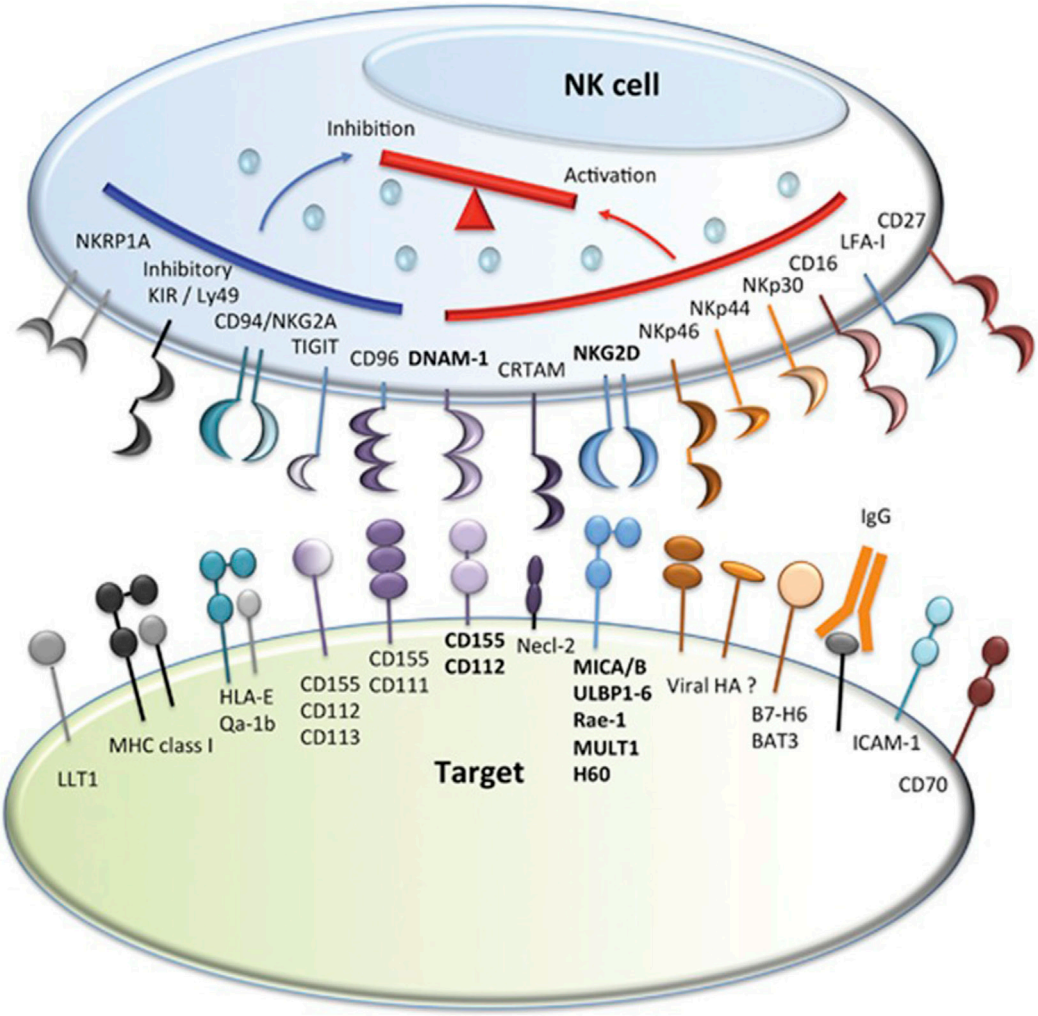

FIGURE 2 | Natural killer (NK) cell inhibitory and activatory receptors and their ligands. Major inhibitory and activating receptors on NK cells and their cognate ligands on target cells. Image adapted from Ref. (46).

transplantation. In CML, cytogenetic or molecular relapses indicate presence of residual disease without clinical signs; DLI have been effective in these patients with responses of >80% (16, 19, 47). In AML, there are a few molecular markers with sufficient sensitivity for diagnosing MRD. Monitoring WT1 gene transcripts has been found to predict relapse and the response to DLI (63) and RUNX1-RUNX1T1 transcript levels in patients with t(8;21) AML (64) pre DLI has been found to be predictive of a higher relapse incidence. MRD in acute leukemia in children and adults has been well documented (65) using a combination of flow cytometry and polymerase chain reaction, the latter for the detection of leukemia-specific fusion transcripts or clone-specific immunoglobulin including T cell receptor genes. In relapsed acute leukemia, a combination of gene transcript levels and four color flow cytometry, MRD monitoring has been found to predict a second relapse post-DLI (66). In myeloma, several groups have studied prophylactic or preemptive DLI (67–69), the rate of durable remissions is low, but secondary treatment is efficacious and survival is excellent.

The successful use of CML in DLI in the 1990s has been substantially reduced due to the reduced number of allo-HSCT for CML, to approximately 1% (70), by the success of tyrosine kinase inhibitors (TKIs) to treat CML. The family of TKI's is capable of restoring complete molecular remission after relapse (71–73). CML relapse, molecular cytogenetic, or hematological has been reported as ranging from 16, 30, and 54%, respectively, using data from the Chronic Malignancies Working Party for the EBMT and based on 500 HSCT transplants from 1968 to 2004. The use of DLI in these cases was most successful if pre DLI factors such as chronic GvHD, cell dose, patient and donor gender mismatch, as previously described was taken into account (31).

In contrast, relapse after allo-HSCT for the other types of leukemia is further dependent in AML, on the age of the patients, disease status pre allo-HSCT, the AML sub types (primary or

secondary), cytogenetic and molecular markers, type of conditioning and stem cell source (74–79). AML patients relapsing after allo-HSCT rarely responded to DLI although remissions have occurred in selected cases (26).

Use of DLI in a large cohort of 399 AML patients, collated from the Acute Leukemia Working Party of the EBMT, was associated with 21% overall patient survival at 2 years, compared with 9% for patients not receiving DLI (33). Better outcome was associated with lower tumor burden at relapse, female gender, favorable cytogenetics, and with patients in hematological remission before DLI or at the time of DLI. From these studies, an algorithm for the clinical use of DLI was developed for use in the treatment of relapsed AML, which included the sequence of cyto reductive chemotherapy or indication of CR1 prior to DLI (80).

Relapse after ALL varies from 30 to 35% depending on whether the patients have undergone a HLA-matched sibling transplant or matched unrelated donor (MUD) transplant (81), and response to DLI has been recorded at 50% with survival rates improved in patients who developed acute GvHD after DLI (82).

COMPLICATIONS OF DLI

Graft-versus-Host Disease

Early experiments in canine, rat, and mice transplant models demonstrated no GvHD following infusion of non-sensitized donor lymphocytes into stable chimerisms (18–21).

This observation led to the concept that DLIs may be used to improve engraftment and accelerate immune reactivity without the occurrence of GvHD in a stable human chimera.

Contrary to the results in animal experiments with dogs and mice, GvHD was seen in humans given DLI (83). There are a great number of differences that may account for this. Unlike human patients, animals used for experiments are of younger age and are kept in protected environments, minimizing chronic infections and immune cross reactivity. More importantly, differences exist in the underlying malignant disease and its impact on alloimmunity as well as prior chemotherapy, depleting lymphocytes and ablating regulatory T cells (84).

Attempts at preventing GvHD included depleting cytotoxic CD8-positive T cells from the transfusion (85), arming T cells with suicide genes (86), and the administration of escalating doses (87), which was widely adopted for the treatment of recurrent CML (88).

An important role in the generation of GvHD after DLI is played by viral infections and/or reactivations of viruses (62, 89). Antiviral and antimicrobial prophylaxis has, therefore, prevented viral infections and improved the response to DLI.

In addition, studies by the Chronic Malignancies Working Party of the EBMT (29, 83, 90) have shown that up to 40% of patients with secondary GvHD post-DLI had a twofold to threefold increased risk of death, compared to patients without GvHD. The best results following DLI are obtained when patients obtain remission without GvHD, thus separating GvL from GvHD in these patients (90–92).

A recent study by Radujkovic et al. aimed to identify pre-DLI factors, which may predict survival in remission without secondary GvHD in patients with relapsing CML. The study identified that the presence of chronic GvHD before DLI and less than 1 year between the allo-HSCT and DLI were associated with inferior survival. The likelihood of survival in remission without GvHD was most prevalent, i.e., 50% at 5 years follow-up, when DLI was given without prior chronic GvHD and greater than 1-year post allo-HSCT for molecular and/or cytogenetic CML relapse.

For hematological relapse, a T cell dose of greater than 50×10^6/kg, the donor–recipient gender mismatch and prior chronic GvHD were the worst prognostic factors.

Initial studies showed that starting the transfusion of donor cells at low cell numbers followed by escalating doses until response or induction of GvHD reduces the incidence and severity of GvHD but preserves a GvL effect (87, 93).

Guglielmi et al. subsequently analyzed 344 CML patients treated by DLI and their study found that the initial cell dose was given based on donor type (HLA-identical sibling or HLA matched volunteer unrelated donor), T cell depletion, GvHD prior to relapse, relapse type (cytogenetic, molecular, and hematological), and year (90). The lower the initial cell dose, the high number of subsequent transfusions were given to achieve a response and the incidence and severity of GvHD and myelosuppression increased with the higher initial cell dose of greater than 0.2×10^6 MNC/kg. Factors such as donor type, gender of donor, disease phase at transplantation, T cell depletion, interval from transplantation to DLI, GvHD prior to relapse and relapse type, all influence outcome post-DLI and potential incidence of GvHD and prolonged survival.

Myelosuppression

Pancytopenia and marrow aplasia have been observed in patients treated with DLI for hematological relapse of CML (92) and transfusion of marrow from the original donors restored hematopoiesis (94). Sometimes, myelosuppression and marrow aplasia were sudden and related to acute GvHD (95). This type of phenomenon can be explained by the incoming donor marrow removing leukemic hematopoiesis prior to donor hematopoiesis being fully established. Lack of recovery in some patients may also be explained by too few stem cells in the donor graft to sustain hematopoiesis.

IMPROVEMENT OF THE RESPONSE

Complete cytogenetic responses were achieved initially in patients with CML who had been treated with massive doses of donor lymphocytes ($>10^8$/kg) and interferon-α (IFN-α) but had failed to respond to IFN-α alone. Responses were better in patients treated with myeloablative conditioning and T cell-depleted grafts than in patients with non-myeloablative conditioning and peripheral blood (PB) stem cells containing high proportions of T cells (29, 96). The better response of myeloid forms of leukemia led to the hypothesis that direct antigen presentation by leukemia-derived DC may play a major role in the activation of donor T cells (91). IFN-α improves the GvL effect of low doses of DLI and the combination of IFN-α and granulocyte macrophage-colony stimulating factor (GM-CSF) improved antigen presentation and generation of cytotoxic

T cells in CML (97). Consequently, patients not responding to IFN-α and DLI responded to the combination of DLI, IFN-α, and GM-CSF. Moreover, T cells of CML patients displayed reduced zeta-chain expression and tended to go into apoptosis, which could be reversed by IFN-α (98). Lymphodepletion prior to DLI may enhance the anti tumor effect of the infused T cells, however, this can cause more GvHD (84).

Future potential improvements may come from the treatment with checkpoint inhibitors in order to increase T cell activation by inhibiting downregulation (99). Preliminary reports have shown feasibility of single doses of ipilimumab with blockade of CTLA4, without producing GvHD (100). An attractive way of treatment may be the use of central memory T cells that maintain a memory immune reaction without producing GvHD (101).

Antigen-Specific T Cells

In acute leukemia, the pace of the disease is too fast to allow the development of immune reactions against the leukemia as observed for CML. Therefore, the generation of specific tumor immune T cells for the rapid elimination of leukemia cells have been investigated, and there are several candidate antigens, which have been used in assessing immune reactions to leukemia and induction of remission.

Leukemia-Associated Antigens (LAAs)

Leukemia-associated antigens are often overexpressed in leukemia blasts and absent in normal tissue such as Wilms tumor 1 (WT-1), preferentially expressed antigen in melanoma (PRAME), melanoma family antigen (MAGE), receptor for hyaluron mediated motility (RHAMM), testis antigens like New York esophageal squamous cell carcinoma-1 cancer-testis antigen (NY3ESO), and granulocyte antigens such as PR1 (a 9 amino acid HLA-A*0201-restricted peptide derived from proteinase3).

The most widely studied antigen is coded by WT-1, a gene involved in Wilms tumor and present in about 77% of AML (102). Cytotoxic T cells against WT-1 kill AML stem cells preventing engraftment in NOD/SCID mice (103). Most immune reactivities found after DLI against any of these antigens are weak. Moreover, immune reactivity against autologous antigens is frequently suppressed by mechanisms of tolerance mediated by, e.g., regulatory T-cells (Tregs) or inhibitory cytokines (104). Peptides presented by foreign HLA antigens can be immunogenic by different configurations (105). It has been shown in a mouse model that T cell receptors cloned from HLA-different T cells can be transduced into autologous T cells to maintain immunity (106). Mispairing of T cell receptor chains with endogenous TCR chains could be avoided by lentiviral (LV) transfer and more recently by silencing endogenous TCR with endonucleases prior to transduction (107). Our own results correlated stable remission after HSCT with the presence of higher proportions of LAA-specific T-cells. The simultaneous detection of two different LAA-specific (CD8-positive T-cells) correlated with a higher chance of long-lasting remission. Moreover, we detected clonally restricted (PRAME-) specific T-cells and, in general, an enrichment of (effector) memory T-cells in cases with stable remission (102).

Our studies focused on patients after HSCT and might, therefore, be in accordance with the finding of PRAME reactive cytotoxic T precursor cells in healthy donors and not in AML patients (108). Encouraging results for PRAME as a target for immunotherapy in leukemia were, however, reported by Rezvani and colleagues (109). Immune response to RHAMM has also been elicited by vaccination (110). At present, lasting success using anti-LAA T cells have not been reported.

Leukemia-Specific Antigens

Leukemia-specific antigens are antigens coded for by a mutational event in the leukemic clone. A unique translocation is the cause of CML, and the peptides derived from this gene fusion are presented by HLA antigens, the most immunogenic by HLA A3. In addition, DC in CML patients have the BCR/ABL translocation and can stimulate allogeneic T cells, inducing a cytotoxic T cell effect (97).

Single cases of enduring responses and immunity to the fusion peptide have been reported with peptide vaccine and interferon-α (104). In AML, many different mutations make the production of a vaccine to leukemia-specific antigens difficult, but cytotoxic T cells against nucleophosmin (NPM1) have been reported (111). NPM1 may be a preferable target, because of its presence on leukemia stem cells. New possibilities may arise from the detection of immune inhibitors of T cell activity, which may be reconstituted by check point inhibitors enabling T cells to react to whatever antigen is recognized. In AML and myeloid malignancies, other mechanisms (33) of T cell suppression may also be important. For example, blockade of CD47 expression on tumor cells, driving macrophage T cell and dendritic cell activation leads to tumor cell killing (33).

Minor Histocompatibility Antigens (mHAs)

Minor histocompatibility antigens are responsible for graft rejection and GvHD in HLA-identical sibling transplants, they may be effective in GvL, if these antigens are expressed on hematopoietic cells. The reaction against hematopoietic cells of the patient is irrelevant, because hematopoiesis is substituted by donor cells. Both CD4-positive and CD8-positive T cells respond to leukemic or minor histocompatibility antigens. In the latter case, cytotoxic T cells have been generated against CML, which have induced remission and shown to be correlated with the presence of cytotoxic T lymphocytes against minor histocompatibility antigens HA1 and HA2 (112). In an EBMT study, responses of patients with recurrent CML were only seen in those with an allogeneic donor, syngeneic twin donors did not respond (29). There are mHAs with a tissue distribution restricted to the hematopoietic system (113) and mHA expressed in all tissues. The strongest mHAs are those coded by the Y chromosome (114). Y chromosome antigens-directed T cell responses show strong antileukemic effects (115), but they may also produce GvHD. mHA also produce peptides presented by both HLA class I and HLA class II, as well as eliciting antibodies against themselves (116). We have investigated Y chromosome genes by comparing leukemic blasts with normal monocytes, and we found four genes overexpressed in AML (102). Peptides of the gene products were loaded onto

T2 cells and cytotoxic T cells could be produced against two of the four antigens. As demonstrated in a canine model, we could demonstrate that female dog-effector-T-cells could be specifically stimulated against male chromosomal (UTY−) gene products (116).

The search for new mHA was expanded to study the reactivity of T cells against antigens and overlapping peptides of genotyped lymphoblastoid cell lines (HapMap-Project) (117). Several new antigens were found, but the majority of reactions did not show significance probably due to additional factors provided by the microenvironment (118).

Production of DC of Leukemia Origin (DC_{leu})

Effective antigen presentation is essential in GvL reactions. GM-CSF has been effective in the production of DC of leukemia origin (DC_{leu}), which were able to induce cytotoxic T cells (119). Inhibition of cytotoxicity was greater with antibody against HLA class I than antibody against HLA class II. Production of DC from AML leukemia blasts was extensively studied by Schmetzer and colleagues (102, 120) (**Figure 3**). It could be shown that DC_{leu} could be successfully generated from blood samples in every AML patient using methods of DC generation containing different mixtures of immune-modulatory factors, GM-CSF, IL-4, TNF-α, FLT3-L, IL-1β, IL-6, PGE2, bacterial lysate of streptococcus pyogenes (PICIBANIL), or calcium ionophore.

Moreover, Schmetzer's group correlated a successful *ex vivo* generation of DC_{leu} from AML blasts before HSCT with a clinical response to HSCT or DLI (102), which may suggest a central role of DC_{leu} in priming antileukemic T cells.

In vivo Schmetzer's group has shown that rats heavily diseased with promyelocyte-like leukemia and treated with DC_{leu}-inducing Kits GM-CSF with Picibanil, prostaglandin 1 or 2 (PGE1 or PGE2) (patent-number 10 2014 014 993) showed a highly

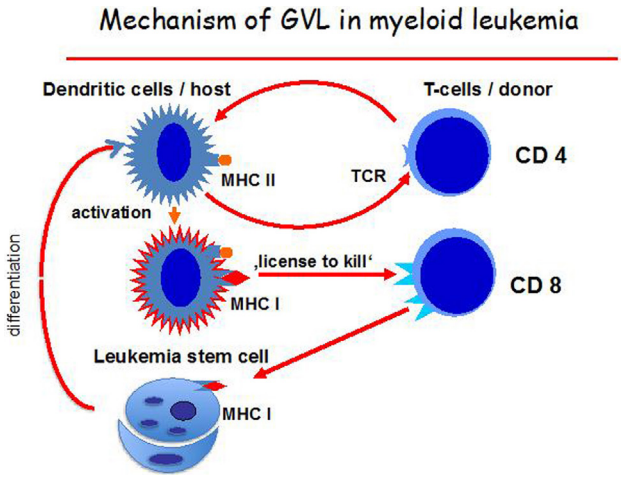

FIGURE 3 | Mechanisms of the graft-versus-leukemia (GvL) effect. Host dendritic cells (DCs), activated by CD4-positive T cells, mature and can then activate CD8-positive T cells *via* a "license to kill" and attack leukemia cells. The leukemia cells themselves can also differentiate into self DC.

significant reduction of blasts, an increase of memory like T-cells, and a decrease of Treg after two Kit-applications in only 9 days, therefore, suggesting that as proof of principle, a DC_{leu} induction *in vivo* could lead to T-cell activation resulting in a specific blast reduction. Our ongoing research focuses on the transfer of this strategy to patients with AML (121) [Christoph Schmid and Helga Schmetzer, personal communication; (102, 122)].

Some patients with AML relapsing after transplantation could be induced into remission by treatment with low-dose cytarabine, mobilized donor cells including PB stem cells and post-grafting GM-CSF (123). GvHD occurred on the day after T cells were administered. The remission rate was doubled compared to the results of an EBMT study and some patients survived in long-term remission without further treatment. Risk (33) factors for failing treatment were early relapse (less than 180 days) and failure to respond to low-dose cytarabine. In the EBMT study, similar risk factors were defined and long-term survival was only seen in patients treated with DLI or second transplants after achieving CR1 (80). Several centers have shown efficacy of repeated treatments with azacitidine and DLI also establishing long-term remission in some patients (124). Azacitidine and cytarabine have the potential to induce differentiation of blasts, new targeted drugs like sorafenib (125) and midostaurin (126) may also be helpful in Fms-related tyrosine kinase 3 (FLT-3)-positive leukemia or panobinostat, an oral deacetylase inhibitor for MM (127, 128).

Both cytokines and targeted drugs enable leukemia blasts to differentiate into DC which, by presenting antigen, provide stimulating signals to donor naïve T cells (129). Both reactive CD8-positive and CD4-positive T cells have been found in patients responding to DLI (130). *In vitro* generated T cell cytotoxicity predominantly consisted of CD8-positive T cells, but CD4-positive cytotoxic T cells were also present (119). Schmetzer et al. have shown that T-cell clonality was more restricted after *ex vivo* DC_{leu} induction compared to blast stimulation, pointing to a role of DC_{leu} to efficiently enrich selected T-cell-clones. Interestingly, T cells with the same Vβ chain of the T cell receptor that was observed *in vivo* were also found in *in vitro* cultures (131).

The production of CD4-positive T cells against HLA-class II restricted minor antigens have the advantage that HLA-class II antigens are only expressed on cells of the hematopoietic system. The GvH reaction of allogeneic T cells, therefore, spares non-hematopoietic organs and is operationally leukemia specific (132). However, there are limitations to this approach, since inflammation of healthy tissue increases the expression of HLA-class II on non-hematopoietic cells and induces GvHD. Like normal hematopoietic stem cells, leukemia stem cells are quiescent and do not express HLA-class II antigens (133). Therefore, HLA-class I restricted CD8-positive T cells may be further required for complete elimination of leukemia stem cells.

The hypothetical mechanism behind the GvL effect is that DCs become activated by donor CD4-positive T cells and, once mature, the DCs activate CD8 positive T cells by a mechanism called "license to kill" (134). These also react against leukemia stem cells until the pool of these cells is depleted. CD8 positive naïve T cells, therefore, become involved in the GvL reaction with antigens involving HLA class II and class I peptides being the optimal target (**Figure 3**). Presumably, repeat interactions of

host/leukemia-derived DCs and donor CD4-positive T cells are necessary for sustained GvL effects. Direct antigen presentation by host/leukemia DCs may also be a further mechanism.

Another possible way the GvL reaction is maintained is by central memory T cells that do not require CD4 help for sustained GvL effects. Precursor cells recognizing LAA and mHA are found in low frequencies in the bone marrow of healthy donors, and these can be expanded by encountering some of these antigens in the patient. Examples of the efficacy of memory T cells are virus-specific T cells that can be selected from immune stem cell donors and transferred to the patient (135). As these cells can expand *in vivo,* the presence of central memory T cells recognizing antigens on leukemia cells in the graft is the most important criteria for successful GvL effects. *Ex vivo* data of Schmetzer and colleagues showing increased memory T cell proportions after T cell stimulation with DC_{leu}, compared to blasts, and reduced naïve T cells support this view (102).

MECHANISMS OF A GvL EFFECT—NK CELLS

T cells and NK cells in the donor graft eliminate residual leukemia cells in the patient by T cell interaction with leukemia-specific antigens or mHA, activated NK cells interact with allogeneic targets lacking killer immune receptors (48, 136) (**Figure 2**).

Natural killer cells are the major players of innate immunity with the fastest reconstitution *in vivo*. NK cells are the earliest lymphocytes recovering after HSCT and due to delayed reconstitution of a functional T cells repertoire, NK cells are a vital lymphocyte subset exerting antileukemic effects and have been linked to reduction in relapse rates or improved disease-free survival (137). Nevertheless, as recently reviewed by Zhao et al., the reconstitution of NK cells is influenced by many factors, including the conditioning regimen, level of T cell depletion, and the use of immune suppression after transplantation (138).

KIR–Ligand Interactions and HSCT Outcome

Many clinical studies have linked NK cells to successful outcomes following HSCT. For instance, the donor KIR genotype plays an important role in the development of infections posttransplant. Recipients of unrelated donor HSCT from donors with an activating (KIR) (B/x) genotype have less infectious (bacterial) complications than those with an A/A KIR genotype, because of the enhanced NK cell function (139). It also has an effect on survival post HSCT; in a small study of HLA-matched T cell-replete sibling transplants, better overall survival was associated with the presence of group B KIR haplotypes in the recipient and the absence in the donor (140). Conversely, three donor B haplotype KIR genes have been reported to be associated with reduced relapse and improved overall survival in a study of HLA-matched T cell-replete sibling transplants (141), and in a cohort transplanted for AML, donor possession of group B KIR haplotypes was associated with improved relapse-free survival but a higher incidence of chronic GvHD (142). The group B KIR haplotype KIR3DS1 in the donor has been found to be associated with decreased acute GvHD in MUD transplantation; however, this effect appears to be unique to this specific B allele (142).

Moreover, in haploidentical HSCT, NK cells may express inhibitory killer immunoglobulin-like receptors that are not engaged by any of the HLA class I alleles present on recipient cells. Such "alloreactive" NK cells greatly contribute both to eradication of leukemia blasts escaping the preparative regimen and to clearance of residual host DCs and T lymphocytes (thus preventing GvHD and graft rejection, respectively) (143).

Furthermore, in umbilical cord blood (UCB) transplants for acute leukemia in first CR1, patients with KIR ligand incompatible donors had improved overall survival (57 versus 40%) and decreased relapse (20 versus 37%) when compared with those without these incompatibilities. Benefits of KIR ligand incompatibility were most striking among patients with AML although UCB recipients with ALL also had a trend toward improved leukemia-free survival (144).

IPH2101 is a human IgG4 monoclonal antibody directed against inhibitory KIRs (KIR2DL-1, -2, and -3), which blocks KIR–ligand interaction and augments NK cell-mediated lysis of HLA-C-expressing tumor cells. A phase I trial of IPH2101 (NCT00552396) was conducted in 32 patients with relapsed/refractory MM suggesting that IPH2101 is safe and tolerable at doses that achieve full inhibitory KIR saturation (145).

In Vitro and *In Vivo* Induction of a GvL Effect

Lentiviral vectors have been successfully used to transduce both T and NK-cell lines. Chimeric antigen receptors (CARs) are synthetic engineered receptors that target surface molecules in their native conformation, independent of MHC and of antigen processing by the target cells (146). For example, CS1 is a surface protein highly expressed on MM cells and is amenable to targeting with CS1-specific CARs. CS1-CAR-transduced NK cells showed stronger cytotoxic activity against CS1-expressing MM cells and showed increased IFN-γ production compared with mock-transduced NK cells. In an orthotopic MM xenograft model, adoptively transferred CS1-CAR-NK-92 cells suppressed the growth of human IM9 myeloma cells and significantly prolonged mouse survival (147). Moreover, CAR-NK cells may be safer compared with that of CAR-T cells, because of lack of *in vivo* clonal expansion and cytokine storm.

Novel research techniques use either all or part of an antibody structure to deliver enhanced effector activity to the tumor site. Bi-specific killer engagers (BiKEs) are constructed with a single-chain Fv against CD16 and a single-chain Fv against a tumor-associated antigen. The mechanisms by which BiKEs potentiate NK effector functions include intracellular calcium mobilization through direct CD16 signaling (148). Fully humanized CD16 × CD33 BiKEs have been shown to trigger NK-cell activation *in vitro* against CD33+ AML cell lines and primary refractory CD33+ AML targets (149). BiKEs enhance degranulation and cytokine production by NK cells derived from patients with MDS and cultured with CD33+ AML cell lines, irrespective

of disease stage and age stratum (150). A potential drawback of this approach is the relatively short half-life of the antibody constructs, with limited trafficking to the tumor site.

Ex Vivo Expansion of NK Cells and Induction of GvL Effects

There is no indication to suggest that human GvHD is linked with NK cell infusions, thus increasing the NK cell dose is one useful approach to improve the antileukemia activity. However, for clinical therapy one significant limitation is that the numbers of NK cells/kg recipient weight obtained by leukophoresisis are relatively small (~2×10^7/kg).

Classically, GMP-compliant NK-cell products have been generated from peripheral blood mononuclear cells collected by apheresis (151). IL-15 was used to promote NK-cell proliferation and survival and has been variably used in GMP-grade laboratory protocols. Different expansion methods rely on human feeder cells including artificial antigen-presenting cells that are modified with costimulatory molecules, such as CD137 ligand and membrane-bound (mb) IL-15 or IL-21. However, expanded NK cells may affect the replicative potential and long-term viability of *in vivo* infusion. For instance, in NK cells, both FAS expression and susceptibility to apoptosis are increased after co-culture with IL-2 or with feeder cells (152). In addition, some receptors required for homing were reduced in expanded NK cells, such as CCR7, a member of the G protein-coupled receptor family. In line with this, NK cells expanded with genetically modified K562 cells were shown to predominantly express a $CD16^+CD56^+$ phenotype, with no detectable CCR7 (153, 154). To obviate this, NK cells have been co-cultured with genetically modified, IL-21/CCR7-expressing K562 cells, in order to transfer CCR7 onto NK cells *via* trogocytosis. CCR7 expression occurred in 80% of expanded NK cells within 1 h (154).

Umbilical cord blood is an emerging source of NK cells for clinical applications and also provides an *in vitro* system to analyze NK development (155). However, NK cells from PB and UCB differentially express cytokine receptors, for instance, IL-15Rα being preferentially detected on UCB NK cells and IL-12Rβ1 and IL-18α receptors being primarily found on PB NK cells (156, 157). This implies that, unlike PB NK cells that are fully activated by IL-2 alone, UCB NK cells may require additional cytokine stimuli (158). For instance, the addition of tacrolimus and low-molecular-weight heparin significantly enhances NK-cell expansion induced by IL-2, IL-15, and anti-CD3 mAbs (159).

Like UCB, human embryonic stem cells (ESCs) as well as induced pluripotent stem cells (iPSCs) are also potential sources of phenotypically mature and functional NK cells. ESCs and iPSCs were first used to produce hematopoietic progenitors with the "spin embryonic body (EB)" method, in which defined numbers of cells were spin-aggregated in serum-free medium. Spin EB-derived cells were then tested in a feeder-free and serum-free system containing NK-cell promoting cytokines, i.e., IL-3, IL-7, IL-15, SCF, and Flt3-L. Importantly, NK cells developed in similar numbers, phenotype, and functional characteristics as those differentiated with the use of murine stromal cells (160).

Several malignant NK cell lines were established and used for clinical trials in some countries, as reviewed elsewhere (161). The adoptive transfer of NK cell lines has theoretical advantages related to lack of expression of inhibitory KIRs, lack of immunogenicity, and ease of expansion. For instance, K562-mb15-41BBL cells were used to expand NK cells transduced with an anti-CD19-BB-ζ CAR and showed enhanced reactivity to CD19+ leukemia cells (162). Similar to K562-mb15-41BBL, K562 genetically modified to express mbIL-21, or to co-express the ligand for 41BB and the NKG2D ligand MICA (K562-4-1BBL-mMICA), have been shown to promote large-scale expansion of NK cells with enhanced antitumor *in vitro* reactivity (163–165). Moreover, EBV-immortalized B-lymphoblastoid cells (EBV-BLCL) are known to strongly support NK cells *in vitro* expansion and antitumor activity (166–168). Escudier and colleagues used 35-Gy-irradiated LAZ 388 EBV-BLCL for the *ex vivo* expansion of NK cells from patients with metastatic renal cell adenocarcinoma. Based on their protocol, a phase I clinical trial is currently investigating technical feasibility and clinical efficacy of large-scale NK infusions (up to 1×10^9/kg) in cancer patients receiving bortezomib administered with the scope of increasing susceptibility of tumor cells to NK-mediated lysis (169, 170).

In addition, Schmetzer and colleagues have shown recently that invariant (i)natural killer T ((i)NKT) and cytokine-induced killer (CIK) cells, where both cell types combine the characteristics of T as well as NK cells, and their subsets are promising cells in the induction of antileukemic reactions. Preliminary findings show that proportions and compositions of these cells provide prognostically relevant data for patients with AML, ALL, and CLL. Moreover Kit-treated AML-blasts (resulting in DC_{leu}) induce a shift not only of T-cells but also of iNKT, NK, and CIK cells counts and proportions, correlating in improved antileukemic activity against AML blasts and implying cross talk between these cells (171).

TREG THERAPY AND ITS EFFECTS IN GvL AND RELAPSE

The effects of Treg therapy for GvHD, GvL, and relapse post-HSCT has been recently summarized by Romano et al. (172). There have been several trials investigating the safety and efficacy of Treg-based therapy, the first recorded trial was reported in 2009 where *in vitro* expanded CD4+ CD25+ CD27− cells were used for the treatment of two patients with either acute GvHD or chronic GvHD (173). The patient with chronic GvHD showed a significant improvement of symptoms and the patient with acute GvHD had a transient improvement, however, due to the very low patient number no conclusions on the effect of Treg therapy on relapse were drawn. Another trial in 2011 reported the use of expanded Tregs from third party UCB in 23 patients with acute GvHD (174), no toxicities were documented and GvHD was reduced with no effect on relapse when compared with 108 historical controls.

Edinger and Hoffmann reported a small phase I safety trial (175) where nine patients at high risk of relapse post-HSCT were treated with freshly isolated donor Tregs, then 8 weeks later, conventional T cells were given to promote GvL activity and, as in the previous trial, there was no increased risk of relapse. In 2014, a group reported a trial in 43 patients with high risk leukemia, using freshly isolated donor Tregs pre-haploidentical

TABLE 1 | Overview of the chapter and summary of main points.

Chapters	Sub headings	Reference	Main points
Introduction		(1–16)	Introducing hematopoietic stem cell transplantation (HSCT), graft-versus-leukemia (GvL), and residual disease
Clinical results using DLI for relapse after hematopoietic cell transplantation		(17–34)	First studies of donor lymphocyte infusion (DLI) for chronic myeloid leukemia (CML) and results for acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL)
The history of the role of T cells and NK cells in the GvL effect		(35–48, 50, 51)	Early studies of the role of T cells and natural killer (NK) cells
Prophylactic and preemptive DLI		(52–81)	Studies in CML; AML and ALL
Complications of DLI	Graft-versus-host disease	(82–92)	Factors affecting GvHD occurrence and DLI
	Myelosuppression	(91, 93, 94)	Myelosuppression can be related to aGvHD
Improvement of the response	Antigen-specific T cells	(29, 90, 95–102)	Overview
	Leukemia-associated antigens (LAAs)	(101–109)	Overview of antigens expressed in leukemia blasts and absent on normal tissue
	Leukemia-specific antigens	(33, 96, 103, 110)	These are antigens coded for by the mutation event in the leukemic clone
	Minor histocompatibility antigens (mHAs)	(29, 111–117)	mHA, responsible for graft rejection and GvHD in HLA identical siblings
	Production of DC of leukemia origin (DC_{leu})	(80, 101, 118–133)	Cytokines and certain drugs cause leukemic blasts to differentiate into DC, which can then stimulate GvL
Mechanisms of a GvL effect—NK cells		(48, 136–138)	Activated donor NK cells induce GvL by interaction with allogeneic targets lacking killer immune receptors
	KIR–ligand interactions and HSCT outcome	(139–145)	Regulate the killing function of NK cells, most are inhibitory, they are pleomorphic and their genotype is important in GvL
	In vitro and *in vivo* induction of a GvL effect	(146–150)	Chimeric antigen receptors (CARs) T cells and CAR NK cells have shown promise and more recently Bi-specific killer engagers have been developed
	Ex vivo expansion of NK cells and induction of GvL effects	(151–170)	NK cells can be expanded from peripheral blood mononuclear cells, umbilical cord blood, ECSs, and also malignant NK cells lines but all have their limitations
Treg therapy and its effect in GvL and relapse		(172–177)	Tregs shown to reduce GvHD and do not induce relapse, but patients may potentially develop non-hematological malignancies

HSCT, to avoid intensive *ex vivo* depletion of T cells in the graft. This protocol showed for the first time that adoptive immunotherapy with Tregs protected against GvHD compared to patients undergoing conventional haploidentical transplants. In addition, the incidence of relapse was reduced, suggesting that Tregs do not target GvL (176). More recently, a trial of the infusion of expanded Tregs and IL-2 for chronic GvHD has been reported. The study showed that the stability and functionality of the Tregs *in vivo* was maintained due to the increase in the number of T cells post-infusion. There was no toxicity nor exacerbation of chronic GvHD or other adverse immune reactions, chronic GvHD responded but the trial had only five patients and two of these patients developed non-hematological malignancies suggesting that Tregs may contribute to a tumor escape mechanism *via* suppression of the immune response (177).

CONCLUSION

Many questions remain to be addressed in order to optimize the GvL effect of DLI for treating and preventing relapse and **Table 1** summarizes the main features of this review. In CML, long-term remissions can readily be obtained by the treatment with low-dose IFN-a and DLI, in AML, long-term remissions may be obtained by a more aggressive approach involving mobilized stem cells and GM-CSF following cytarabine or repeated treatments with targeted drugs like azacytidine, sorfenib, midostaurin, immune-modulatory/blastmodulatory Kits, and DLI. Better results may be achieved with prophylactic or preemptive DLI and/or treatment with blast-modulating Kits combining myeloid cell-differentiating factor GM-CSF with "danger"-signaling and DC-maturation-inducing factors (e.g., PGE1, PGE2, or Picibanil) addressing myeloid blasts and converting them to DC_{leu}—resulting in an *in vivo* stimulation of antileukemic T-cells. In addition to measuring minimal residual disease (MRD) or mixed chimerism, prophylaxis for viral infections may be required to avoid triggering of GvHD.

In myeloma, antigen presentation may be a problem and dendritic cell vaccines as well as low-dose lenalidomide may be helpful for sustained remissions. Antibodies-engaging effector cells are still to be studied in the allogeneic situation. Finally,

the role of CD4-positive T cells and their interaction with CD8-positive T cells remains to be demonstrated on leukemia stem cells.

Clinical therapy with NK cells has been inspired by recognition of their potent antileukemia activity. The studies discussed above provide a solid basis for development of NK cell trials for leukemia therapy while minimizing risks (151). To advance NK cell therapies, both further study of basic NK biology (including iNKT and CIK cells) as well as a better understanding of interactions with other immune cells will be required (171). Unmanipulated bone marrow followed by CD6-depleted mobilized blood cells produced long-term remissions in advanced cases of acute leukemia; CD6-depleted PBSC provides NK cells, stem cells, and a minority of suppressive CD8-positive cells (178). Recently, excellent results have been reported in ALL and lymphoma patients with HLA-haploidentical transplants and high-dose cyclophosphamide after transplantation (179, 180). Even HLA haploidentical DLIs were possible in cases of relapse with excellent results in Hodgkin's disease (181).

AUTHOR CONTRIBUTIONS

All authors contributed to the manuscript and AD, HJ-K, and HS reviewed the contents.

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REFERENCES

1. Thomas E, Storb R, Clift RA, Fefer A, Johnson FL, Neiman PE, et al. Bone-marrow transplantation (first of two parts). *N Engl J Med* (1975) 292(16): 832–43. doi:10.1056/NEJM197504172921605
2. Santos GW, Sensenbrenner LL, Burke PJ, Mullins GM, Blas WB, Tutschka PJ, et al. The use of cyclophosphamide for clinical marrow transplantation. *Transplant Proc* (1972) 4(4):559–64.
3. Thomas ED, Epstein RB. Bone marrow transplantation in acute leukemia. *Cancer Res* (1965) 25(9):1521–4.
4. Sensenbrenner LL, Steele AA, Santos GW. Recovery of hematologic competence without engraftment following attempted bone marrow transplantation for aplastic anemia: report of a case with diffusion chamber studies. *Exp Hematol* (1977) 5(1):51–8.
5. Thomas ED, Buckner CD, Banaji M, Clift RA, Fefer A, Flournoy N, et al. One hundred patients with acute leukemia treated by chemotherapy, total body irradiation, and allogeneic marrow transplantation. *Blood* (1977) 49(4):511–33.
6. Santos GW, Tutschka PJ, Brookmeyer R, Saral R, Beschorner WE, Bias WB, et al. Marrow transplantation for acute nonlymphocytic leukemia after treatment with busulfan and cyclophosphamide. *N Engl J Med* (1983) 309(22):1347–53. doi:10.1056/NEJM198312013092202
7. Barnes DW, Corp MJ, Loutit JF, Neal FE. Treatment of murine leukaemia with X rays and homologous bone marrow; preliminary communication. *Br Med J* (1956) 2(4993):626–7. doi:10.1136/bmj.2.4993.626
8. Mathé G, Amiel JL, Schwarzenberg L, Cattan A, Schneider M, Devries MJ, et al. Successful allogenic bone marrow transplantation in man: chimerism, induced specific tolerance and possible anti-leukemic effects. *Blood* (1965) 25:179–96.
9. Mery AM, Sekiguchi M, Vaubel WE, Amiel JL, Mathe G. Investigation on a test of histocompatibility for allogeneic grafts. 3. A study in man. *Transplantation* (1966) 4(2):206–8.
10. Matsukura M, Mery AM, Amiel JL, Mathe G. Investigation on a test of histocompatibility for allogeneic grafts. II. A study on rabbits. *Transplantation* (1963) 1:61–4. doi:10.1097/00007890-196301010-00007
11. Epstein RB, Storb R, Clift RA, Thomas ED. Transplantation of stored allogeneic bone marrow in dogs selected by histocompatibility typing. *Transplantation* (1969) 8(4):496–501. doi:10.1097/00007890-196910000-00022
12. Epstein RB, Storb R, Ragde H, Thomas ED. Cytotoxic typing antisera for marrow grafting in littermate dogs. *Transplantation* (1968) 6(1):45–58. doi:10.1097/00007890-196801000-00005
13. Horowitz MM, Gale RP, Sondel PM, Goldman JM, Kersey J, Kolb HJ, et al. Graft-versus-leukemia reactions after bone marrow transplantation. *Blood* (1990) 75(3):555–62.
14. Dazzi F, Fozza C. Disease relapse after haematopoietic stem cell transplantation: risk factors and treatment. *Best Pract Res Clin Haematol* (2007) 20(2):311–27. doi:10.1016/j.beha.2006.10.002
15. Savani BN, Mielke S, Reddy N, Goodman S, Jagasia M, Rezvani K. Management of relapse after allo-SCT for AML and the role of second transplantation. *Bone Marrow Transplant* (2009) 44(12):769–77. doi:10.1038/bmt.2009.300
16. Woolfrey AE, Anasetti C, Storer B, Doney K, Milner LA, Sievers EL, et al. Factors associated with outcome after unrelated marrow transplantation for treatment of acute lymphoblastic leukemia in children. *Blood* (2002) 99(6):2002. doi:10.1182/blood.V99.6.2002
17. Sullivan KM, Storb R, Buckner CD, Fefer A, Fisher L, Weiden PL, et al. Graft-versus-host disease as adoptive immunotherapy in patients with advanced hematologic neoplasms. *N Engl J Med* (1989) 320(13):828–34. doi:10.1056/NEJM198903303201303
18. Weiden PL, Storb R, Tsoi MS, Graham TC, Lerner KG, Thomas ED. Infusion of donor lymphocytes into stable canine radiation chimeras: implications for mechanism of transplantation tolerance. *J Immunol* (1976) 116(5):1212–9.
19. Field EO, Gibbs JE. Reduced sensitivity of F1 hybrid rats to rechallenge with parental strain spleen cells. *Clin Exp Immunol* (1966) 1(2):195–205.
20. Thompson JS, Simmons EL, Crawford MK, Severson CD. Studies on immunological unresponsiveness during secondary disease III. Effect of donor strain on acquisition of mutual tolerance. *Transplantation* (1969) 7(6):447–58. doi:10.1097/00007890-196906000-00001
21. Vos O, Weyzen WW. "Killing effect" of injected lymphnode cells in homologous radiation chimeras. *Transplant Bull* (1962) 30:111–7. doi:10.1097/00006534-196210000-00042
22. Kolb HJ, Mittermuller J, Clemm C, Holler E, Ledderose G, Brehm G, et al. Donor leukocyte transfusions for treatment of recurrent chronic myelogenous leukemia in marrow transplant patients. *Blood* (1990) 76(12):2462–5.
23. Truitt RL, Atasoylu AA. Impact of pretransplant conditioning and donor T cells on chimerism, graft-versus-host disease, graft-versus-leukemia reactivity, and tolerance after bone marrow transplantation. *Blood* (1991) 77(11):2515–23.
24. Drobyski WR, Roth MS, Thibodeau SN, Gottschall JL. Molecular remission occurring after donor leukocyte infusions for the treatment of relapsed chronic myelogenous leukemia after allogeneic bone marrow transplantation. *Bone Marrow Transplant* (1992) 10(3):301–4.
25. Naparstek E, Or R, Nagler A, Cividalli G, Engelhard D, Aker M, et al. T-cell-depleted allogeneic bone marrow transplantation for acute leukaemia using Campath-1 antibodies and post-transplant administration of donor's peripheral blood lymphocytes for prevention of relapse. *Br J Haematol* (1995) 89(3):506–15. doi:10.1111/j.1365-2141.1995.tb08356.x
26. Collins RH Jr, Shpilberg O, Drobyski WR, Porter DL, Giralt S, Champlin R, et al. Donor leukocyte infusions in 140 patients with relapsed malignancy after allogeneic bone marrow transplantation. *J Clin Oncol* (1997) 15(2):433–44. doi:10.1200/JCO.1997.15.2.433

27. Porter DL, Collins RH Jr, Shpilberg O, Drobyski WR, Connors JM, Sproles A, et al. Long-term follow-up of patients who achieved complete remission after donor leukocyte infusions. *Biol Blood Marrow Transplant* (1999) 5(4):253–61. doi:10.1053/bbmt.1999.v5.pm10465105
28. Dazzi F, Szydlo RM, Cross NC, Craddock C, Kaeda J, Kanfer E, et al. Durability of responses following donor lymphocyte infusions for patients who relapse after allogeneic stem cell transplantation for chronic myeloid leukemia. *Blood* (2000) 96(8):2712–6.
29. Kolb HJ, Schattenberg A, Goldman JM, Hertenstein B, Jacobsen N, Arcese W, et al. Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. *Blood* (1995) 86(5):2041–50.
30. Shiobara S, Takahasi S, Yabe H, Maruta I, Kodera Y. [Therapeutic effect of donor leukocyte transfusion in relapsing marrow transplants in Japan]. *Rinsho Ketsueki* (1997) 38(11):1162–9.
31. Radujkovic A, Guglielmi C, Bergantini S, Iacobelli S, van Biezen A, Milojkovic D, et al. Donor lymphocyte infusions for chronic myeloid leukemia relapsing after allogeneic stem cell transplantation: may we predict graft-versus-leukemia without graft-versus-host disease? *Biol Blood Marrow Transplant* (2015) 21(7):1230–6. doi:10.1016/j.bbmt.2015.03.012
32. Zeiser R, Bertz H, Spyridonidis A, Houet L, Finke J. Donor lymphocyte infusions for multiple myeloma: clinical results and novel perspectives. *Bone Marrow Transplant* (2004) 34(11):923–8. doi:10.1038/sj.bmt.1704670
33. Schmid C, Labopin M, Nagler A, Bornhauser M, Finke J, Fassas A, et al. Donor lymphocyte infusion in the treatment of first hematological relapse after allogeneic stem-cell transplantation in adults with acute myeloid leukemia: a retrospective risk factors analysis and comparison with other strategies by the EBMT Acute Leukemia Working Party. *J Clin Oncol* (2007) 25(31):4938–45. doi:10.1200/JCO.2007.11.6053
34. Kolb HJ. Graft-versus-leukemia effects of transplantation and donor lymphocytes. *Blood* (2008) 112(12):4371–83. doi:10.1182/blood-2008-03-077974
35. Marmont AM, Horowitz MM, Gale RP, Sobocinski K, Ash RC, van Bekkum DW, et al. T-cell depletion of HLA-identical transplants in leukemia. *Blood* (1991) 78(8):2120–30.
36. Ballester OF, Fang T, Raptis A, Ballester G, Wilcox P, Hiemenz J, et al. Adoptive immunotherapy with donor lymphocyte infusions and interleukin-2 after high-dose therapy and autologous stem cell rescue for multiple myeloma. *Bone Marrow Transplant* (2004) 34(5):419–23. doi:10.1038/sj.bmt.1704617
37. Daguindau E, Lioure B, Buzyn A, Robin M, Faucher C, Kuentz M, et al. Evidence for anti-tumour effect of allogeneic haematopoietic SCT in cases without sustained donor engraftment. *Bone Marrow Transplant* (2010) 45(1):177–80. doi:10.1038/bmt.2009.96
38. Kiessling R, Wigzell H. An analysis of the murine NK cell as to structure, function and biological relevance. *Immunol Rev* (1979) 44:165–208. doi:10.1111/j.1600-065X.1979.tb00270.x
39. Karre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature* (1986) 319(6055):675–8. doi:10.1038/319675a0
40. Ljunggren HG, Karre K. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol Today* (1990) 11(7):237–44. doi:10.1016/0167-5699(90)90097-S
41. Wagtmann N, Biassoni R, Cantoni C, Verdiani S, Malnati MS, Vitale M, et al. Molecular clones of the p58 NK cell receptor reveal immunoglobulin-related molecules with diversity in both the extra- and intracellular domains. *Immunity* (1995) 2(5):439–49. doi:10.1016/1074-7613(95)90025-X
42. Colonna M, Samaridis J. Cloning of immunoglobulin-superfamily members associated with HLA-C and HLA-B recognition by human natural killer cells. *Science* (1995) 268(5209):405–8. doi:10.1126/science.7716543
43. Lee N, Llano M, Carretero M, Ishitani A, Navarro F, Lopez-Botet M, et al. HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A. *Proc Natl Acad Sci U S A* (1998) 95(9):5199–204. doi:10.1073/pnas.95.9.5199
44. Borrego F, Ulbrecht M, Weiss EH, Coligan JE, Brooks AG. Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis. *J Exp Med* (1998) 187(5):813–8. doi:10.1084/jem.187.5.813
45. Braud VM, Allan DSJ, O'Callaghan CA, Soderstrom K, D'Andrea A, Ogg GS, et al. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature* (1998) 391(6669):795–9. doi:10.1038/35869
46. Chan CJ, Smyth MJ, Martinet L. Molecular mechanisms of natural killer cell activation in response to cellular stress. *Cell Death Differ* (2014) 21(1):5–14. doi:10.1038/cdd.2013.26
47. Caligiuri MA. Human natural killer cells. *Blood* (2008) 112(3):461–9. doi:10.1182/blood-2007-09-077438
48. Velardi A. Role of KIRs and KIR ligands in hematopoietic transplantation. *Curr Opin Immunol* (2008) 20(5):581–7. doi:10.1016/j.coi.2008.07.004
49. Morris V. *NK Cell Activation through KIR-HLA Interactions Decreases Leukemia Relapse after Transplant*. (Vol. 2). Science Spotlight [Internet] (2012).
50. Benjamin JE, Gill S, Negrin RS. Biology and clinical effects of natural killer cells in allogeneic transplantation. *Curr Opin Oncol* (2010) 22(2):130–7. doi:10.1097/CCO.0b013e328335a559
51. Baron F, Petersdorf EW, Gooley T, Sandmaier BM, Malkki M, Chauncey TR, et al. What is the role for donor natural killer cells after nonmyeloablative conditioning? *Biol Blood Marrow Transplant* (2009) 15(5):580–8. doi:10.1016/j.bbmt.2009.01.018
52. Kolb HJ, Gunther W, Schumm M, Holler E, Wilmanns W, Thierfelder S. Adoptive immunotherapy in canine chimeras. *Transplantation* (1997) 63(3):430–6. doi:10.1097/00007890-199702150-00017
53. Schmid C, Schleuning M, Ledderose G, Tischer J, Kolb HJ. Sequential regimen of chemotherapy, reduced-intensity conditioning for allogeneic stem-cell transplantation, and prophylactic donor lymphocyte transfusion in high-risk acute myeloid leukemia and myelodysplastic syndrome. *J Clin Oncol* (2005) 23(24):5675–87. doi:10.1200/JCO.2005.07.061
54. Jedlickova Z, Schmid C, Koenecke C, Hertenstein B, Baurmann H, Schwerdtfeger R, et al. Long-term results of adjuvant donor lymphocyte transfusion in AML after allogeneic stem cell transplantation. *Bone Marrow Transplant* (2016) 51(5):663–7. doi:10.1038/bmt.2015.234
55. Hasskarl J, Zerweck A, Wasch R, Ihorst G, Bertz H, Finke J. Induction of graft versus malignancy effect after unrelated allogeneic PBSCT using donor lymphocyte infusions derived from frozen aliquots of the original graft. *Bone Marrow Transplant* (2012) 47(2):277–82. doi:10.1038/bmt.2011.45
56. Eefting M, Halkes CJ, de Wreede LC, van Pelt CM, Kersting S, Marijt EW, et al. Myeloablative T cell-depleted alloSCT with early sequential prophylactic donor lymphocyte infusion is an efficient and safe post-remission treatment for adult ALL. *Bone Marrow Transplant* (2014) 49(2):287–91. doi:10.1038/bmt.2013.111
57. Krishnamurthy P, Potter VT, Barber LD, Kulasekararaj AG, Lim ZY, Pearce RM, et al. Outcome of donor lymphocyte infusion after T cell-depleted allogeneic hematopoietic stem cell transplantation for acute myelogenous leukemia and myelodysplastic syndromes. *Biol Blood Marrow Transplant* (2013) 19(4):562–8. doi:10.1016/j.bbmt.2012.12.013
58. Marks DI, Lush R, Cavenagh J, Milligan DW, Schey S, Parker A, et al. The toxicity and efficacy of donor lymphocyte infusions given after reduced-intensity conditioning allogeneic stem cell transplantation. *Blood* (2002) 100(9):3108–14. doi:10.1182/blood-2002-02-0506
59. de Lima M, Bonamino M, Vasconcelos Z, Colares M, Diamond H, Zalcberg I, et al. Prophylactic donor lymphocyte infusions after moderately ablative chemotherapy and stem cell transplantation for hematological malignancies: high remission rate among poor prognosis patients at the expense of graft-versus-host disease. *Bone Marrow Transplant* (2001) 27(1):73–8. doi:10.1038/sj.bmt.1702726
60. Liga M, Triantafyllou E, Tiniakou M, Lambropoulou P, Karakantza M, Zoumbos NC, et al. High alloreactivity of low-dose prophylactic donor lymphocyte infusion in patients with acute leukemia undergoing allogeneic hematopoietic cell transplantation with an alemtuzumab-containing conditioning regimen. *Biol Blood Marrow Transplant* (2013) 19(1):75–81. doi:10.1016/j.bbmt.2012.07.021
61. Thomson KJ, Morris EC, Milligan D, Parker AN, Hunter AE, Cook G, et al. T-cell-depleted reduced-intensity transplantation followed by donor leukocyte infusions to promote graft-versus-lymphoma activity results in excellent long-term survival in patients with multiply relapsed follicular lymphoma. *J Clin Oncol* (2010) 28(23):3695–700. doi:10.1200/JCO.2009.26.9100
62. Stevanovic S, van Bergen CA, van Luxemburg-Heijs SA, van der Zouwen B, Jordanova ES, Kruisselbrink AB, et al. HLA class II upregulation during viral

infection leads to HLA-DP-directed graft-versus-host disease after CD4+ donor lymphocyte infusion. *Blood* (2013) 122(11):1963–73. doi:10.1182/blood-2012-12-470872
63. Candoni A, Tiribelli M, Toffoletti E, Cilloni D, Chiarvesio A, Michelutti A, et al. Quantitative assessment of WT1 gene expression after allogeneic stem cell transplantation is a useful tool for monitoring minimal residual disease in acute myeloid leukemia. *Eur J Haematol* (2009) 82(1):61–8. doi:10.1111/j.1600-0609.2008.01158.x
64. Qin YZ, Wang Y, Xu LP, Zhang XH, Chen H, Han W, et al. The dynamics of RUNX1-RUNX1T1 transcript levels after allogeneic hematopoietic stem cell transplantation predict relapse in patients with t(8;21) acute myeloid leukemia. *J Hematol Oncol* (2017) 10(1):44. doi:10.1186/s13045-017-0414-2
65. Brüggemann M, Raff T, Kneba M. Has MRD monitoring superseded other prognostic factors in adult ALL? *Blood* (2012) 120(23):4470–81. doi:10.1182/blood-2012-06-379040
66. Yan CH, Wang JZ, Liu DH, Xu LP, Chen H, Liu KY, et al. Chemotherapy followed by modified donor lymphocyte infusion as a treatment for relapsed acute leukemia after haploidentical hematopoietic stem cell transplantation without in vitro T-cell depletion: superior outcomes compared with chemotherapy alone and an analysis of prognostic factors. *Eur J Haematol* (2013) 91(4):304–14. doi:10.1111/ejh.12168
67. Alyea E, Weller E, Schlossman R, Canning C, Webb I, Doss D, et al. T-cell-depleted allogeneic bone marrow transplantation followed by donor lymphocyte infusion in patients with multiple myeloma: induction of graft-versus-myeloma effect. *Blood* (2001) 98(4):934–9. doi:10.1182/blood.V98.4.934
68. Bellucci R, Alyea EP, Weller E, Chillemi A, Hochberg E, Wu CJ, et al. Immunologic effects of prophylactic donor lymphocyte infusion after allogeneic marrow transplantation for multiple myeloma. *Blood* (2002) 99(12):4610–7. doi:10.1182/blood.V99.12.4610
69. Levenga H, Levison-Keating S, Schattenberg AV, Dolstra H, Schaap N, Raymakers RA. Multiple myeloma patients receiving pre-emptive donor lymphocyte infusion after partial T-cell-depleted allogeneic stem cell transplantation show a long progression-free survival. *Bone Marrow Transplant* (2007) 40(4):355–9. doi:10.1038/sj.bmt.1705742
70. Passweg JR, Baldomero H, Bader P, Bonini C, Cesaro S, Dreger P, et al. Hematopoietic stem cell transplantation in Europe 2014: more than 40 000 transplants annually. *Bone Marrow Transplant* (2016) 51(6):786–92. doi:10.1038/bmt.2016.20
71. Olavarria E, Craddock C, Dazzi F, Marin D, Marktel S, Apperley JF, et al. Imatinib mesylate (STI571) in the treatment of relapse of chronic myeloid leukemia after allogeneic stem cell transplantation. *Blood* (2002) 99(10):3861–2. doi:10.1182/blood.V99.10.3861
72. Hess G, Bunjes D, Siegert W, Schwerdtfeger R, Ledderose G, Wassmann B, et al. Sustained complete molecular remissions after treatment with imatinib-mesylate in patients with failure after allogeneic stem cell transplantation for chronic myelogenous leukemia: results of a prospective phase II open-label multicenter study. *J Clin Oncol* (2005) 23(30):7583–93. doi:10.1200/JCO.2005.01.3110
73. Apperley JF. Managing the patient with chronic myeloid leukemia through and after allogeneic stem cell transplantation. *Hematology Am Soc Hematol Educ Program* (2006) 2006(1):226–32. doi:10.1182/asheducation-2006.1.226
74. Craddock CF. Full-intensity and reduced-intensity allogeneic stem cell transplantation in AML. *Bone Marrow Transplant* (2008) 41(5):415–23. doi:10.1038/sj.bmt.1705975
75. Meijer E, Cornelissen JJ. Allogeneic stem cell transplantation in acute myeloid leukemia in first or subsequent remission: weighing prognostic markers predicting relapse and risk factors for non-relapse mortality. *Semin Oncol* (2008) 35(4):449–57. doi:10.1053/j.seminoncol.2008.04.015
76. Barrett AJ, Savani BN. Stem cell transplantation with reduced-intensity conditioning regimens: a review of ten years experience with new transplant concepts and new therapeutic agents. *Leukemia* (2006) 20(10):1661–72. doi:10.1038/sj.leu.2404334
77. Giralt SA, Champlin RE. Leukemia relapse after allogeneic bone marrow transplantation: a review. *Blood* (1994) 84(11):3603–12.
78. Barrett AJ, Savani BN. Allogeneic stem cell transplantation for myelodysplastic syndrome. *Semin Hematol* (2008) 45(1):49–59. doi:10.1053/j.seminhematol.2007.10.005
79. Dombret H, Raffoux E, Gardin C. Acute myeloid leukemia in the elderly. *Semin Oncol* (2008) 35(4):430–8. doi:10.1053/j.seminoncol.2008.04.013
80. Schmid C, Labopin M, Nagler A, Niederwieser D, Castagna L, Tabrizi R, et al. Treatment, risk factors, and outcome of adults with relapsed AML after reduced intensity conditioning for allogeneic stem cell transplantation. *Blood* (2012) 119(6):1599–606. doi:10.1182/blood-2011-08-375840
81. Mohty M. *Haematopoietic Stem Cell Transplantation: The EBMT Handbook*. 6th ed. EBMT (2012). p. 331–41.
82. Yegin ZA, Özkurt ZN, Aki ŞZ, Sucak GT. Donor lymphocyte infusion for leukemia relapse after hematopoietic stem cell transplantation. *Transfus Apher Sci* (2010) 42(3):239–45. doi:10.1016/j.transci.2010.03.011
83. Chalandon Y, Passweg JR, Schmid C, Olavarria E, Dazzi F, Simula MP, et al. Outcome of patients developing GVHD after DLI given to treat CML relapse: a study by the Chronic Leukemia Working Party of the EBMT. *Bone Marrow Transplant* (2010) 45(3):558–64. doi:10.1038/bmt.2009.177
84. Miller JS, Weisdorf DJ, Burns LJ, Slungaard A, Wagner JE, Verneris MR, et al. Lymphodepletion followed by donor lymphocyte infusion (DLI) causes significantly more acute graft-versus-host disease than DLI alone. *Blood* (2007) 110(7):2761–3. doi:10.1182/blood-2007-05-090340
85. Giralt S, Hester J, Huh Y, Hirsch-Ginsberg C, Rondon G, Seong D, et al. CD8-depleted donor lymphocyte infusion as treatment for relapsed chronic myelogenous leukemia after allogeneic bone marrow transplantation. *Blood* (1995) 86(11):4337–43.
86. Bordignon C, Bonini C, Verzeletti S, Nobili N, Maggioni D, Traversari C, et al. Transfer of the HSV-tk gene into donor peripheral blood lymphocytes for in vivo modulation of donor anti-tumor immunity after allogeneic bone marrow transplantation. *Hum Gene Ther* (1995) 6(6):813–9. doi:10.1089/hum.1995.6.6-813
87. Mackinnon S, Papadopoulos EB, Carabasi MH, Reich L, Collins NH, Boulad F, et al. Adoptive immunotherapy evaluating escalating doses of donor leukocytes for relapse of chronic myeloid leukemia after bone marrow transplantation: separation of graft-versus-leukemia responses from graft-versus-host disease. *Blood* (1995) 86(4):1261–8.
88. Dazzi F, Szydlo RM, Goldman JM. Donor lymphocyte infusions for relapse of chronic myeloid leukemia after allogeneic stem cell transplant: where we now stand. *Exp Hematol* (1999) 27(10):1477–86. doi:10.1016/S0301-472X(99)00096-X
89. Peggs KS. Attack of the T-cell clones. *Blood* (2013) 122(11):1847–8. doi:10.1182/blood-2013-07-511022
90. Guglielmi C, Arcese W, Dazzi F, Brand R, Bunjes D, Verdonck LF, et al. Donor lymphocyte infusion for relapsed chronic myelogenous leukemia: prognostic relevance of the initial cell dose. *Blood* (2002) 100(2):397–405. doi:10.1182/blood.V100.2.397
91. Kolb HJ, Schmid C, Barrett AJ, Schendel DJ. Graft-versus-leukemia reactions in allogeneic chimeras. *Blood* (2004) 103(3):767–76. doi:10.1182/blood-2003-02-0342
92. van Rhee F, Lin F, Cullis JO, Spencer A, Cross NC, Chase A, et al. Relapse of chronic myeloid leukemia after allogeneic bone marrow transplant: the case for giving donor leukocyte transfusions before the onset of hematologic relapse. *Blood* (1994) 83(11):3377–83.
93. Dazzi F, Szydlo RM, Craddock C, Cross NCP, Kaeda J, Chase A, et al. Comparison of single-dose and escalating-dose regimens of donor lymphocyte infusion for relapse after allografting for chronic myeloid leukemia. *Blood* (2000) 95(1):67–71.
94. Drobyski WR, Keever CA, Roth MS, Koethe S, Hanson G, McFadden P, et al. Salvage immunotherapy using donor leukocyte infusions as treatment for relapsed chronic myelogenous leukemia after allogeneic bone marrow transplantation: efficacy and toxicity of a defined T-cell dose. *Blood* (1993) 82(8):2310–8.
95. Porter DL, Roth MS, McGarigle C, Ferrara J, Antin JH. Induction of graft-versus-host disease as immunotherapy for relapsed chronic myeloid leukemia. *N Engl J Med* (1994) 330(2):100–6. doi:10.1056/NEJM199401133300204
96. Beelen DW, Elmaagacli A, Muller KD, Hirche H, Schaefer UW. Influence of intestinal bacterial decontamination using metronidazole and ciprofloxacin or ciprofloxacin alone on the development of acute graft-versus-host disease after marrow transplantation in patients with hematologic malignancies: final results and long-term follow-up of an open-label prospective randomized trial. *Blood* (1999) 93(10):3267–75.

97. Chen X, Regn S, Raffegerst S, Kolb HJ, Roskrow M. Interferon alpha in combination with GM-CSF induces the differentiation of leukaemic antigen-presenting cells that have the capacity to stimulate a specific anti-leukaemic cytotoxic T-cell response from patients with chronic myeloid leukaemia. *Br J Haematol* (2000) 111(2):596–607. doi:10.1111/j.1365-2141.2000.02361.x
98. Chen X, Woiciechowsky A, Raffegerst S, Schendel D, Kolb HJ, Roskrow M. Impaired expression of the CD3-zeta chain in peripheral blood T cells of patients with chronic myeloid leukaemia results in an increased susceptibility to apoptosis. *Br J Haematol* (2000) 111(3):817–25. doi:10.1046/j.1365-2141.2000.02415.x
99. Bashey A, Medina B, Corringham S, Pasek M, Carrier E, Vrooman L, et al. CTLA4 blockade with ipilimumab to treat relapse of malignancy after allogeneic hematopoietic cell transplantation. *Blood* (2009) 113(7):1581–8. doi:10.1182/blood-2008-07-168468
100. Ohta A, Sitkovsky M. Extracellular adenosine-mediated modulation of regulatory T cells. *Front Immunol* (2014) 5:304. doi:10.3389/fimmu.2014.00304
101. Anderson BE, Zheng H, Taylor PA, Matte-Martone C, McNiff JM, Jain D, et al. Memory T cells in GVHD and GVL. *Biol Blood Marrow Transplant* (2008) 14(1 Suppl 1):19–20.
102. Schmetzer HM, Schmid C. The T-Cells' role in antileukemic reactions-perspectives for future therapies. In: Demirer T, editor. *In -New Advances in Stem Cell Transplantation*. INTECH Open Access Publisher (2012). Chapter 4. p. 59–82.
103. Gao L, Bellantuono I, Elsasser A, Marley SB, Gordon MY, Goldman JM, et al. Selective elimination of leukemic CD34(+) progenitor cells by cytotoxic T lymphocytes specific for WT1. *Blood* (2000) 95(7):2198–203.
104. Bocchia M, Defina M, Aprile L, Ippoliti M, Crupi R, Rondoni M, et al. Complete molecular response in CML after p210 BCR-ABL1-derived peptide vaccination. *Nat Rev Clin Oncol* (2010) 7(10):600–3. doi:10.1038/nrclinonc.2010.141
105. Rammensee HG, Rotzschke O, Falk K. Self tolerance of natural MHC class I ligands. *Int Rev Immunol* (1993) 10(4):291–300. doi:10.3109/08830189309061704
106. Stauss HJ, Thomas S, Cesco-Gaspere M, Hart DP, Xue SA, Holler A, et al. WT1-specific T cell receptor gene therapy: improving TCR function in transduced T cells. *Blood Cells Mol Dis* (2008) 40(1):113–6. doi:10.1016/j.bcmd.2007.06.018
107. Provasi E, Genovese P, Lombardo A, Magnani Z, Liu PQ, Reik A, et al. Editing T cell specificity towards leukemia by zinc finger nucleases and lentiviral gene transfer. *Nat Med* (2012) 18(5):807–15. doi:10.1038/nm.2700
108. van den Ancker W, Ruben JM, Westers TM, Wulandari D, Bontkes HJ, Hooijberg E, et al. Priming of PRAME- and WT1-specific CD8+ T cells in healthy donors but not in AML patients in complete remission: implications for immunotherapy. *Oncoimmunology* (2013) 2(4):e23971. doi:10.4161/onci.23971
109. Rezvani K, Yong AS, Tawab A, Jafarpour B, Eniafe R, Mielke S, et al. Ex vivo characterization of polyclonal memory CD8+ T-cell responses to PRAME-specific peptides in patients with acute lymphoblastic leukemia and acute and chronic myeloid leukemia. *Blood* (2009) 113(10):2245–55. doi:10.1182/blood-2008-03-144071
110. Schmitt M, Schmitt A, Rojewski MT, Chen J, Giannopoulos K, Fei F, et al. RHAMM-R3 peptide vaccination in patients with acute myeloid leukemia, myelodysplastic syndrome, and multiple myeloma elicits immunologic and clinical responses. *Blood* (2008) 111(3):1357–65. doi:10.1182/blood-2007-07-099366
111. Greiner J, Ono Y, Hofmann S, Schmitt A, Mehring E, Gotz M, et al. Mutated regions of nucleophosmin 1 elicit both CD4(+) and CD8(+) T-cell responses in patients with acute myeloid leukemia. *Blood* (2012) 120(6):1282–9. doi:10.1182/blood-2011-11-394395
112. Falkenburg JH, van de Corput L, Marijt EW, Willemze R. Minor histocompatibility antigens in human stem cell transplantation. *Exp Hematol* (2003) 31(9):743–51. doi:10.1016/S0301-472X(03)00190-5
113. Marijt WA, Heemskerk MH, Kloosterboer FM, Goulmy E, Kester MG, van der Hoorn MA, et al. Hematopoiesis-restricted minor histocompatibility antigens HA-1- or HA-2-specific T cells can induce complete remissions of relapsed leukemia. *Proc Natl Acad Sci U S A* (2003) 100(5):2742–7. doi:10.1073/pnas.0530192100
114. Goulmy E, Termijtelen A, Bradley BA, van Rood JJ. Alloimmunity to human H-Y. *Lancet* (1976) 2(7996):1206. doi:10.1016/S0140-6736(76)91727-X
115. Randolph SS, Gooley TA, Warren EH, Appelbaum FR, Riddell SR. Female donors contribute to a selective graft-versus-leukemia effect in male recipients of HLA-matched, related hematopoietic stem cell transplants. *Blood* (2004) 103(1):347–52. doi:10.1182/blood-2003-07-2603
116. Bund D, Buhmann R, Gökmen F, Zorn J, Kolb HJ, Schmetzer HM. Minor histocompatibility antigen UTY as target for graft-versus-leukemia and graft-versus-haematopoiesis in the canine model. *Scand J Immunol* (2013) 77(1):39–53. doi:10.1111/sji.12011
117. Bleakley M, Riddell SR. Exploiting T cells specific for human minor histocompatibility antigens for therapy of leukemia. *Immunol Cell Biol* (2011) 89(3):396–407. doi:10.1038/icb.2010.124
118. Griffioen M, van Bergen CAM, Falkenburg JHF. Autosomal minor histocompatibility antigens: how genetic variants create diversity in immune targets. *Front Immunol* (2016) 7:100. doi:10.3389/fimmu.2016.00100
119. Woiciechowsky A, Regn S, Kolb HJ, Roskrow M. Leukemic dendritic cells generated in the presence of FLT3 ligand have the capacity to stimulate an autologous leukemia-specific cytotoxic T cell response from patients with acute myeloid leukemia. *Leukemia* (2001) 15(2):246–55. doi:10.1038/sj.leu.2402013
120. Schmetzer HM, Kremser A, Loibl J, Kroell T, Kolb HJ. Quantification of ex vivo generated dendritic cells (DC) and leukemia-derived DC contributes to estimate the quality of DC, to detect optimal DC-generating methods or to optimize DC-mediated T-cell-activation-procedures ex vivo or in vivo 9. *Leukemia* (2007) 21(6):1338–41. doi:10.1038/sj.leu.2404639
121. Kolb HJ, Rank A, Chen X, Woiciechowsky A, Roskrow M, Schmid C, et al. In-vivo generation of leukaemia-derived dendritic cells. *Best Pract Res Clin Haematol* (2004) 17(3):439–51. doi:10.1016/S1521-6926(04)00051-9
122. Schwarzinger I, Valent P, Koller U, Marosi C, Schneider B, Haas O, et al. Prognostic significance of surface marker expression on blasts of patients with de novo acute myeloblastic leukemia. *J Clin Oncol* (1990) 8(3):423–30. doi:10.1200/JCO.1990.8.3.423
123. Schmid C, Schleuning M, Aschan J, Ringden O, Hahn J, Holler E, et al. Low-dose ARAC, donor cells, and GM-CSF for treatment of recurrent acute myeloid leukemia after allogeneic stem cell transplantation. *Leukemia* (2004) 18(8):1430–3. doi:10.1038/sj.leu.2403412
124. Schroeder T, Czibere A, Platzbecker U, Bug G, Uharek L, Luft T, et al. Azacitidine and donor lymphocyte infusions as first salvage therapy for relapse of AML or MDS after allogeneic stem cell transplantation. *Leukemia* (2013) 27(6):1229–35. doi:10.1038/leu.2013.7
125. Metzelder SK, Schroeder T, Finck A, Scholl S, Fey M, Gotze K, et al. High activity of sorafenib in FLT3-ITD-positive acute myeloid leukemia synergizes with allo-immune effects to induce sustained responses. *Leukemia* (2012) 26(11):2353–9. doi:10.1038/leu.2012.105
126. Stone RM, Fischer T, Paquette R, Schiller G, Schiffer CA, Ehninger G, et al. Phase IB study of the FLT3 kinase inhibitor midostaurin with chemotherapy in younger newly diagnosed adult patients with acute myeloid leukemia. *Leukemia* (2012) 26(9):2061–8. doi:10.1038/leu.2012.115
127. Laubach JP, Moreau P, San-Miguel JF, Richardson PG. Panobinostat for the treatment of multiple myeloma. *Clin Cancer Res* (2015) 21(21):4767–73. doi:10.1158/1078-0432.CCR-15-0530
128. Xu WS, Parmigiani RB, Marks PA. Histone deacetylase inhibitors: molecular mechanisms of action. *Oncogene* (2007) 26(37):5541–52. doi:10.1038/sj.onc.1210620
129. Steinman RM, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med* (1973) 137(5):1142–62. doi:10.1084/jem.137.5.1142
130. Steger B, Milosevic S, Doessinger G, Reuther S, Liepert A, Braeu M, et al. CD4(+)and CD8(+)T-cell reactions against leukemia-associated- or minor-histocompatibility-antigens in AML-patients after allogeneic SCT. *Immunobiology* (2014) 219(4):247–60. doi:10.1016/j.imbio.2013.10.008
131. Reuther S, Schmetzer H, Schuster FR, Krell P, Grabrucker C, Liepert A, et al. In vitro-induced response patterns of antileukemic T cells: characterization by spectratyping and immunophenotyping. *Clin Exp Med* (2013) 13(1):29–48. doi:10.1007/s10238-012-0180-y
132. Stumpf AN, van der Meijden ED, van Bergen CA, Willemze R, Falkenburg JH, Griffioen M. Identification of 4 new HLA-DR-restricted minor histocompatibility antigens as hematopoietic targets in antitumor immunity. *Blood* (2009) 114(17):3684–92. doi:10.1182/blood-2009-03-208017

133. Srour EF, Brandt JE, Briddell RA, Leemhuis T, van Besien K, Hoffman R. Human CD34+ HLA-DR-bone marrow cells contain progenitor cells capable of self-renewal, multilineage differentiation, and long-term in vitro hematopoiesis. *Blood Cells* (1991) 17(2):287–95.
134. Ridge JP, Di Rosa F, Matzinger P. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature* (1998) 393(6684):474–8. doi:10.1038/30989
135. Moosmann A, Bigalke I, Tischer J, Schirrmann L, Kasten J, Tippmer S, et al. Effective and long-term control of EBV PTLD after transfer of peptide-selected T cells. *Blood* (2010) 115(14):2960–70. doi:10.1182/blood-2009-08-236356
136. Barrett AJ. Understanding and harnessing the graft-versus-leukaemia effect. *Br J Haematol* (2008) 142(6):877–88. doi:10.1111/j.1365-2141.2008.07260.x
137. Kumar S, Chen MG, Gastineau DA, Gertz MA, Inwards DJ, Lacy MQ, et al. Lymphocyte recovery after allogeneic bone marrow transplantation predicts risk of relapse in acute lymphoblastic leukemia. *Leukemia* (2003) 17(9):1865–70. doi:10.1038/sj.leu.2403055
138. Zhao XY, Chang YJ, Huang XJ. Conflicting impact of alloreactive NK cells on transplantation outcomes after haploidentical transplantation: do the reconstitution kinetics of natural killer cells create these differences? *Biol Blood Marrow Transplant* (2011) 17(10):1436–42. doi:10.1016/j.bbmt.2011.05.020
139. Tomblyn M, Young JA, Haagenson MD, Klein JP, Trachtenberg EA, Storek J, et al. Decreased infections in recipients of unrelated donor hematopoietic cell transplantation from donors with an activating KIR genotype. *Biol Blood Marrow Transplant* (2010) 16(8):1155–61. doi:10.1016/j.bbmt.2010.02.024
140. McQueen KL, Dorighi KM, Guethlein LA, Wong R, Sanjanwala B, Parham P. Donor-recipient combinations of group A and B KIR haplotypes and HLA class I ligand affect the outcome of HLA-matched, sibling donor hematopoietic cell transplantation. *Hum Immunol* (2007) 68(5):309–23. doi:10.1016/j.humimm.2007.01.019
141. Stringaris K, Adams S, Uribe M, Eniafe R, Wu CO, Savani BN, et al. Donor KIR Genes 2DL5A, 2DS1 and 3DS1 are associated with a reduced rate of leukemia relapse after HLA-identical sibling stem cell transplantation for acute myeloid leukemia but not other hematologic malignancies. *Biol Blood Marrow Transplant* (2010) 16(9):1257–64. doi:10.1016/j.bbmt.2010.03.004
142. Venstrom JM, Gooley TA, Spellman S, Pring J, Malkki M, Dupont B, et al. Donor activating KIR3DS1 is associated with decreased acute GVHD in unrelated allogeneic hematopoietic stem cell transplantation. *Blood* (2010) 115(15):3162–5. doi:10.1182/blood-2009-08-236943
143. Moretta L, Locatelli F, Pende D, Marcenaro E, Mingari MC, Moretta A. Killer Ig-like receptor-mediated control of natural killer cell alloreactivity in haploidentical hematopoietic stem cell transplantation. *Blood* (2011) 117(3):764–71. doi:10.1182/blood-2010-08-264085
144. Willemze R, Rodrigues CA, Labopin M, Sanz G, Michel G, Socie G, et al. KIR-ligand incompatibility in the graft-versus-host direction improves outcomes after umbilical cord blood transplantation for acute leukemia. *Leukemia* (2009) 23(3):492–500. doi:10.1038/leu.2008.365
145. Benson DM Jr, Hofmeister CC, Padmanabhan S, Suvannasankha A, Jagannath S, Abonour R, et al. A phase 1 trial of the anti-KIR antibody IPH2101 in patients with relapsed/refractory multiple myeloma. *Blood* (2012) 120(22):4324–33. doi:10.1182/blood-2012-06-438028
146. Maus MV, Grupp SA, Porter DL, June CH. Antibody-modified T cells: CARs take the front seat for hematologic malignancies. *Blood* (2014) 123(17):2625–35. doi:10.1182/blood-2013-11-492231
147. Chu J, Deng Y, Benson DM, He S, Hughes T, Zhang J, et al. CS1-specific chimeric antigen receptor (CAR)-engineered natural killer cells enhance in vitro and in vivo antitumor activity against human multiple myeloma. *Leukemia* (2014) 28(4):917–27. doi:10.1038/leu.2013.279
148. Gleason MK, Verneris MR, Todhunter DA, Zhang B, McCullar V, Zhou SX, et al. Bispecific and trispecific killer cell engagers directly activate human NK cells through CD16 signaling and induce cytotoxicity and cytokine production. *Mol Cancer Ther* (2012) 11(12):2674–84. doi:10.1158/1535-7163.MCT-12-0692
149. Wiernik A, Foley B, Zhang B, Verneris MR, Warlick E, Gleason MK, et al. Targeting natural killer cells to acute myeloid leukemia in vitro with a CD16 x 33 bispecific killer cell engager and ADAM17 inhibition. *Clin Cancer Res* (2013) 19(14):3844–55. doi:10.1158/1078-0432.CCR-13-0505
150. Gleason MK, Ross JA, Warlick ED, Lund TC, Verneris MR, Wiernik A, et al. CD16xCD33 bispecific killer cell engager (BiKE) activates NK cells against primary MDS and MDSC CD33+ targets. *Blood* (2014) 123(19):3016–26. doi:10.1182/blood-2013-10-533398
151. Pittari G, Filippini P, Gentilcore G, Grivel JC, Rutella S. Revving up natural killer cells and cytokine-induced killer cells against hematological malignancies. *Front Immunol* (2015) 6:230. doi:10.3389/fimmu.2015.00230
152. Ortaldo JR, Winkler-Pickett RT, Nagata S, Ware CF. Fas involvement in human NK cell apoptosis: lack of a requirement for CD16-mediated events. *J Leukoc Biol* (1997) 61(2):209–15.
153. Somanchi SS, Senyukov VV, Denman CJ, Lee DA. Expansion, purification, and functional assessment of human peripheral blood NK cells. *J Vis Exp* (2011) (48):e2540. doi:10.3791/2540
154. Somanchi SS, Somanchi A, Cooper LJ, Lee DA. Engineering lymph node homing of ex vivo-expanded human natural killer cells via trogocytosis of the chemokine receptor CCR7. *Blood* (2012) 119(22):5164–72. doi:10.1182/blood-2011-11-389924
155. Rutella S, Bonanno G, Marone M, De Ritis D, Mariotti A, Voso MT, et al. Identification of a novel subpopulation of human cord blood CD34-CD133-CD7-CD45+lineage-cells capable of lymphoid/NK cell differentiation after in vitro exposure to IL-15. *J Immunol* (2003) 171(6):2977–88. doi:10.4049/jimmunol.171.6.2977
156. Alnabhan R, Madrigal A, Saudemont A. Differential activation of cord blood and peripheral blood natural killer cells by cytokines. *Cytotherapy* (2015) 17(1):73–85. doi:10.1016/j.jcyt.2014.08.003
157. Shaim H, Yvon E. Cord blood: a promising source of allogeneic natural killer cells for immunotherapy. *Cytotherapy* (2015) 17(1):1–2. doi:10.1016/j.jcyt.2014.12.001
158. Tomchuck SL, Leung WH, Dallas MH. Enhanced cytotoxic function of natural killer and CD3+CD56+ cells in cord blood after culture. *Biol Blood Marrow Transplant* (2015) 21(1):39–49. doi:10.1016/j.bbmt.2014.10.014
159. Tanaka J, Sugita J, Shiratori S, Shigematu A, Asanuma S, Fujimoto K, et al. Expansion of NK cells from cord blood with antileukemic activity using GMP-compliant substances without feeder cells. *Leukemia* (2012) 26(5):1149–52. doi:10.1038/leu.2011.345
160. Knorr DA, Ni Z, Hermanson D, Hexum MK, Bendzick L, Cooper LJ, et al. Clinical-scale derivation of natural killer cells from human pluripotent stem cells for cancer therapy. *Stem Cells Transl Med* (2013) 2(4):274–83. doi:10.5966/sctm.2012-0084
161. Cheng M, Zhang J, Jiang W, Chen Y, Tian Z. Natural killer cell lines in tumor immunotherapy. *Front Med* (2012) 6(1):56–66. doi:10.1007/s11684-012-0177-7
162. Imai C, Iwamoto S, Campana D. Genetic modification of primary natural killer cells overcomes inhibitory signals and induces specific killing of leukemic cells. *Blood* (2005) 106(1):376–83. doi:10.1182/blood-2004-12-4797
163. Liu Y, Wu HW, Sheard MA, Sposto R, Somanchi SS, Cooper LJ, et al. Growth and activation of natural killer cells ex vivo from children with neuroblastoma for adoptive cell therapy. *Clin Cancer Res* (2013) 19(8):2132–43. doi:10.1158/1078-0432.CCR-12-1243
164. Jiang B, Wu X, Li XN, Yang X, Zhou Y, Yan H, et al. Expansion of NK cells by engineered K562 cells co-expressing 4-1BBL and mMICA, combined with soluble IL-21. *Cell Immunol* (2014) 290(1):10–20. doi:10.1016/j.cellimm.2014.04.011
165. Denman CJ, Senyukov VV, Somanchi SS, Phatarpekar PV, Kopp LM, Johnson JL, et al. Membrane-bound IL-21 promotes sustained ex vivo proliferation of human natural killer cells. *PLoS One* (2012) 7(1):e30264. doi:10.1371/journal.pone.0030264
166. Hercend T, Meuer S, Reinherz EL, Schlossman SF, Ritz J. Generation of a cloned NK cell line derived from the "null cell" fraction of human peripheral blood. *J Immunol* (1982) 129(3):1299–305.
167. Perussia B, Ramoni C, Anegon I, Cuturi MC, Faust J, Trinchieri G. Preferential proliferation of natural killer cells among peripheral blood mononuclear cells cocultured with B lymphoblastoid cell lines. *Nat Immun Cell Growth Regul* (1987) 6(4):171–88.
168. Rabinowich H, Sedlmayr P, Herberman RB, Whiteside TL. Increased proliferation, lytic activity, and purity of human natural killer cells cocultured with mitogen-activated feeder cells. *Cell Immunol* (1991) 135(2):454–70. doi:10.1016/0008-8749(91)90290-R
169. Lundqvist A, Abrams SI, Schrump DS, Alvarez G, Suffredini D, Berg M, et al. Bortezomib and depsipeptide sensitize tumors to tumor necrosis factor-

related apoptosis-inducing ligand: a novel method to potentiate natural killer cell tumor cytotoxicity. *Cancer Res* (2006) 66(14):7317–25. doi:10.1158/0008-5472.CAN-06-0680
170. Lundqvist A, Su S, Rao S, Childs R. Cutting edge: bortezomib-treated tumors sensitized to NK cell apoptosis paradoxically acquire resistance to antigen-specific T cells. *J Immunol* (2010) 184(3):1139–42. doi:10.4049/jimmunol.0902856
171. Boeck C, Amberger D, Doraneh-Gard F, Sutanto W, Guenther T, Schmol J. Significance and frequencies, compositions and/or antileukemic activity of (DC–stimulated) invariant NKT-, NK and CIK cells on the outcome of patients with AML, ALL and CLL. *J Immunotherapy* (2017) (in press).
172. Romano M, Tung SL, Smyth LA, Lombardi G. Treg therapy in transplantation: a general overview. *Transpl Int* (2017). doi:10.1111/tri.12909
173. Trzonkowski P, Bieniaszewska M, Juścińska J, Dobyszuk A, Krzystyniak A, Marek N, et al. First-in-man clinical results of the treatment of patients with graft versus host disease with human ex vivo expanded CD4+CD25+CD127− T regulatory cells. *Clin Immunol* (2009) 133(1):22–6. doi:10.1016/j.clim.2009.06.001
174. Brunstein CG, Miller JS, Cao Q, McKenna DH, Hippen KL, Curtsinger J, et al. Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. *Blood* (2011) 117(3):1061–70. doi:10.1182/blood-2010-07-293795
175. Edinger M, Hoffmann P. Regulatory T cells in stem cell transplantation: strategies and first clinical experiences. *Curr Opin Immunol* (2011) 23(5):679–84. doi:10.1016/j.coi.2011.06.006
176. Martelli MF, Di Ianni M, Ruggeri L, Falzetti F, Carotti A, Terenzi A, et al. HLA-haploidentical transplantation with regulatory and conventional T-cell adoptive immunotherapy prevents acute leukemia relapse. *Blood* (2014) 124(4):638–44. doi:10.1182/blood-2014-03-564401
177. Theil A, Tuve S, Oelschlagel U, Maiwald A, Dohler D, Ossmann D, et al. Adoptive transfer of allogeneic regulatory T cells into patients with chronic graft-versus-host disease. *Cytotherapy* (2015) 17(4):473–86. doi:10.1016/j.jcyt.2014.11.005
178. Kolb H-J, Bigalke I, Simoes B, Falk C, Tischer J, Hill W, et al. CD6-depleted mobilized stem cells for modification of HVG and GVH reactions after HLA-haploidentical marrow transplantation. *Blood* (2004) 104(11):978.
179. Burroughs LM, O'Donnell PV, Sandmaier BM, Storer BE, Luznik L, Symons HJ, et al. Comparison of outcomes of HLA-matched related, unrelated, or HLA-haploidentical related hematopoietic cell transplantation following non-myeloablative conditioning for relapsed or refractory Hodgkin lymphoma. *Biol Blood Marrow Transplant* (2008) 14(11):1279–87. doi:10.1016/j.bbmt.2008.08.014
180. Raiola A, Dominietto A, Varaldo R, Ghiso A, Galaverna F, Bramanti S, et al. Unmanipulated haploidentical BMT following non-myeloablative conditioning and post-transplantation CY for advanced Hodgkin's lymphoma. *Bone Marrow Transplant* (2014) 49(2):190–4. doi:10.1038/bmt.2013.166
181. Ghiso A, Raiola AM, Gualandi F, Dominietto A, Varaldo R, Van Lint MT, et al. DLI after haploidentical BMT with post-transplant CY. *Bone Marrow Transplant* (2015) 50(1):56–61. doi:10.1038/bmt.2014.217

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REVIEW
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Immune Reconstitution after Allogeneic Hematopoietic Stem Cell Transplantation

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The timely reconstitution and regain of function of a donor-derived immune system is of utmost importance for the recovery and long-term survival of patients after allogeneic hematopoietic stem cell transplantation (HSCT). Of note, new developments such as umbilical cord blood or haploidentical grafts were associated with prolonged immunodeficiency due to delayed immune reconstitution, raising the need for better understanding and enhancing the process of immune reconstitution and finding strategies to further optimize these transplant procedures. Immune reconstitution post-HSCT occurs in several phases, innate immunity being the first to regain function. The slow T cell reconstitution is regarded as primarily responsible for deleterious infections with latent viruses or fungi, occurrence of graft-versus-host disease, and relapse. Here we aim to summarize the major steps of the adaptive immune reconstitution and will discuss the importance of immune balance in patients after HSCT.

Keywords: hematopoietic stem cell transplantation, immune reconstitution, infection, graft-versus-leukemia effect, graft-versus-host disease

INTRODUCTION

The reconstitution of different immune cell subsets after allogeneic hematopoietic stem cell transplantation (HSCT) (**Figure 1**) occurs at different time points summarized in **Table 1**. After conditioning therapy, patients undergo an "aplastic phase" (severe neutropenia or pre-engraftment phase) until neutrophils recover. The total nucleated cell (TNC) dose and $CD34^+$ cell dose within the graft source are important factors contributing to the rate of engraftment and outcome after HSCT. Umbilical cord blood (UCB) grafts contain lower TNC levels compared to bone marrow transplant (BMT) and peripheral blood stem cell transplant (PBSCT), what increase the time of neutrophil engraftment from ~14 days after PBSCT and 21 days after BMT to 30 days after UCB transplantation (1, 2). Moreover, recent study showed that high TNC cell dose was associated with improved overall survival (OS), decreased relapse, and increased incidence of chronic graft-versus-host disease (GvHD) in patients receiving PBSCT (3). On the other hand, it has been presented that patients with higher $CD34^+$ dose within PBSCT had faster platelet engraftment, but lower OS and increased relapse (4).

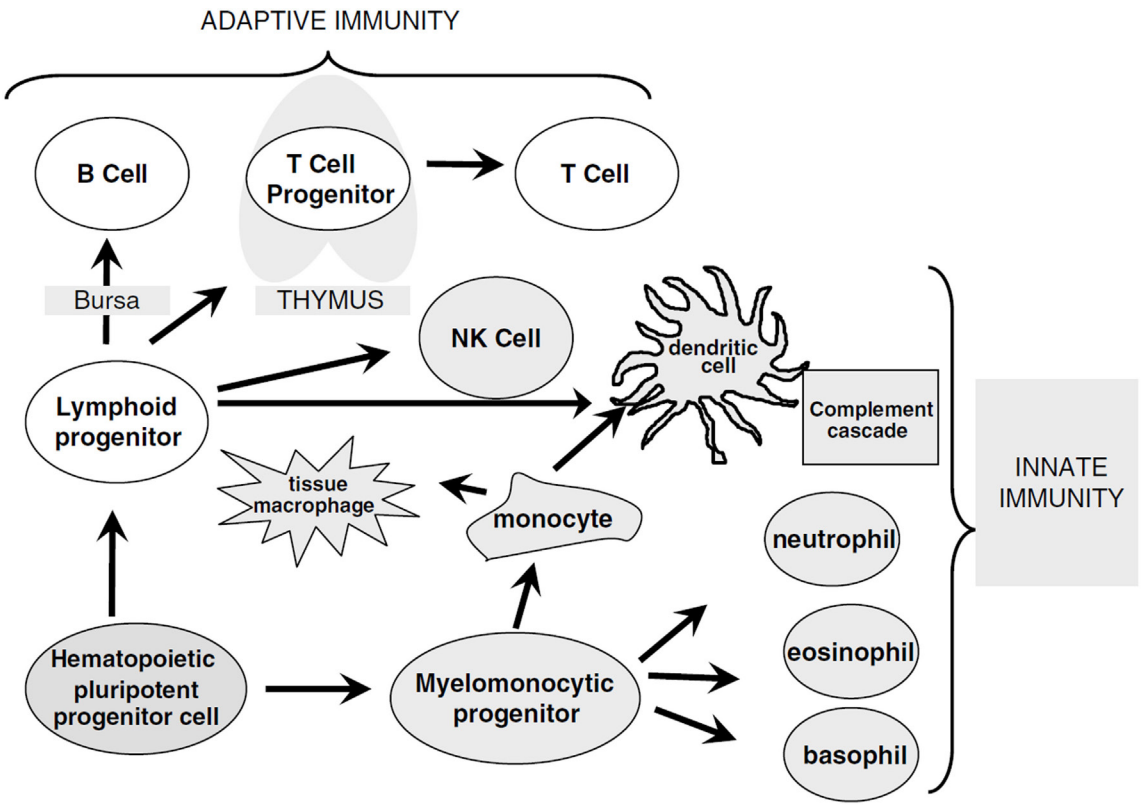

FIGURE 1 | Overview of immune cell differentiation. The figure shows the different types of immune cells and their development from different precursors. The reconstitution of innate immunity occurs rapidly within 20–30 days after allogeneic HSCT while reconstitution of adaptive immunity is delayed following HSCT and can require up to 1 year. Natural killer (NK) cells, monocytes, granulocytes, and dendritic cells are derived from myelomonocytic progenitor cells. B and T cells differentiate from lymphoid progenitor cells and require specialized microenvironments in order to efficiently differentiate from primitive progenitors, and typically show delayed and incomplete recovery. Reprinted by permission from Macmillan Publishers Ltd.: Bone Marrow Transplantation (5), copyright (2005).

TABLE 1 | Immune reconstitution after allogeneic HSCT.

Immune cells	Duration after allogeneic HSCT
Neutrophils >0.5 × 10^9/L	~14 days for PBSC, ~21 days for BM, and ~30 days for CB
NK cells	30–100 days
T cells	100 days
$CD19^+$ B cells	1–2 years

PBSC, peripheral blood stems cells; BM, bone marrow; CB, cord blood; NK cells, natural killer cells.

The infections encountered during the pre-engraftment phase consist primarily of bacterial and fungal infections that are reasonably well controlled by medications given for prophylaxis and treatment (6) (**Figure 2**). The first 100 days after HSCT (engraftment phase) are characterized by cellular immunodeficiencies due to a reduced number of natural killer (NK) cells of the innate immune system and T cells of the adaptive immune system. This renders patients especially susceptible to viral reactivations including cytomegalovirus (CMV) and Epstein–Barr virus (EBV) as well as viral diseases (7, 8).

The recovery of the T cell compartment relies on peripheral expansion of memory T cells, driven by cytokines as well as allogeneic antigens encountered in the host, and is followed by the production of naive T cells in the thymus (5). $CD4^+$ T cells reconstitute later than $CD8^+$ T cells and depend more on thymic generation of $CD4^+CD45RA^+$ naive T cells after HSCT explaining the reported inversion of the CD4/CD8 ratio (9). About 3 months after HSCT, $CD4^+$ T cell numbers of about 200/μL have been observed (10). T cell receptor (TCR) rearrangement excision DNA circles (TRECs) have been investigated as surrogate parameters for reconstitution of thymus-derived $CD4^+CD45RA^+$ naive T cells (11). TREC levels remain low until 3–6 months after allogeneic HSCT (5). A special subgroup of $CD4^+$ cells are regulatory T cells (Tregs), which may be important for a better outcome after allogenic HSCT (12). Tregs suppress the activity of effector T cells, thus reducing inflammation and promoting immune homeostasis after allogenic HSCT (13). Clinical, preclinical, and experimental models have shown that Treg reconstitution plays a critical role in amelioration of GvHD while preserving the graft-versus-leukemia (GvL) effect (14, 15). Increasing age is associated with thymic atrophy and loss of function (16). Cycling of mature lymphocytes maintains numbers of mature T cells by homeostatic peripheral expansion (5). Naive $CD4^+$ and $CD8^+$ T cells rely on interleukin-7 (IL-7) and TCR engagement for survival and expansion (17). $CD8^+CD27^+$

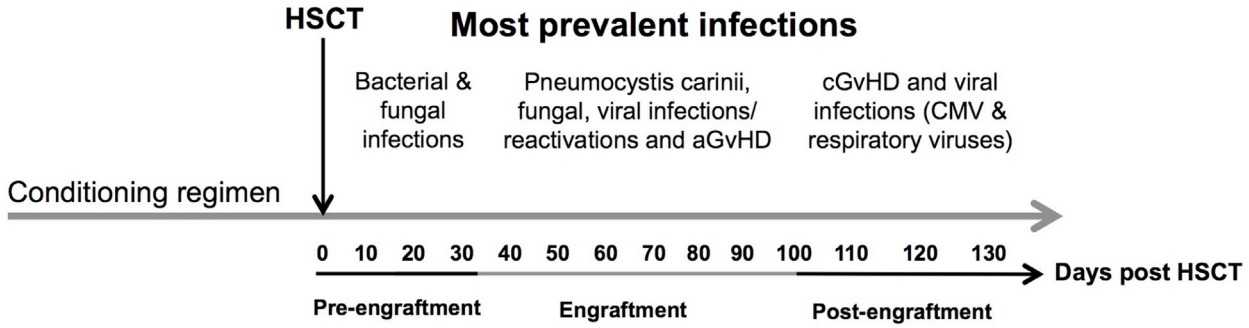

FIGURE 2 | Time line of complications after allogeneic HSCT. The figure shows the most prevalent complications after HSCT according to the three phases of engraftment. Concomitant infectious complications consisting of bacterial, fungal, and viral infections are shown according to their occurrence as well as association with acute and chronic GvHD during different phases of follow-up: (1) pre-engraftment, (2) engraftment, and (3) post-engraftment phase. Abbreviations: CMV, cytomegalovirus; aGvHD, acute graft-versus-host disease; cGvHD, chronic graft-versus-host disease.

memory T cells can be maintained and expanded by cytokine signals alone involving IL-7 and interleukin-15 (IL-15) (18). In older patients, the lack of $CD4^+CD45RA^+$ naive T cells with a broad TCR repertoire leads to an increased risk for opportunistic infections and probably also to increased risk of leukemic relapse (19, 20). The lack of $CD4^+CD45RA^+$ naive T cells is additionally aggravated by GvHD (21, 22).

The B cell compartment representing the humoral immunity is the slowest to reconstitute and may take up to 5 years after allogeneic HSCT. Transitional $CD19^+CD21^{low}CD38^{high}$ B cells are the first B cells emigrating from the bone marrow (BM) and are elevated in the peripheral blood (PB) in the first months after HSCT before their percentage progressively decreases, while the proportion of more mature B cell subpopulations increases (23). The lack of $CD19^+CD27^+$ memory B cells, decreased levels of circulating immunoglobulins, impaired immunoglobulin class switching, and a loss of complexity in immunoglobulin gene rearrangement patterns leave allogeneic HSCT patients vulnerable to encapsulated bacteria such as *Streptococcus pneumoniae* and *Haemophilus influenzae* (1, 24). In this review, we summarize the reconstitution of the adaptive immunity and discuss the importance of achieving immune balance after HSCT.

ADAPTIVE IMMUNITY

Immune Reconstitution of B Cells after Allogeneic Hematopoietic Stem Cell Transplantation

Patients undergoing HSCT often experience late recovery of B cell numbers leading to a defect of B cell mediated immunity. Generally, B cell numbers recover to normal counts within 12 months after HSCT (25), although complete recovery may take up to 2 years. In the first few months, very few circulating B cells have been observed (25, 26) and within 1–2 years following HSCT, B cell numbers reach levels exceeding normal adult individual ones followed by gradual decline, similarly to the normal ontogeny in young children (26). First B cells emerging into the periphery are $CD19^+CD21^{low}CD38^{high}$ transitional B cells, which subsequently decrease in percentages while mature $CD19^+CD21^{high}CD27^-$ naive B cells are being replenished (1, 23). Transitional B cells were first described as $CD24^{high}CD38^{high}$ (23). Later on, another marker of transitional B cells was identified, distinguishing between T1 and T2 transitional cells. T1 cells were reported as $CD21^{low}$ and described as the first B cell population emigrating from the BM, which subsequently differentiate toward $CD21^+$, T2 phenotype and serve as precursors of the $CD19^+CD21^{high}CD27^-$ naive B cell pool in PB and tissues (27). Complete reconstitution of the B cell compartment includes the recovery of both $CD19^+CD21^{high}CD27^-$ naive and $CD19^+CD27^+$ memory B cells. Reconstitution of memory B cells occurs upon environmental or vaccine-based antigen exposure and requires $CD4^+$ T cell help (28). Complete $CD19^+CD27^+$ memory B cell development may take up to 5 years after HSCT (26). In the study by Corre and colleagues, numbers of $CD19^+CD21^{high}CD27^-$ naive B cells normalized by 6 months and reached above normal values around 24 months after myeloablative conditioning for allogeneic HSCT (29). $CD19^+CD27^+$ memory B cells remained persistently low during the 2 years of follow-up (29). Other authors similarly reported relatively fast naive B cell reconstitution followed by delayed memory B cell recovery (30, 31). In addition, early expansion of $CD19^+CD5^+$ B cells has been reported (29, 32), a subset described as pre-naive circulating B cells representing a distinct intermediate phenotype between transitional and naive B cells (33). These cells showed only partial responses to B cell receptor (BCR) stimulation and CD40 ligation, but similarly to $CD19^+CD21^{high}CD27^-$ naive B cells, these were capable to differentiate into plasma cells and had the ability to function as antigen-presenting cells (APCs) (33).

In the first 2 years following allogeneic HSCT, B cell function remains compromised. Different B cell subpopulations often reconstitute over a different period of time contributing to a defective humoral response. Delayed T cell recovery and the reversed CD4/CD8 ratio may also contribute to low circulating B cell numbers following HSCT (26). Furthermore, $CD19^+CD27^+$ memory B cells can be influenced by low T helper cells as they

require their help for isotype switching (26). In addition, somatic hypermutation seems to be diminished even in the presence of normal donor $CD4^+$ T cell numbers, implying an environmental defect (26, 34). Normal levels of serum IgM are usually measurable 3–6 months after HSCT (35, 36), followed by normalization of serum IgG1/IgG3, IgG2/IgG4, and IgA similar to that observed during normal development in the first years of life (37). However, in some patients, long-term antibody class deficiencies have been reported (38). The immunoglobuline heavy chain (IgH) repertoire is often characterized by delayed class switching and oligoclonal dominance with specific rearrangements dominating at different time points in these patients (36, 39). Measurement of B lymphocyte repertoire diversity by analysis of IgH complementarity determining region 3 (CDR3) revealed limited variation of IgH CDR3 repertoire in $CD19^+CD27^+$ memory B lymphocytes compared to $CD19^+CD21^{high}CD27^-$ naive B cells at 3 and 6 months after allogeneic HSCT. Decrease in $CD19^+CD27^+$ memory B cell IgH CDR3 repertoire, but not $CD19^+CD21^{high}CD27^-$ naive B cell one, was also observed when compared to healthy controls suggesting a role of $CD19^+CD27^+$ memory B cells in oligoclonal restriction (35). Both $CD19^+CD27^+$ memory B cells and $CD19^+CD21^{high}CD27^-$ naive B cells reach normal diversity, comparable to healthy individuals, 12 months after HSCT (35).

Different settings of HSCT may also influence B cell recovery. Patients receiving antithymocyte globulin-fresenius (ATG-F) presented delayed $CD19^+$ B cell recovery up to 5 months after HSCT compared to non-ATG-F patients (40). ATG is a potent immunosuppressant administrated before HSCT to prevent graft rejection and to reduce incidence of acute and chronic GvHD in patients receiving grafts from unrelated donors (40, 41). Absolute $CD19^+$ B cells normalized 1 year after HSCT in both groups. ATG-treated patients had significantly worse $CD19^+CD21^{high}CD27^-$ naive B cell and $CD19^+CD27^+$ memory B cell regeneration within the first month after HSCT indicating a negative impact of ATG on B cell immune reconstitution (40). Depending on the brand, ATG may also have immunomodulatory effects on B cells (42). Slow B cell recovery has been observed in patients receiving non-myeloablative conditioning compared to those given myeloablative therapy, with reduced B cell numbers observed in most patients up to 12 months after non-myeloablative therapy for HSCT (43). However, these findings may in part be explained by older patient age and higher incidence of acute GvHD in this patient cohort (43). Both acute and chronic GvHD have been associated with delayed B cell reconstitution, and reduction or lack of B cell precursors in the BM has been observed in these patients compared to patients without GvHD (44). In a study on 93 allograft recipients, the number of BM B cell precursors on day 30 after HSCT was significantly lower in patients later developing grades 2–4 acute GvHD compared to patients with grades 0–1 disease (44). Moreover, patients developing extensive chronic GvHD within 1 year after transplantation had lower percentages of B cell precursors on day 365 compared with patients without chronic GvHD or with limited chronic GvHD (44). However, the effect of acute and chronic GvHD could not be separated from the possible influence of glucocorticoid treatment in this study due to low patient numbers suggesting B cell deficiency after transplantation may in part be a result of inhibition of B lymphopoiesis by GvHD and/or its treatment (44). In addition, a decrease of absolute $CD19^+$ B cells in patients at first diagnosis of chronic GvHD and a disturbance of B cell homeostasis in patients with active chronic GvHD have been observed (45, 46). Stem cell source may also influence numbers of circulating B cells with higher counts detected in recipients of peripheral blood stem cells (PBSC); however, this observation may be attributed to the higher amount of mature B cells in PBSC grafts compared with BM (44, 47, 48).

Even patients who show recovery of overall $CD19^+$ B cell numbers are not considered fully immunocompetent and as a result of decreased B cell function, impaired vaccine responses to infectious antigens have been observed (26). Lack of $CD19^+CD27^+$ memory B cells, decrease of circulating immunoglobulins, and impaired immunoglobulin gene rearrangement render these patients susceptible to encapsulated bacteria and viruses (1, 24).

Immune Reconstitution of T Cells and Their Role after HSCT

$CD4^+$ and $CD8^+$ T cells reconstitute within the first year after HSCT and enable defense against viral or fungal infections, as well as maintaining the GvL effect. A subset of $CD4^+$ T cells are so called regulatory T cells (Tregs). In the next paragraph, we aim to summarize their development and function in patients after allogeneic HSCT.

Regulatory T Cells in Immune Reconstitution and Their Impact after HSCT

Regulatory T Cells

Tregs are a subset of $CD4^+$ T cells whose function is to suppress immune responses and maintain self-tolerance (49). A transcription factor called FoxP3, a member of the fork head family of transcription factors, is critical for the development and function of Tregs and is used as a definite marker to identify Tregs (49, 50). Tregs are a functionally mature subpopulation of T cells and can also be induced from $CD4^+CD45RA^+$ naive T cells in the periphery (51). Natural Tregs (nTregs) are derived from the thymus and are characterized by the co-expression of CD4, high expression of CD25 and FoxP3 (52). Induced or adaptive Tregs (iTregs) are generated in peripheral lymphoid organs in the presence of transforming growth factor beta (TGF-β) (53) (**Figure 3**).

Some recent studies have shown that nTregs are more stable than iTregs in relation to their differential DNA methylation profiles and other epigenetic regulations of FoxP3 (54, 55).

Tregs in Immune Balance

Tregs can downregulate immune responses by (a) production of inhibitory cytokines and (b) a contact-mediated effect on APCs. Tregs produce the anti-inflammatory cytokine interleukin-10 (IL-10) that inhibits production of interleukin-12 (IL-12) by activated dendritic cells (DCs) and macrophages (56, 57). IL-10 also inhibits the expression of co-stimulators and

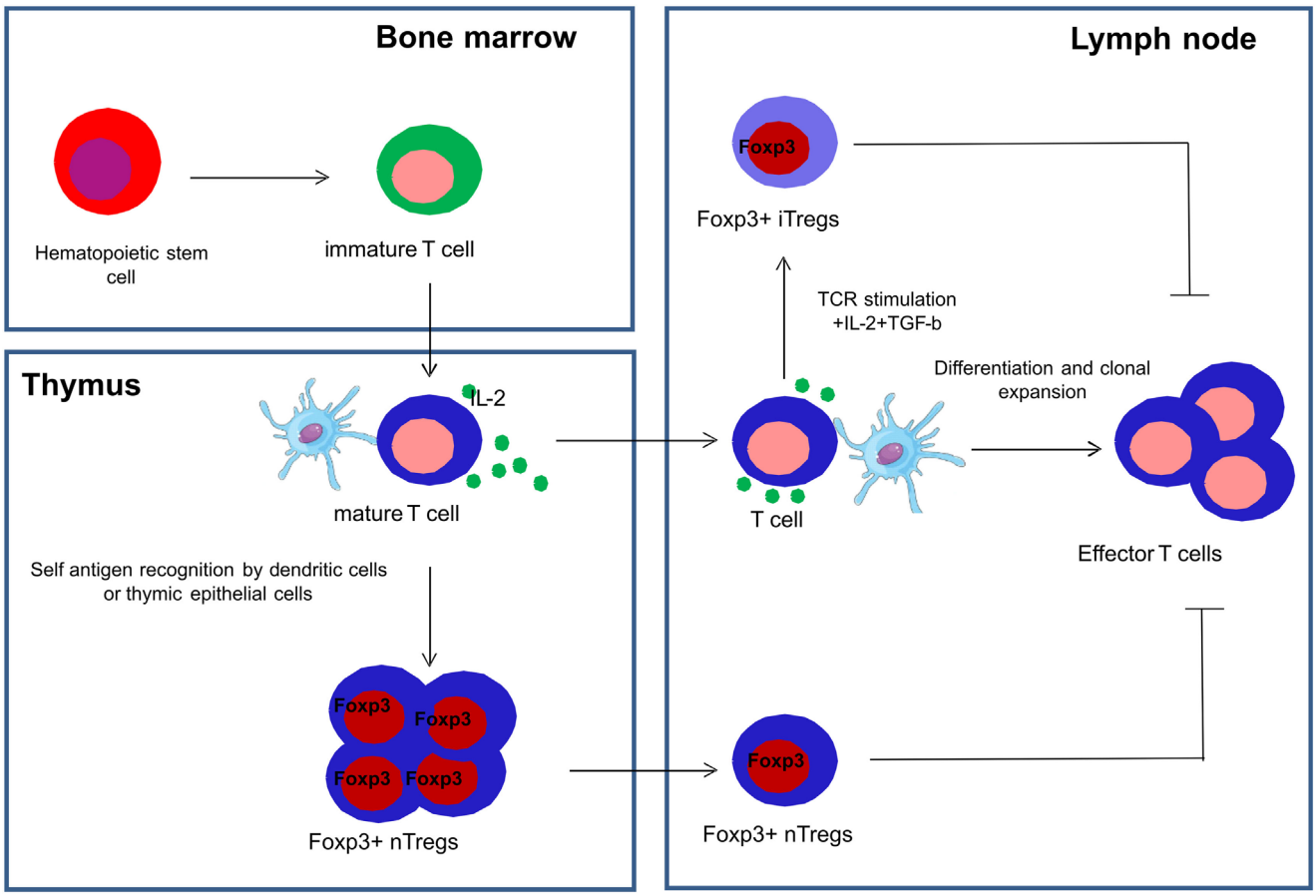

FIGURE 3 | Development of natural and induced regulatory T cells. Natural regulatory T cells (nTregs) are derived from the thymus and are characterized by the co-expression of CD4, high expression of CD25 and FoxP3, and are collectively represented as $CD4^+CD25^+FoxP3^+$ Tregs. Induced or adaptive regulatory T cells (iTregs) are generated in the peripheral lymphoid organs in the presence of transforming growth factor beta (TGF-β) and interleukin-2 (IL-2).

major histocompatibility complex (MHC) class II molecules on DCs and macrophages and thus inducing tolerance within the immune system (56–58). Another anti-inflammatory cytokine produced by Tregs, TGF-β, inhibits the proliferation and effector functions of T cells and the activation of macrophages (59, 60). TGF-β also regulates the differentiation of functionally distinct subsets of T cells, stimulates production of immunoglobulin A (IgA) antibodies, promotes tissue repair after local immune and inflammatory reactions subside, and confers Treg-mediated immune reconstitution (56–58, 61). Tregs play a major role in regulation of epithelial inflammation and are strongly influenced by the interaction with the epithelial microbial environment (62, 63).

Tregs in Animal Models of Hematopoietic Stem Cell and Solid Organ Transplantation

Tregs play an indispensable role in both solid organ transplant tolerance and in allograft tolerance after HSCT. In rodents and humans, a subpopulation of thymus-derived naive $CD4^+$ T cells that co-express the IL-2R alpha chain, CD25, have potent suppressive activity (64). Tregs mediate transplantation tolerance in experimental models of skin and/or solid organ transplantation (65) as well as tolerance to BM allografts (66). By allogeneic HSCT, malignant and non-malignant hematological disorders can be cured, but at the same time, treatment efficacy is limited due to occurrence of GvHD (67). Regulatory T cells have received considerable attention in recent years due to their ability to suppress the proliferation of conventional T cells when added to donor grafts and prevention of GvHD in animal models (68). Using a mouse model, Edinger and colleagues have shown that $CD4^+CD25^+$ Tregs suppress GvHD after BMT without abrogating the GvL or graft-versus-tumor (GvT) effect (14) supporting the importance of Tregs in allogenic HSCT. Furthermore, Nguyen and colleagues demonstrated that the adoptive transfer of Tregs preserved thymic and lymphoid architecture of the host and hence accelerated posttransplant T cell immune reconstitution in a murine GvHD model (69).

Taylor and colleagues demonstrated that *in vivo* depletion of $CD25^+$ T cells and depletion of $CD25^+$ T cells in the transplant inoculum, worsened GvHD whereas adoptive transfer of $CD4^+$ $CD25^+$ nTregs together with the BM graft ameliorated GvHD (70).

While an increasing number of publications have focused on the biology of $CD4^+CD25^+Foxp3^+CD45RO^{lo}$ naive Tregs in GvHD, less attention has been given to iTregs, probably due to the lack of proven cell surface marker that differentiate nTregs from iTregs. Fantini and colleagues demonstrated that iTregs can be generated from $CD4^+$ T cells in the presence of TGF-β and can be expanded in culture (71). On the other hand, Koenecke and colleagues showed that administration of *in vitro* generated iTregs along with BM grafts containing alloreactive donor T cells did not provide any significant protection from lethal GvHD, due to limited *in vivo* survival of these cells (72). They also demonstrated that iTregs lost their Foxp3 expression, along with a loss of suppressive function early after transplantation, thus making iTregs unsuitable for use in a therapeutic approach (72) if administered as an external cellular product. Not only iTregs but also nTregs have been shown to loose Foxp3 expression in a STAT3-dependent manner and can revert to a proinflammatory phenotype under inflammatory conditions (73). Therefore, inflammation seems to affect Foxp3 expression in both natural and induced Tregs.

Although Tregs have long been assumed to be solely a subset of the $CD4^+$ T cell compartment, a $CD8^+$ Treg population has been recently described and shown to be capable of suppressing T cell responses (74). In terms of GvHD, Robb and colleagues reported that $CD8^+Foxp3^+$ Tregs suppressed GvHD and attenuated GvHD mortality after BMT in a mouse model (75). Interestingly, $CD8^+Foxp3^+$ cells were more suppressive than $CD4^+Foxp3^+$ cells (75). Using a rat model, Xystrakis and colleagues provided a first report on $CD8^+$ Tregs conferring their regulatory properties *via* a cell to cell contact dependent mechanism to prevent GvHD and thus confirming $CD8^+Foxp3^+$ Tregs in a second species (76). Clinical studies on $CD8^+$ Tregs at a functional level are scarce to date. However, Zheng and colleagues reported that human $CD8^+$ Tregs potentially inhibit GvHD without compromising general immunity and GvL activity in humanized mouse models (77). Taken together, these findings provide an insight into the efficacy of both $CD4^+$ and $CD8^+$ Tregs as potential novel therapeutic approaches in clinic.

Tregs in Clinical Hematopoietic Stem Cell Transplantation

Many researchers have focused on evaluating Treg cell numbers after HSCT, since they play an important role in the amelioration of GvHD. Using PB of patients after transplantation, Li and colleagues demonstrated that the frequency of $CD4^+CD25^+$ Tregs was significantly downregulated in patients with severe acute or chronic GvHD (78). They also showed that a decreased level of $CD4^+CD25^+$ Tregs correlated with increased severity of GvHD (78). While the majority of studies focused on blood derived Tregs, there is little information on Tregs isolated from intestinal tissues due to the lack of availability of repeated gut biopsies. Using immunoenzymatic labeling, Rieger and colleagues were the first to demonstrate that infiltrating Tregs decreased the signs of acute and chronic GvHD in intestinal mucosa (79). They showed that patients with acute and chronic GvHD had a complete lack of counter regulation indicated by a $Foxp3^+$/$CD8^+$ T cell ratio identical to that of healthy individuals, while this ratio was increased in patients without GvHD (79). These results have been discussed controversially in the literature since Lord and colleagues demonstrated that $Foxp3^+$ T cells were not decreased in PB or gastrointestinal tissues and that the frequency of Tregs did not correspond to disease incidence or severity (80). On the contrary, these investigators reported that $Foxp3^+$ T cells were significantly upregulated in GvHD-afflicted intestinal mucosa when compared to non-GvHD mucosal tissues (80). This finding was further supported by Ratajczak and colleagues who observed an increased proportion of $CD4^+Foxp3^+$ T cells in patients with grades 2–4 compared to grades 0–1 acute GvHD (81). One possible explanation for these conflicting results may be the difficulty to discriminate natural and induced Tregs. It is possible that nTregs are decreased in GvHD while iTregs may be increased in order to compensate for the exaggerated inflammation during GvHD. Imanguli and colleagues observed an upregulation of functional markers such as $CD3^+$, $CD4^+$, $CD27^+$, $ICOS^+$, and $CD39^+$ in Tregs that traffic into tissue including skin and oral mucosa exerting a suppressive function in patients with chronic GvHD (82). Interestingly, normal numbers of activated $CD45^-Foxp3^{hi}$ Tregs were observed in tissue and PB of patients with chronic GvHD whereas naive or resting $CD45RA^+Foxp3^+$ Tregs that presumably control chronic GvHD effector cells were reduced compared to patients without chronic GvHD.

Tregs in GvHD: First-in-Man Clinical Trial

Studies in mouse models of GvHD have provided information on the suppressive nature of Tregs and their potential to suppress and ameliorate GvHD without impairing the GvL effect. The first clinical trial using Tregs to suppress acute/chronic GvHD in patients were completed recently. This "first-in-man-study" reported the adoptive transfer of *ex vivo* expanded $CD4^+CD25^+CD127^-$ Tregs in one patient with chronic GvHD and another with acute GvHD after HSCT with an HLA-identical sibling donor (83). Transfer of Tregs resulted in a reduction of the steroid dose administered, increased levels of circulating Tregs, and a decrease in inflammatory cytokine levels in the PB (83). Another "first-in-man-study" was reported after double UCBT in 23 patients, who received *in vitro* expanded $0.1–30 \times 10^5$ UCB $CD4^+CD25^+CD127^-$ Tregs per kilogram derived from partially HLA-matched third-party UCB units (15). There was a significant reduction in the incidence of acute GvHD grades II–IV (43 versus 61%, $P = 0.05$) when compared to 108 historical controls without transfusion of Tregs. No toxicities, infections, relapse, or early mortality were observed suggesting that UCB Tregs could be beneficial for preventing acute GvHD (15). Furthermore, Di Ianni and colleagues reported a clinical trial in 28 patients receiving adoptively transferred $CD4^+CD25^+CD127^-$ Tregs after T-cell-depleted haploidentical HSCT without further immunosuppression (13). Only 2 out of 28 patients developed grades II–IV acute GvHD and no chronic GvHD was observed. They showed that adoptive transfer of freshly isolated donor-derived Tregs 4 days before inoculating the $CD34^+$ stem cells prevented acute and chronic GvHD in the absence of further immunosuppression. Tregs promoted lymphoid reconstitution, improvement of

immunity to opportunistic pathogens (no CMV-related death of patients) without abrogating the GvL effect (13). In addition, Hoffmann reported *in vitro* expansion of highly purified polyclonal human $CD4^+CD25^{high}$ Tregs through the use of artificial APCs for repeated stimulation *via* CD3 and CD28 in the presence of high-dose IL-2 (84). These cells not only maintained their phenotype and expressed suppressive activity but also maintained the expression of the lymph node homing receptors L-selectin and CCR7 (84). Furthermore, the same group reported results of a small phase I safety and feasibility trial where freshly isolated donor-derived $CD4^+CD25^{high}$ Tregs were infused into nine patients with high risk for leukemic relapse after cessation of systemic GvHD prophylaxis (12). After 8 weeks, additional $CD4^+$ T cells were administered to promote GvL activity. Patients showed no signs of GvHD nor opportunistic infections or early disease relapse supporting the safety and efficacy of Treg transfusion (12). This has led to a phase II clinical trial for the treatment of patients with steroid-refractory acute GvHD using freshly isolated $CD4^+CD25^{high}$ Tregs that is currently ongoing. Taken together, these early trials suggest that Tregs could be a novel approach for prophylaxis and treatment of patients with acute GvHD in larger clinical trials. The impact of Treg transfusion on the immune reconstitution has to be further investigated.

Induction of Regulatory T Cells after HSCT

Tregs induce tolerance and maintain immune homeostasis (51). A major challenge of Treg cell therapy is their relative scarcity in PB (0.5–1% of $CD4^+CD25^{high}$ T cells) (85). In 2011, Hippen and colleagues presented two individual reports regarding the generation of induced Tregs on a large scale (86) and *ex vivo* expansion of natural Tregs (86). Both methods focus on the development of expansion protocols for either type of Tregs to obtain higher yields for clinical trials on treatment or prevention of GvHD (86). In patients with chronic GvHD, Matsuoka and co-workers reported that daily administration of low-dose IL-2 induced selective expansion of functional $CD4^+CD25^+CD127^-$ Tregs, improved chronic GvHD, restored $CD4^+$ T cell homeostasis, and promoted the reestablishment of immune tolerance (87). Koreth et al. reported the case of 29 chronic GvHD patients that the administration of subcutaneous low dose IL-2 rapidly induced preferential and sustained Tregs expansion without any immune impairment (88). This suggests that low-dose IL-2 could be a potential therapy to restore immune balance after HSCT. Another approach to manipulate Tregs *in vivo* was reported by Furusawa and colleagues (89). Clostridial products, like short chain fatty acids (SCFA) or mainly butyrate, can induce the differentiation of colonic Tregs *in vitro* and *in vivo* in mouse models (89). This points toward the necessity of host–microbiome interaction to establish immunological tolerance and homeostasis in the gut. Moreover, Mathewson and colleagues reported that restoring clostridial metabolites or the strain itself modulated intestinal epithelial cell integrity and mitigated GvHD in mice (63). Taken together, these findings strongly suggest that the right balance of gut microbiome may be crucial to induce Tregs for intestinal tolerance.

$CD4^+$ and $CD8^+$ T Cell Reconstitution

Memory T cells [central memory (T_{CM}) and effector memory (T_{EM})], tissue resident memory cells (T_{RM}), and effector cells (T_{EFF}) cells are essential to control viral reactivations after allogeneic HSCT. Upon encountering antigens, memory cells differentiate to T_{EFF} and lyse the infected cells and secret proinflammatory cytokines [e.g., IFN-γ and tumor necrosis factor-α (TNF-α)] (90). Immune surveillance of T_{CM} occurs trafficking through secondary lymphoid organs, T_{EM} and T_{EFF}, through non-lymphoid organs (91). In contrary, T_{RM} cells reside at various sites (e.g., liver, lungs, gut, and skin) and provide immediate antiviral response (cytotoxicity and secretion of IFN-γ) without trafficking (92). The reconstitution of $CD4^+CD45RA^+$ naive T cells, providing the broad range of TCR repertoire needed to control infections and to avoid the reappearance of leukemic cells, is essential after allogeneic HSCT (11, 93). The conditioning regimens applied, increasing patient age and occurrence of acute and chronic GvHD, have devastating effects on thymic function after HSCT (28, 93–95). Reconstitution of $CD4^+CD45RA^+$ naive T cells can be demonstrated by measuring TRECs. Immune reconstitution of $CD4^+$ and $CD8^+$ T cells is also essential for maintaining a GvL effect (1). Reconstitution of $CD8^+$ T cells is faster than that of $CD4^+$ T cells, which usually occurs around day +100 or later and is indicated by the inversion of the $CD4^+/CD8^+$ T cell ratio (1) early after HSCT (**Table 1**). The time period until complete reconstitution of $CD4^+$ T cells can take up to 2 years after allogenic HSCT (96).

Major Factors Influencing T Cell Immune Reconstitution: GvHD and Immunosuppressive Treatment

Acute GvHD is one of the severe complications occurring early after HSCT contributing substantially to non-relapse mortality (NRM). Development of acute GvHD is influenced by human leukocyte antigen (HLA) disparities or gender mismatches between donor and recipient, the intensity of the conditioning regimen applied, CMV reactivation, and the stem cell source (97, 98). Acute GvHD can also occur in the HLA-identical transplant setting (siblings or matched unrelated donors) due to minor histocompatibility antigen differences between donor and recipient (98). Acute GvHD is an immune response directed against the host immune system, tissues, and organs (99, 100). GvHD by itself can inhibit T cell functions by limiting TCR diversity, T cell development, and dysfunction in cytokine production, most likely through damage of the BM and/or thymus, apoptosis, and release of cytokines in a so-called "cytokine storm" (101).

Bone marrow gives rise to all hematopoietic lineages and is the homing site for memory cells of the adaptive immunity (102). Recently, BM has been established as an additional target of alloreactivity observed during GvHD leading to the depletion of both hematopoietic progenitors and niche-forming cells (103), resulting in disrupted hematopoiesis and delayed immune reconstitution (104). Along with the BM, the thymus plays an important role in the maturation of hematopoietic precursors and T cell development (93). Acute GvHD substantially decreases

thymic output and thus recovery of CD4+ T cells and diversified T cell repertoires (93). Acute GvHD leads to a further skewing of the TCR repertoires of both CD4+ and CD8+ T cells as well as antigen-specific T cells (99). Both T lymphopenia and inadequate repertoire of CD4+ and CD8+ T cells for at least 1 year after transplant foster recurrent infections with latent viruses.

In addition, treatment of patients with acute GvHD with corticosteroids or other immunosuppressive drugs increases the risk of viral reactivations (98, 105). It has been reported that the risk of CMV infections is directly related to the dose and duration of steroid administration (106). Administration of high doses of steroids was shown to be an independent risk factor for impaired functional recovery of CMV-specific CD4+ and CD8+ T cells (106). Moreover, Özdemir and colleagues reported that steroids induced a significant impairment of CD8+ T cells for producing TNF-α (107).

T Cell Depletion of the Stem Cell Graft

Although T cell depletion (TCD) of the stem cell graft reduces GvHD, it is associated with delayed immune reconstitution, infectious complications, and an increased risk of relapse (108). Thus, *ex vivo* T-cell depletion by either CD34+ cell selection or CD3+/CD19+ cell depletion has not been routinely performed and repletion protocols have been widely studied [e.g., HSV-Tk-transduced T cell transfer, other donor lymphocyte infusion-based protocols (109, 110)]. *In vivo* T cell reducing or impairing agents include ATG [e.g., ATG-Fresenius; Germany, or thymoglobulin (thymo); Genzyme; USA] or anti-CD52 antibody (alemtuzumab or campath), a particularly powerful reagent for immunosuppression (108, 111). ATG administration leads to prolonged immunosuppression of both CD4+ T cells and CD4+CD25+CD127− Treg cells (111) and appears to have less severe effect on immune reconstitution when compared to campath (112).

Stem Cell Source

The source of stem cells can impact on both complications as well as time to immune reconstitution after allogeneic HSCT (**Table 2**). Investigators reported that the source of stem cells is a predictive factor for recovery of CMV-specific cytotoxic T lymphocytes (CTL) (10). Recipients of PBSCs had improved functional CMV–CTL recovery and earlier CMV-specific CD4+ T cell reconstitution than patients given BM grafts (106, 113). These findings can be explained by the fact that PBSC grafts compared to BM contain more lymphocytes and higher numbers of CD4+CD45RO+ memory T cells (114).

TABLE 2 | Stem cell source influences immune reconstitution and complications after HSCT.

Complication	PBSCs	BM	CB
aGvHD	++	+	+/−
Infections	+	+	++
Viral reactivations	++	++	+/−
Relapse	+/−	++	++

The table summarizes the influence of different stem cell sources on the immune reconstitution and selected complications after HSCT. The degree of association is indicated by plus (+).

aGvHD, acute graft-versus-host disease; PBSCs, peripheral blood stem cells; BM, bone marrow; CB, cord blood; ++, high; +, moderate; and +/−, low.

Influence of CD4+ and CD8+ T Cells on GvL

Graft-versus-leukemia is defined as an immune response directed against leukemia/tumor cells of the recipient after allogeneic HSCT. Over the years, several studies have shown that CD4+ and CD8+ T cells play an important role in establishing a GvL effect through various mechanisms such as cytotoxic T cells releasing granzyme B and apoptosis mediated by FAS ligands (115). GvL is often associated with GvHD, but GvL responses against, e.g., minor histocompatibility antigens solely expressed on hematopoietic cells (mHA1) may be specifically directed against leukemic cells or the recipients' hematopoietic cells. The precise role of CD4+ and CD8+ T cells for achievement of a GvL effect is not clearly understood today (115, 116). Complete depletion of T cells by CD34+ cell selection leads to a high incidence of relapse, resulting in death in about 20–50% of patients (117). T cell repletion or donor lymphocyte infusions (DLI) can prevent relapse, but can lead to a higher probability of acute and chronic GvHD (118, 119). Several protocols tried to circumvent the problem of increased acute and chronic GvHD by delayed add-back of genetically modified T cells (109) or other manipulations of the donor's lymphocytes such as selection of either CD4+ or CD8+ T cells prior to transfusion (120, 121).

Virus-Specific Immune Reconstitution (Antigen-Specific Cytotoxic T Cells) after HSCT

T cells are the most important effector cells in the control of viral infections. Thus, T cell reconstitution after allogeneic HSCT has a significant impact on the control of infectious complications. The first phase of virus-specific T cell reconstitution and expansion early after HSCT depends on the transfer of mature (effector, memory, or naive) virus-specific T cells within the donor graft and the resident antigen-specific cells (10, 122). Viral infections occur mostly between engraftment and day +90 posttransplant (123). However, also late (after day +90) and recurrent CMV reactivations have been observed, which have been associated with impaired reconstitution or function of antiviral immunity (106). CMV is a latent virus, which belongs to the family of herpesviruses and is one among the common viral pathogens that can reactivate after HSCT. It reactivates in about 60–70% of CMV-seropositive patients, and the primary infection affects 20–30% of CMV seronegative recipients transplanted from CMV-seropositive donors (124). Uncontrolled CMV reactivations can lead to a life-threatening, multi-organ CMV disease such as retinitis, gastroenteritis, or pneumonia (125–127). Advances in CMV monitoring, preemptive antiviral therapy, and quantification of CMV–CTLs are crucial in the prevention of CMV disease (128). The most important risk factors for CMV infection include recipient CMV-seropositivity, TCD of the graft, and acute GvHD (123). Early reconstitution of antiviral immunity remains an essential issue for the control

of CMV reactivations after HSCT. The recovery of both $CD8^+$ and $CD4^+$ CMV-specific T cells may be a marker for protection against CMV reactivations (129).

Epstein–Barr virus infection is also a frequent viral complication after allogeneic HSCT, which may progress to EBV-associated posttransplant lymphoproliferative disease (PTLD) that causes unspecific symptoms such as fever and lymphadenopathy with a high viral load in the PB (130). These complications are mediated by several risk factors including TCD combined with reduced intensity conditioning (RIC) leading to impaired anti-EBV T cell-mediated immunity and persistence of residual recipient B cells (131). In addition, HLA disparity and acute GvHD have also been known to increase the risk of PTLD due to the delayed or impaired specific immune reconstitution (132).

However, other viral pathogens such as adenovirus (ADV), human herpes virus 6 (HHV6), BK-polyoma virus (BKV), and respiratory viruses occur less frequently in adult patients in comparison to CMV and EBV after allogeneic HSCT (133). The control of these viruses again depends upon the reconstitution of antiviral immunity.

Antigen-Specific T Cell Reconstitution and Immunity Against CMV

Among the viruses mentioned above, T cell immune reconstitution against CMV has been studied most intensively and will be described in more detail below, as an example for virus (or any antigen)-specific T cell reconstitution and expansion. Apart from the above mentioned factors influencing T cell reconstitution (TCD of the graft; stem cell source, occurrence of acute or chronic GvHD), CMV serostatus of patient and donor is one of the most important variables influencing CMV-specific T cell immune reconstitution. CMV-seropositive recipients and donors (R+D+) have much faster reconstitution of CMV–CTLs (prior to day +50) and a subclinical CMV reactivation can even boost this development (106). On the other hand, CMV-seropositive recipients transplanted from CMV-seronegative donors (R+D−) lack the protective donor-derived immunity and hence have delayed recovery of antiviral immunity (between days +120 and +150) and a higher risk for recurrent CMV reactivations (134). In **Figure 4**, examples of patients with typical CMV–CTL immune reconstitution for R+D+ and R+D− groups are shown to demonstrate the impact of CMV serostatus on CMV–CTL immune reconstitution. Additionally, it has been shown, that CMV–CTLs of recipient origin can survive the conditioning regimen and can add to the protection against CMV, especially in R+D− patients (135).

Does the Quantity or Quality of CMV-Specific T Cells Matter?

Recent technological developments in cellular immunology have aided in the understanding of antigen-specific T cell responses and the antiviral immunity after HSCT. With the instigation of multimer (e.g., tetramer and streptamer) technology, antigen-specific T cells are readily detected and isolated without stimulation (137–139). In order to study those cell functions, we can choose from a broad variety of assays including cytokine secretion assays, ELISPOT, intracellular staining, which require stimulation of cells with viral lysates, viral proteins, or peptides (137). As for CMV, immunity toward CMV immunodominant epitopes, which include pp65 and IE-1 antigens have been most intensively studied (140, 141).

Initiation of multimer technology allowed the investigation of CMV–CTLs in patients after allogeneic HSCT in order to search for a protective threshold (113, 137, 142). A chronological overview of selected publications on monitoring of CMV-specific T cell responses after HSCT, with the focus on the protective numbers of CMV–CTLs is provided in **Table 3**. It has been shown that the inability to control CMV reactivation following HSCT

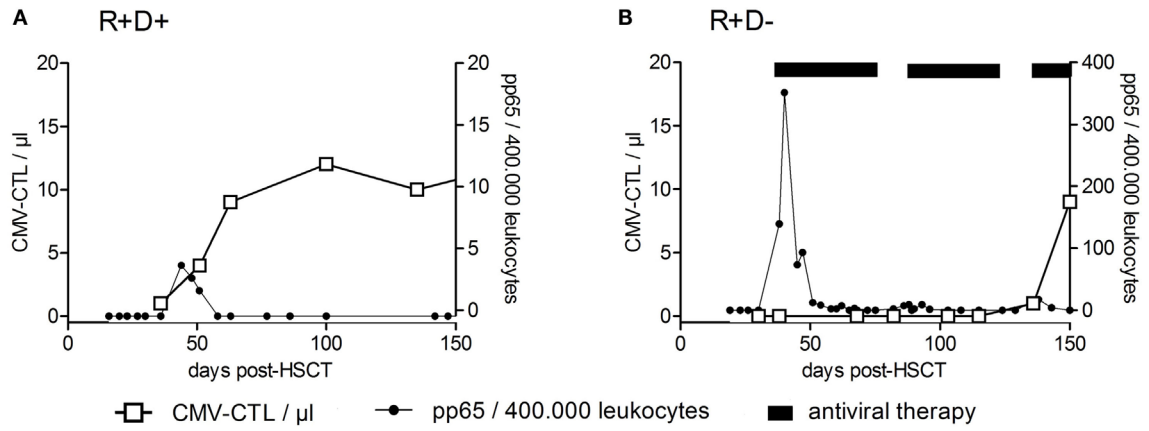

FIGURE 4 | Recovery of CMV-specific cytotoxic T lymphocytes after HSCT. Examples of reconstitution of CMV-specific cytotoxic T lymphocytes (CTLs) after HSCT for CMV-seropositive recipients transplanted from CMV-seropositive donors (R+/D+) **(A)** and CMV-seropositive recipients transplanted from CMV-seronegative donors (R+D−) **(B)** are shown. CMV–CTL numbers per microliter of whole blood (left *y*-axis) were plotted against the time after HSCT (days). The right *y*-axis shows the number of pp65-positive cells/400,000 leukocytes (detection of CMV-reactivation). The CMV R+D+ patient had a CMV-reactivation by day +39 and responded by an expansion of CMV–CTLs. No significant reconstitution of CMV–CTLs within the CMV R+D− patient was detected until day +100 despite the early CMV reactivation. Adapted from Ref. (136).

TABLE 3 | Selected publications on monitoring of CMV-specific T cell responses after HSCT.

Reference	Key information
Altman et al. (146)	First use of MHC tetramers to enumerate and characterize antigen-specific T cells
Cwynarski et al. (113)	Protection from CMV reactivation with ≥10 CMV–CTL cells/μL blood
Gratama et al. (142)	(1) Failure to recover HLA-A*02-NLV–CMV–CTLs is associated with the development of CMV disease (2) Number of HLA-A*02-NLV–CMV–CTLs in the grafts administered to CMV-seropositive HSCT recipients is inversely correlated with the number of recurrent CMV infections
Aubert et al. (147)	Less than 20 cells/μL of HLA-A*02 CMV–CTLs predicted episodes of viral replication
Chen et al. (148)	More than 10–20 cells/μL CMV–CTLs conferred protection against CMV reactivation
Özdemir et al. (107)	Inability to control CMV reactivation is caused by impaired function of CMV–CTLs rather than an inability to recover sufficient numbers of CMV-specific T cells
Lacey et al. (149)	CMV-specific cellular immune responses restricted by HLA-B*07 dominated those restricted by HLA-A*02
Akiyama et al. (150)	Frequency of HLA-A*24 CMVpp65 tetramer-positive staining correlated with cytotoxicity and IFN-γ production
Bunde et al. (151)	High frequencies of IFN-γ producing IE-1, but not pp65-specific $CD8^+$ T cells, correlated with protection from CMV disease
Lilleri et al. (152)	Levels of $CD4^+$ T cells below 1 cell/μL and of $CD8^+$ T cells less than 3 cells/μL did not protect against recurrent CMV infection
Gratama et al. (153)	(1) CMV–CTLs provided protection against recurrent CMV reactivations (2) CMV disease appeared to be prevented by the IE-1-specific subset rather than the pp65-specific $CD8^+$ T cell subset
Koehl et al. (154)	(1) Numbers of CMV–CTLs differ significantly depending on the HLA type (2) Number of CMV–CTLs below 10 cells/μL does not correlate with susceptibility for CMV reactivation
Giest et al. (155)	HLA-A*24/pp65- and HLA-B*35/pp65-CTLs correlated with protection from CMV reactivation at significantly lower cell levels than HLA-A*01/pp50- and HLA-A*02/pp65-CTLs
Gratama, et al. (156)	Less than 7 cells/μL of CMV–CTLs during the first 65 days after transplantation was a significant risk factor for CMV-related complications
Borchers et al. (134)	(1) Presence of CMV–CTLs before day +50 and their expansion after reactivation protected against recurrent CMV reactivations (2) CMV–CTL reconstitution was delayed in the CMV R+D− group
Lilleri et al. (157)	Combination of CMV–CTL monitoring and viral monitoring can be used to direct preemptive treatment with antiviral drugs
Borchers et al. (136)	(1) 1 cell/μL of CMV–CTLs between days +50 and +75 marked the beginning of immune response against CMV in the CMV R+D+ group (2) Expansion of CMV (3) Sequential monitoring of CMV

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MHC, major histocompatibility complex; CMV–CTL, cytomegalovirus cytotoxic T lymphocytes; HLA, human leukocyte antigen; IFNγ, interferon gamma; IE-1, immediate early-1.

is due to the impaired function of antigen-specific $CD8^+$ T cells rather than an inability to recover sufficient numbers of CMV-specific T cells (143). Although CMV–$CD8^+$ CTLs have been considered as the main antiviral effector cells, CMV-specific $CD4^+$ T cells have been shown to play a crucial role in expansion and activation of CMV–CTLs, maintaining a long-term and efficient immunity against CMV (129). It has been reported that $CD4^+$ and CD8+ CMV-specific T cells follow similar patterns of reconstitution (144), and their functional reconstitution is correlated with the absolute $CD4^+$ or $CD8^+$ T cell numbers (106, 145). So far, there is no threshold for protective levels of CMV-specific T cells applicable for all patients. Sequential monitoring of individual patients for the kinetics of CMV–CTL recovers, their ability to produce cytokines and expansion upon reactivation, are currently being used to detect recurrent CMV reactivations (136) (**Figure 4**). In summary, both the quantity and quality of immune reconstitution are important for preventing viral infection after allogeneic HSCT.

CONCLUSION AND FUTURE OUTLOOK

Reconstitution of the donor-derived immune system is essential for control of infectious complications, modulation of GvHD, and relapse control, thus contributing to long-term survival. In this review, we have described the major events in immune cell reconstitution, considering the most important cell types, their approximate time of reconstitution, and their interaction after HSCT. The recovery of the innate immunity is vital, especially in the absence of $CD4^+CD45RO^+$ memory and $CD4^+CD45RA^+$ naive T cells.

Today, the understanding of $CD4^+CD25^+CD127^-$ regulatory T cells has advanced significantly in both preclinical and clinical models for GvHD. The remaining challenge is to generate large amounts of $CD4^+CD25^+CD127^-$ Tregs with high purity and stable Foxp3-expression in a cost effective way. A further clinical problem is the optimal time point of Treg application. If Tregs are administered to treat patients with steroid-refractory GvHD, there may be a substantial delay between production and application and, thus, lack of feasibility and treatment success. Furthermore, the impact of ongoing systemic immunosuppression on Treg cell function has to be considered in clinical trials. A further aspect to be solved in the future is to optimize tissue conditions for survival and expansion of T regs, as these cells are under the strong control of local microbiota especially in the main target tissues of GvHD.

AUTHOR CONTRIBUTIONS

EW, EH, and HG designed the review and revised it critically for important intellectual content. JO, MKJ, SG, and PV provided the draft, summarized available data, and selected the references. All the authors approved the final version of the manuscript.

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REFERENCES

1. Seggewiss R, Einsele H. Immune reconstitution after allogeneic transplantation and expanding options for immunomodulation: an update. *Blood* (2010) 115(19):3861–8. doi:10.1182/blood-2009-12-234096
2. Danby R, Rocha V. Improving engraftment and immune reconstitution in umbilical cord blood transplantation. *Front Immunol* (2014) 5:68. doi:10.3389/fimmu.2014.00068
3. Martin PS, Li S, Nikiforow S, Alyea EP, Antin JH, Armand P, et al. Infused total nucleated cell dose is a better predictor of transplant outcomes than CD34(+) cell number in reduced-intensity mobilized peripheral blood allogeneic hematopoietic cell transplantation. *Haematologica* (2016) 101(4):499–505. doi:10.3324/haematol.2015.134841
4. Remberger M, Törlén J, Ringdén O, Engström M, Watz E, Uhlin M, et al. Effect of total nucleated and CD34+ cell dose on outcome after allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant* (2015) 21(5):889–93. doi:10.1016/j.bbmt.2015.01.025
5. Fry TJ, Mackall CL. Immune reconstitution following hematopoietic progenitor cell transplantation: challenges for the future. *Bone Marrow Transplant* (2005) 35(Suppl 1):S53–7. doi:10.1038/sj.bmt.1704848
6. Moss P, Rickinson A. Cellular immunotherapy for viral infection after HSC transplantation. *Nat Rev Immunol* (2005) 5(1):9–20. doi:10.1038/nri1526
7. Chatzidimitriou D, Gavriilaki E, Sakellari I, Diza E. Hematopoietic cell transplantation and emerging viral infections. *J Med Virol* (2010) 82(3):528–38. doi:10.1002/jmv.21696
8. Gennery AR, Maggina P. Infection following haematopoietic stem cell transplantation. *Paediatr Child Health* (2014) 24(6):236–41. doi:10.1016/j.paed.2013.11.005
9. Huttunen P, Taskinen M, Siitonen S, Saarinen-Pihkala UM. Impact of very early CD4+/CD8+ T cell counts on the occurrence of acute graft-versus-host disease and NK cell counts on outcome after pediatric allogeneic hematopoietic stem cell transplantation. *Pediatr Blood Cancer* (2015) 62(3):522–8. doi:10.1002/pbc.25347
10. Luo X-H, Chang Y-J, Huang X-J. Improving cytomegalovirus-specific T cell reconstitution after haploidentical stem cell transplantation. *J Immunol Res* (2014) 2014:12. doi:10.1155/2014/631951
11. Toubert A, Glauzy S, Douay C, Clave E. Thymus and immune reconstitution after allogeneic hematopoietic stem cell transplantation in humans: never say never again. *Tissue Antigens* (2012) 79(2):83–9. doi:10.1111/j.1399-0039.2011.01820.x
12. Edinger M, Hoffmann P. Regulatory T cells in stem cell transplantation: strategies and first clinical experiences. *Curr Opin Immunol* (2011) 23(5):679–84. doi:10.1016/j.coi.2011.06.006
13. Di Ianni M, Falzetti F, Carotti A, Terenzi A, Castellino F, Bonifacio E, et al. Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. *Blood* (2011) 117(14):3921–8. doi:10.1182/blood-2010-10-311894
14. Edinger M, Hoffmann P, Ermann J, Drago K, Fathman CG, Strober S, et al. CD4+ CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. *Nat Med* (2003) 9(9):1144–50. doi:10.1038/nm915
15. Brunstein CG, Miller JS, Cao Q, McKenna DH, Hippen KL, Curtsinger J, et al. Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. *Blood* (2011) 117(3):1061–70. doi:10.1182/blood-2010-07-293795
16. Hakim FT, Memon SA, Cepeda R, Jones EC, Chow CK, Kasten-Sportes C, et al. Age-dependent incidence, time course, and consequences of thymic renewal in adults. *J Clin Invest* (2005) 115(4):930–9. doi:10.1172/JCI200522492
17. Bolotin E, Smogorzewska M, Smith S, Widmer M, Weinberg K. Enhancement of thymopoiesis after bone marrow transplant by in vivo interleukin-7. *Blood* (1996) 88(5):1887–94.
18. Guimond M, Fry TJ, Mackall CL. Cytokine signals in T-cell homeostasis. *J Immunother* (2005) 28(4):289–94. doi:10.1097/01.cji.0000165356.03924.e7
19. van Heijst JWJ, Ceberio I, Lipuma LB, Samilo DW, Wasilewski GD, Gonzales AMR, et al. Quantitative assessment of T-cell repertoire recovery after hematopoietic stem cell transplantation. *Nat Med* (2013) 19(3):372–7. doi:10.1038/nm.3100
20. Yew PY, Alachkar H, Yamaguchi R, Kiyotani K, Fang H, Yap KL, et al. Quantitative characterization of T-cell repertoire in allogeneic hematopoietic stem cell transplant recipients. *Bone Marrow Transplant* (2015) 50(9):1227–34. doi:10.1038/bmt.2015.133
21. Storek J, Joseph A, Dawson MA, Douek DC, Storer B, Maloney DG. Factors influencing T-lymphopoiesis after allogeneic hematopoietic cell transplantation. *Transplantation* (2002) 73(7):1154–8. doi:10.1097/00007890-200204150-00026
22. Storek J, Geddes M, Khan F, Huard B, Helg C, Chalandon Y, et al. Reconstitution of the immune system after hematopoietic stem cell transplantation in humans. *Semin Immunopathol* (2008) 30(4):425–37. doi:10.1007/s00281-008-0132-5
23. Marie-Cardine A, Divay F, Dutot I, Green A, Perdrix A, Boyer O, et al. Transitional B cells in humans: characterization and insight from B lymphocyte reconstitution after hematopoietic stem cell transplantation. *Clin Immunol* (2008) 127(1):14–25. doi:10.1016/j.clim.2007.11.013
24. D'Orsogna LJ, Wright MP, Krueger RG, McKinnon EJ, Buffery SI, Witt CS, et al. Allogeneic hematopoietic stem cell transplantation recipients have defects of both switched and igm memory B cells. *Biol Blood Marrow Transplant* (2009) 15(7):795–803. doi:10.1016/j.bbmt.2008.11.024
25. Park BG, Park CJ, Jang S, Chi HS, Kim DY, Lee JH, et al. Reconstitution of lymphocyte subpopulations after hematopoietic stem cell transplantation: comparison of hematologic malignancies and donor types in event-free patients. *Leuk Res* (2015) 39(12):1334–41. doi:10.1016/j.leukres.2015.09.010
26. Williams KM, Gress RE. Immune reconstitution and implications for immunotherapy following haematopoietic stem cell transplantation. *Best Pract Res Clin Haematol* (2008) 21(3):579–96. doi:10.1016/j.beha.2008.06.003
27. Suryani S, Fulcher DA, Santner-Nanan B, Nanan R, Wong M, Shaw PJ, et al. Differential expression of CD21 identifies developmentally and functionally distinct subsets of human transitional B cells. *Blood* (2010) 115(3):519–29. doi:10.1182/blood-2009-07-234799
28. Mackall C, Fry T, Gress R, Peggs K, Storek J, Toubert A. Background to hematopoietic cell transplantation, including post transplant immune recovery. *Bone Marrow Transplant* (2009) 44(8):457–62. doi:10.1038/bmt.2009.255
29. Corre E, Carmagnat M, Busson M, de Latour RP, Robin M, Ribaud P, et al. Long-term immune deficiency after allogeneic stem cell transplantation: B-cell deficiency is associated with late infections. *Haematologica* (2010) 95(6):1025–9. doi:10.3324/haematol.2009.018853
30. Storek J, Saxon A. Reconstitution of B cell immunity following bone marrow transplantation. *Bone Marrow Transplant* (1992) 9(6):395–408.
31. Storek J, Ferrara S, Ku N, Giorgi JV, Champlin RE, Saxon A. B cell reconstitution after human bone marrow transplantation: recapitulation of ontogeny? *Bone Marrow Transplant* (1993) 12(4):387–98.
32. Moins-Teisserenc H, Busson M, Herda A, Apete S, Peffault de Latour R, Robin M, et al. CD19+CD5+ B cells and B1-like cells following allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant* (2013) 19(6):988–91. doi:10.1016/j.bbmt.2013.03.006
33. Lee J, Kuchen S, Fischer R, Chang S, Lipsky PE. Identification and characterization of a human CD5+ pre-naive B cell population. *J Immunol* (2009) 182(7):4116–26. doi:10.4049/jimmunol.0803391

34. Glas AM, van Montfort EH, Storek J, Green EG, Drissen RP, Bechtold VJ, et al. B-cell-autonomous somatic mutation deficit following bone marrow transplant. *Blood* (2000) 96(3):1064–9.
35. Omazic B, Lundkvist I, Mattsson J, Permert J, Nasman-Bjork I. Memory B lymphocytes determine repertoire oligoclonality early after haematopoietic stem cell transplantation. *Clin Exp Immunol* (2003) 134(1):159–66. doi:10.1046/j.1365-2249.2003.02260.x
36. Small TN, Robinson WH, Miklos DB. B cells and transplantation: an educational resource. *Biol Blood Marrow Transplant* (2009) 15(1 Suppl):104–13. doi:10.1016/j.bbmt.2008.10.016
37. Bemark M, Holmqvist J, Abrahamsson J, Mellgren K. Translational mini-review series on B cell subsets in disease. Reconstitution after haematopoietic stem cell transplantation – revelation of B cell developmental pathways and lineage phenotypes. *Clin Exp Immunol* (2012) 167(1):15–25. doi:10.1111/j.1365-2249.2011.04469.x
38. Aucouturier P, Barra A, Intrator L, Cordonnier C, Schulz D, Duarte F, et al. Long lasting IgG subclass and antibacterial polysaccharide antibody deficiency after allogeneic bone marrow transplantation. *Blood* (1987) 70(3):779–85.
39. Nasman-Bjork I, Lundkvist I. Oligoclonal dominance of immunoglobulin VH3 rearrangements following allogeneic bone marrow transplantation. *Bone Marrow Transplant* (1998) 21(12):1223–30. doi:10.1038/sj.bmt.1701261
40. Roll P, Muhammad K, Stuhler G, Grigoleit U, Einsele H, Tony HP. Effect of ATG-F on B-cell reconstitution after hematopoietic stem cell transplantation. *Eur J Haematol* (2015) 95(6):514–23. doi:10.1111/ejh.12524
41. Fuji S, Kim SW, Yano S, Hagiwara S, Nakamae H, Hidaka M, et al. A prospective multicenter study of unrelated bone marrow transplants using a reduced-intensity conditioning regimen with low-dose ATG-F. *Bone Marrow Transplant* (2015) 51(3):451–3. doi:10.1038/bmt.2015.268
42. Zand MS. B-cell activity of polyclonal antithymocyte globulins. *Transplantation* (2006) 82(11):1387–95. doi:10.1097/01.tp.0000244063.05338.27
43. Petersen SL, Ryder LP, Bjork P, Madsen HO, Heilmann C, Jacobsen N, et al. A comparison of T-, B- and NK-cell reconstitution following conventional or nonmyeloablative conditioning and transplantation with bone marrow or peripheral blood stem cells from human leucocyte antigen identical sibling donors. *Bone Marrow Transplant* (2003) 32(1):65–72. doi:10.1038/sj.bmt.1704084
44. Storek J, Wells D, Dawson MA, Storer B, Maloney DG. Factors influencing B lymphopoiesis after allogeneic hematopoietic cell transplantation. *Blood* (2001) 98(2):489–91. doi:10.1182/blood.V98.2.489
45. Greinix HT, Pohlreich D, Kouba M, Kormoczi U, Lohmann I, Feldmann K, et al. Elevated numbers of immature/transitional CD21- B lymphocytes and deficiency of memory CD27+ B cells identify patients with active chronic graft-versus-host disease. *Biol Blood Marrow Transplant* (2008) 14(2):208–19. doi:10.1016/j.bbmt.2007.10.009
46. Greinix HT, Kuzmina Z, Weigl R, Kormoczi U, Rottal A, Wolff D, et al. CD19+CD21low B cells and CD4+CD45RA+CD31+ T cells correlate with first diagnosis of chronic graft-versus-host disease. *Biol Blood Marrow Transplant* (2015) 21(2):250–8. doi:10.1016/j.bbmt.2014.11.010
47. Storek J, Witherspoon RP, Maloney DG, Chauncey TR, Storb R. Improved reconstitution of CD4 T cells and B cells but worsened reconstitution of serum IgG levels after allogeneic transplantation of blood stem cells instead of marrow. *Blood* (1997) 89(10):3891–3.
48. Storek J, Dawson MA, Storer B, Stevens-Ayers T, Maloney DG, Marr KA, et al. Immune reconstitution after allogeneic marrow transplantation compared with blood stem cell transplantation. *Blood* (2001) 97(11):3380–9. doi:10.1182/blood.V97.11.3380
49. Fontenot JD, Rudensky AY. A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3. *Nat Immunol* (2005) 6(4):331–7. doi:10.1038/ni1179
50. Feuerer M, Hill JA, Mathis D, Benoist C. Foxp3+ regulatory T cells: differentiation, specification, subphenotypes. *Nat Immunol* (2009) 10(7):689–95. doi:10.1038/ni.1760
51. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell* (2008) 133(5):775–87. doi:10.1016/j.cell.2008.05.009
52. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* (2003) 299(5609):1057–61. doi:10.1126/science.1079490
53. Cobbold SP, Castejon R, Adams E, Zelenika D, Graca L, Humm S, et al. Induction of foxP3+ regulatory T cells in the periphery of T cell receptor transgenic mice tolerized to transplants. *J Immunol* (2004) 172(10):6003–10. doi:10.4049/jimmunol.172.10.6003
54. Baron U, Floess S, Wieczorek G, Baumann K, Grützkau A, Dong J, et al. DNA demethylation in the human FOXP3 locus discriminates regulatory T cells from activated FOXP3+ conventional T cells. *Eur J Immunol* (2007) 37(9):2378–89. doi:10.1002/eji.200737594
55. Zheng Y, Josefowicz S, Chaudhry A, Peng XP, Forbush K, Rudensky AY. Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. *Nature* (2010) 463(7282):808–12. doi:10.1038/nature08750
56. Maloy KJ, Powrie F. Regulatory T cells in the control of immune pathology. *Nat Immunol* (2001) 2(9):816–22. doi:10.1038/ni0901-816
57. Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. *Nat Rev Immunol* (2008) 8(7):523–32. doi:10.1038/nri2343
58. Campbell DJ, Koch MA. Phenotypical and functional specialization of FOXP3+ regulatory T cells. *Nat Rev Immunol* (2011) 11(2):119–30. doi:10.1038/nri2916
59. Kehrl JH, Wakefield LM, Roberts AB, Jakowlew S, Alvarez-Mon M, Derynck R, et al. Production of transforming growth factor beta by human T lymphocytes and its potential role in the regulation of T cell growth. *J Exp Med* (1986) 163(5):1037–50. doi:10.1084/jem.163.5.1037
60. Haak-Frendscho M, Wynn T, Czuprynski C, Paulnock D. Transforming growth factor-beta 1 inhibits activation of macrophage cell line RAW 264.7 for cell killing. *Clin Exp Immunol* (1990) 82(2):404. doi:10.1111/j.1365-2249.1990.tb05461.x
61. Belkaid Y, Rouse BT. Natural regulatory T cells in infectious disease. *Nat Immunol* (2005) 6(4):353–60. doi:10.1038/ni1181
62. Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, Liu H, et al. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature* (2013) 504(7480):451–5. doi:10.1038/nature12726
63. Mathewson ND, Jenq R, Mathew AV, Koenigsknecht M, Hanash A, Toubai T, et al. Gut microbiome-derived metabolites modulate intestinal epithelial cell damage and mitigate graft-versus-host disease. *Nat Immunol* (2016) 17(5):505–13. doi:10.1038/ni.3400
64. Shevach EM. CD4+ CD25+ suppressor T cells: more questions than answers. *Nat Rev Immunol* (2002) 2(6):389–400. doi:10.1038/nri821
65. Kingsley CI, Karim M, Bushell AR, Wood KJ. CD25+ CD4+ regulatory T cells prevent graft rejection: CTLA-4-and IL-10-dependent immunoregulation of alloresponses. *J Immunol* (2002) 168(3):1080–6. doi:10.4049/jimmunol.168.3.1080
66. Joffre O, Gorsse N, Romagnoli P, Hudrisier D, Van Meerwijk JP. Induction of antigen-specific tolerance to bone marrow allografts with CD4+ CD25+ T lymphocytes. *Blood* (2004) 103(11):4216–21. doi:10.1182/blood-2004-01-0005
67. Ferrara JL, Levine JE, Reddy P, Holler E. Graft-versus-host disease. *Lancet* (2009) 373(9674):1550–61. doi:10.1016/S0140-6736(09)60237-3
68. Cohen JL, Boyer O. The role of CD4+ CD25 hi regulatory T cells in the physiopathogeny of graft-versus-host disease. *Curr Opin Immunol* (2006) 18(5):580–5. doi:10.1016/j.coi.2006.07.007
69. Nguyen VH, Shashidhar S, Chang DS, Ho L, Kambham N, Bachmann M, et al. The impact of regulatory T cells on T-cell immunity following hematopoietic cell transplantation. *Blood* (2008) 111(2):945–53. doi:10.1182/blood-2007-07-103895
70. Taylor PA, Lees CJ, Blazar BR. The infusion of ex vivo activated and expanded CD4+ CD25+ immune regulatory cells inhibits graft-versus-host disease lethality. *Blood* (2002) 99(10):3493–9. doi:10.1182/blood.V99.10.3493
71. Fantini MC, Becker C, Monteleone G, Pallone F, Galle PR, Neurath MF. Cutting edge: TGF-β induces a regulatory phenotype in CD4+ CD25− T cells through Foxp3 induction and down-regulation of Smad7. *J Immunol* (2004) 172(9):5149–53. doi:10.4049/jimmunol.172.9.5149
72. Koenecke C, Czeloth N, Bubke A, Schmitz S, Kissenpfennig A, Malissen B, et al. Alloantigen-specific de novo-induced Foxp3+ Treg revert in vivo and do not protect from experimental GVHD. *Eur J Immunol* (2009) 39(11):3091–6. doi:10.1002/eji.200939432
73. Laurence A, Amarnath S, Mariotti J, Kim YC, Foley J, Eckhaus M, et al. STAT3 transcription factor promotes instability of nTreg cells and limits generation

of iTreg cells during acute murine graft-versus-host disease. *Immunity* (2012) 37(2):209–22. doi:10.1016/j.immuni.2012.05.027
74. Ligocki AJ, Niederkorn JY. Advances on non-CD4 + Foxp3+ T regulatory cells: CD8+, type 1, and double negative t regulatory cells in organ transplantation. *Transplantation* (2015) 99(8):1553–9. doi:10.1097/TP.0000000000000813
75. Robb RJ, Lineburg KE, Kuns RD, Wilson YA, Raffelt NC, Olver SD, et al. Identification and expansion of highly suppressive CD8+ FoxP3+ regulatory T cells after experimental allogeneic bone marrow transplantation. *Blood* (2012) 119(24):5898–908. doi:10.1182/blood-2011-12-396119
76. Xystrakis E, Dejean AS, Bernard I, Druet P, Liblau R, Gonzalez-Dunia D, et al. Identification of a novel natural regulatory CD8 T-cell subset and analysis of its mechanism of regulation. *Blood* (2004) 104(10):3294–301. doi:10.1182/blood-2004-03-1214
77. Zheng J, Liu Y, Liu Y, Liu M, Xiang Z, Lam K-T, et al. Human CD8+ regulatory T cells inhibit GVHD and preserve general immunity in humanized mice. *Sci Transl Med* (2013) 5(168):ra169. doi:10.1126/scitranslmed.3004943
78. Li Q, Zhai Z, Xu X, Shen Y, Zhang A, Sun Z, et al. Decrease of CD4+ CD25+ regulatory T cells and TGF-β at early immune reconstitution is associated to the onset and severity of graft-versus-host disease following allogeneic haematogenesis stem cell transplantation. *Leuk Res* (2010) 34(9):1158–68. doi:10.1016/j.leukres.2010.03.017
79. Rieger K, Loddenkemper C, Maul J, Fietz T, Wolff D, Terpe H, et al. Mucosal FOXP3+ regulatory T cells are numerically deficient in acute and chronic GvHD. *Blood* (2006) 107(4):1717–23. doi:10.1182/blood-2005-06-2529
80. Lord JD, Hackman RC, Gooley TA, Wood BL, Moklebust AC, Hockenbery DM, et al. Blood and gastric FOXP3+ T cells are not decreased in human gastric graft-versus-host disease. *Biol Blood Marrow Transplant* (2011) 17(4):486–96. doi:10.1016/j.bbmt.2010.09.015
81. Ratajczak P, Janin A, De Latour RP, Leboeuf C, Desveaux A, Keyvanfar K, et al. Th17/Treg ratio in human graft-versus-host disease. *Blood* (2010) 116(7):1165–71. doi:10.1182/blood-2009-12-255810
82. Imanguli M, Cowen E, Rose J, Dhamala S, Swaim W, Lafond S, et al. Comparative analysis of FoxP3(+); regulatory T cells in the target tissues and blood in chronic graft versus host disease. *Leukemia* (2014) 28(10):2016–27. doi:10.1038/leu.2014.92
83. Trzonkowski P, Bieniaszewska M, Juścińska J, Dobyszuk A, Krzystyniak A, Marek N, et al. First-in-man clinical results of the treatment of patients with graft versus host disease with human ex vivo expanded CD4+ CD25+ CD127− T regulatory cells. *Clin Immunol* (2009) 133(1):22–6. doi:10.1016/j.clim.2009.06.001
84. Hoffmann P, Eder R, Kunz-Schughart LA, Andreesen R, Edinger M. Large-scale in vitro expansion of polyclonal human CD4+ CD25high regulatory T cells. *Blood* (2004) 104(3):895–903. doi:10.1182/blood-2004-01-0086
85. Strauss L, Whiteside TL, Knights A, Bergmann C, Knuth A, Zippelius A. Selective survival of naturally occurring human CD4+ CD25+ Foxp3+ regulatory T cells cultured with rapamycin. *J Immunol* (2007) 178(1):320–9. doi:10.4049/jimmunol.178.1.320
86. Hippen K, Merkel S, Schirm D, Nelson C, Tennis N, Riley J, et al. Generation and large-scale expansion of human inducible regulatory T cells that suppress graft-versus-host disease. *Am J Transplant* (2011) 11(6):1148–57. doi:10.1111/j.1600-6143.2011.03558.x
87. Matsuoka K-I, Koreth J, Kim HT, Bascug G, McDonough S, Kawano Y, et al. Low-dose interleukin-2 therapy restores regulatory T cell homeostasis in patients with chronic graft-versus-host disease. *Sci Transl Med* (2013) 5(179):ra143–79. doi:10.1126/scitranslmed.3005265
88. Koreth J, Matsuoka K, Kim HT, McDonough SM, Bindra B, Alyea EP III, et al. Interleukin-2 and regulatory T cells in graft-versus-host disease. *N Engl J Med* (2011) 365(22):2055–66. doi:10.1056/NEJMoa1108188
89. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, et al. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature* (2013) 504(7480):446–50. doi:10.1038/nature12721
90. Mahnke YD, Brodie TM, Sallusto F, Roederer M, Lugli E. The who's who of T-cell differentiation: human memory T-cell subsets. *Eur J Immunol* (2013) 43(11):2797–809. doi:10.1002/eji.201343751
91. von Andrian UH, Mempel TR. Homing and cellular traffic in lymph nodes. *Nat Rev Immunol* (2003) 3(11):867–78. doi:10.1038/nri1222
92. Smith CJ, Quinn M, Snyder CM. CMV-specific CD8 T cell differentiation and localization: implications for adoptive therapies. *Front Immunol* (2016) 7:352. doi:10.3389/fimmu.2016.00352
93. Clave E, Busson M, Douay C, Peffault de Latour R, Berrou J, Rabian C, et al. Acute graft-versus-host disease transiently impairs thymic output in young patients after allogeneic hematopoietic stem cell transplantation. *Blood* (2009) 113(25):6477–84. doi:10.1182/blood-2008-09-176594
94. Fujimaki K, Maruta A, Yoshida M, Kodama F, Matsuzaki M, Fujisawa S, et al. Immune reconstitution assessed during five years after allogeneic bone marrow transplantation. *Bone Marrow Transplant* (2001) 27(12):1275–81. doi:10.1038/sj.bmt.1703056
95. Le RQ, Melenhorst JJ, Battiwalla M, Hill B, Memon S, Savani BN, et al. Evolution of the donor T-cell repertoire in recipients in the second decade after allogeneic stem cell transplantation. *Blood* (2011) 117(19):5250–6. doi:10.1182/blood-2011-01-329706
96. Heining C, Spyridonidis A, Bernhardt E, Schulte-Monting J, Behringer D, Grullich C, et al. Lymphocyte reconstitution following allogeneic hematopoietic stem cell transplantation: a retrospective study including 148 patients. *Bone Marrow Transplant* (2007) 39(10):613–22. doi:10.1038/sj.bmt.1705648
97. Jacobsohn DA, Vogelsang GB. Acute graft versus host disease. *Orphanet J Rare Dis* (2007) 2:35. doi:10.1186/1750-1172-2-35
98. Cantoni N, Hirsch HH, Khanna N, Gerull S, Buser A, Bucher C, et al. Evidence for a bidirectional relationship between cytomegalovirus replication and acute graft-versus-host disease. *Biol Blood Marrow Transplant* (2010) 16(9):1309–14. doi:10.1016/j.bbmt.2010.03.020
99. Auletta JJ, Lazarus HM. Immune restoration following hematopoietic stem cell transplantation: an evolving target. *Bone Marrow Transplant* (2005) 35(9):835–57. doi:10.1038/sj.bmt.1704966
100. Blazar BR, Murphy WJ, Abedi M. Advances in graft-versus-host disease biology and therapy. *Nat Rev Immunol* (2012) 12(6):443–58. doi:10.1038/nri3212
101. Ferrara JL, Cooke KR, Teshima T. The pathophysiology of acute graft-versus-host disease. *Int J Hematol* (2003) 78(3):181–7. doi:10.1007/BF02983793
102. Szyska M, Na I-K. Bone marrow GvHD after allogeneic hematopoietic stem cell transplantation. *Front Immunol* (2016) 7:118. doi:10.3389/fimmu.2016.00118
103. Shono Y, Ueha S, Wang Y, Abe J, Kurachi M, Matsuno Y, et al. Bone marrow graft-versus-host disease: early destruction of hematopoietic niche after MHC-mismatched hematopoietic stem cell transplantation. *Blood* (2010) 115(26):5401–11. doi:10.1182/blood-2009-11-253559
104. Müller AMS, Linderman JA, Florek M, Miklos D, Shizuru JA. Allogeneic T cells impair engraftment and hematopoiesis after stem cell transplantation. *Proc Natl Acad Sci U S A* (2010) 107(33):14721–6. doi:10.1073/pnas.1009220107
105. McCarthy AL, Malik Peiris JS, Taylor CE, Green MA, Sviland L, Pearson AD, et al. Increase in severity of graft versus host disease by cytomegalovirus. *J Clin Pathol* (1992) 45(6):542–4. doi:10.1136/jcp.45.6.542
106. Hakki M, Riddell SR, Storek J, Carter RA, Stevens-Ayers T, Sudour P, et al. Immune reconstitution to cytomegalovirus after allogeneic hematopoietic stem cell transplantation: impact of host factors, drug therapy, and subclinical reactivation. *Blood* (2003) 102(8):3060–7. doi:10.1182/blood-2002-11-3472
107. Özdemir E, St. John LS, Gillespie G, Rowland-Jones S, Champlin RE, Molldrem JJ, et al. Cytomegalovirus reactivation following allogeneic stem cell transplantation is associated with the presence of dysfunctional antigen-specific CD8+ T cells. *Blood* (2002) 100(10):3690–7. doi:10.1182/blood-2002-05-1387
108. Marek A, Stern M, Chalandon Y, Ansari M, Ozsahin H, Gungor T, et al. The impact of T-cell depletion techniques on the outcome after haploidentical hematopoietic SCT. *Bone Marrow Transplant* (2014) 49(1):55–61. doi:10.1038/bmt.2013.132
109. Ciceri F, Bonini C, Stanghellini MT, Bondanza A, Traversari C, Salomoni M, et al. Infusion of suicide-gene-engineered donor lymphocytes after family haploidentical haemopoietic stem-cell transplantation for leukaemia (the TK007 trial): a non-randomised phase I-II study. *Lancet Oncol* (2009) 10(5):489–500. doi:10.1016/S1470-2045(09)70074-9
110. Weissinger EM, Borchers S, Silvani A, Provasi E, Radrizzani M, Beckmann IK, et al. Long term follow up of patients after allogeneic stem cell transplantation

and transfusion of HSV-TK transduced T-cells. *Front Pharmacol* (2015) 6:76. doi:10.3389/fphar.2015.00076

111. Na I-K, Wittenbecher F, Dziubianau M, Herholz A, Mensen A, Kunkel D, et al. Rabbit antithymocyte globulin (Thymoglobulin(®)) impairs the thymic output of both conventional and regulatory CD4(+) T cells after allogeneic hematopoietic stem cell transplantation in adult patients. *Haematologica* (2013) 98(1):23–30. doi:10.3324/haematol.2012.067611
112. Juliusson G, Theorin N, Karlsson K, Frodin U, Malm C. Subcutaneous alemtuzumab vs ATG in adjusted conditioning for allogeneic transplantation: influence of Campath dose on lymphoid recovery, mixed chimerism and survival. *Bone Marrow Transplant* (2006) 37(5):503–10. doi:10.1038/sj.bmt.1705263
113. Cwynarski K, Ainsworth J, Cobbold M, Wagner S, Mahendra P, Apperley J, et al. Direct visualization of cytomegalovirus-specific T-cell reconstitution after allogeneic stem cell transplantation. *Blood* (2001) 97(5):1232–40. doi:10.1182/blood.V97.5.1232
114. Jansen J, Hanks S, Thompson JM, Dugan MJ, Akard LP. Transplantation of hematopoietic stem cells from the peripheral blood. *J Cell Mol Med* (2005) 9(1):37–50. doi:10.1111/j.1582-4934.2005.tb00335.x
115. Barrett AJ. Mechanisms of the graft-versus-leukemia reaction. *Stem Cells* (1997) 15(4):248–58. doi:10.1002/stem.150248
116. Chakraverty R, Eom H-S, Sachs J, Buchli J, Cotter P, Hsu R, et al. Host MHC class II+ antigen-presenting cells and CD4 cells are required for CD8-mediated graft-versus-leukemia responses following delayed donor leukocyte infusions. *Blood* (2006) 108(6):2106–13. doi:10.1182/blood-2006-03-007427
117. Nikiforow S, Alyea EP. Maximizing GVL in allogeneic transplantation: role of donor lymphocyte infusions. *Hematology Am Soc Hematol Educ Program* (2014) 2014(1):570–5. doi:10.1182/asheducation-2014.1.570
118. Kolb H, Schattenberg A, Goldman J, Hertenstein B, Jacobsen N, Arcese W, et al. Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. European group for blood and marrow transplantation working party chronic leukemia. *Blood* (1995) 86(5):2041–50.
119. Kolb H-J, Schmid C, Barrett AJ, Schendel DJ. Graft-versus-leukemia reactions in allogeneic chimeras. *Blood* (2004) 103(3):767–76. doi:10.1182/blood-2003-02-0342
120. Meyer RG, Britten CM, Wehler D, Bender K, Hess G, Konur A, et al. Prophylactic transfer of CD8-depleted donor lymphocytes after T-cell-depleted reduced-intensity transplantation. *Blood* (2007) 109(1):374–82. doi:10.1182/blood-2006-03-005769
121. Meyer RG, Wagner EM, Konur A, Bender K, Schmitt T, Hemmerling J, et al. Donor CD4 T cells convert mixed to full donor T-cell chimerism and replenish the CD52-positive T-cell pool after alemtuzumab-based T-cell-depleted allo-transplantation. *Bone Marrow Transplant* (2010) 45(4):668–74. doi:10.1038/bmt.2009.212
122. Zhou W, Longmate J, Lacey SF, Palmer JM, Gallez-Hawkins G, Thao L, et al. Impact of donor CMV status on viral infection and reconstitution of multifunction CMV-specific T cells in CMV-positive transplant recipients. *Blood* (2009) 113(25):6465–76. doi:10.1182/blood-2009-02-203307
123. Ljungman P, Hakki M, Boeckh M. Cytomegalovirus in hematopoietic stem cell transplant recipients. *Hematol Oncol Clin North Am* (2011) 25(1):151–69. doi:10.1016/j.hoc.2010.11.011
124. Boeckh M, Ljungman P. How we treat cytomegalovirus in hematopoietic cell transplant recipients. *Blood* (2009) 113(23):5711–9. doi:10.1182/blood-2008-10-143560
125. Mori T, Mori S, Kanda Y, Yakushiji K, Mineishi S, Takaue Y, et al. Clinical significance of cytomegalovirus (CMV) antigenemia in the prediction and diagnosis of CMV gastrointestinal disease after allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant* (2003) 33(4):431–4. doi:10.1038/sj.bmt.1704369
126. Eid AJ, Bakri SJ, Kijpittayarit S, Razonable RR. Clinical features and outcomes of cytomegalovirus retinitis after transplantation. *Transpl Infect Dis* (2008) 10(1):13–8. doi:10.1111/j.1399-3062.2007.00241.x
127. Travi G, Pergam SA. Cytomegalovirus pneumonia in hematopoietic stem cell recipients. *J Intensive Care Med* (2014) 29(4):200–12. doi:10.1177/0885066613476454
128. Ljungman P, Perez-Bercoff L, Jonsson J, Avetisyan G, Sparrelid E, Aschan J, et al. Risk factors for the development of cytomegalovirus disease after allogeneic stem cell transplantation. *Haematologica* (2006) 91(1):78–83.
129. Pourgheysari B, Piper KP, McLarnon A, Arrazi J, Bruton R, Clark F, et al. Early reconstitution of effector memory CD4+ CMV-specific T cells protects against CMV reactivation following allogeneic SCT. *Bone Marrow Transplant* (2008) 43(11):853–61. doi:10.1038/bmt.2008.403
130. Gärtner BC, Schäfer H, Marggraff K, Eisele G, Schäfer M, Roemer K, et al. Evaluation of use of Epstein-Barr viral load in patients after allogeneic stem cell transplantation to diagnose and monitor posttransplant lymphoproliferative disease. *J Clin Microbiol* (2002) 40(2):351–8. doi:10.1128/JCM.40.2.351-358.2002
131. Peric Z, Cahu X, Chevallier P, Brissot E, Malard F, Guillaume T, et al. Features of Epstein-Barr virus (EBV) reactivation after reduced intensity conditioning allogeneic hematopoietic stem cell transplantation. *Leukemia* (2011) 25(6):932–8. doi:10.1038/leu.2011.26
132. Uhlin M, Wikell H, Sundin M, Blennow O, Maeurer M, Ringden O, et al. Risk factors for Epstein-Barr virus-related post-transplant lymphoproliferative disease after allogeneic hematopoietic stem cell transplantation. *Haematologica* (2014) 99(2):346–52. doi:10.3324/haematol.2013.087338
133. Maschmeyer G, Ljungman P. Infections in hematopoietic stem cell transplant recipients. In: Safdar A, editor. *Principles and Practice of Cancer Infectious Diseases*. Springer (2011). p. 17–25.
134. Borchers S, Luther S, Lips U, Hahn N, Kontsendorn J, Stadler M, et al. Tetramer monitoring to assess risk factors for recurrent cytomegalovirus reactivation and reconstitution of antiviral immunity post allogeneic hematopoietic stem cell transplantation. *Transpl Infect Dis* (2011) 13(3):222–36. doi:10.1111/j.1399-3062.2011.00626.x
135. Chalandon Y, Degermann S, Villard J, Arlettaz L, Kaiser L, Vischer S, et al. Pretransplantation CMV-specific T cells protect recipients of T-cell-depleted grafts against CMV-related complications. *Blood* (2006) 107(1):389–96. doi:10.1182/blood-2005-07-2746
136. Borchers S, Bremm M, Lehrnbecher T, Dammann E, Pabst B, Wölk B, et al. Sequential anti-cytomegalovirus response monitoring may allow prediction of cytomegalovirus reactivation after allogeneic stem cell transplantation. *PLoS One* (2012) 7(12):e50248. doi:10.1371/journal.pone.0050248
137. Borchers S, Ogonek J, Varanasi PR, Tischer S, Bremm M, Eiz-Vesper B, et al. Multimer monitoring of CMV-specific T cells in research and in clinical applications. *Diagn Microbiol Infect Dis* (2014) 78(3):201–12. doi:10.1016/j.diagmicrobio.2013.11.007
138. Odendahl M, Grigoleit GU, Bönig H, Neuenhahn M, Albrecht J, Anderl F, et al. Clinical-scale isolation of 'minimally manipulated' cytomegalovirus-specific donor lymphocytes for the treatment of refractory cytomegalovirus disease. *Cytotherapy* (2014) 16(9):1245–56. doi:10.1016/j.jcyt.2014.05.023
139. Kumaresan P, Figliola M, Moyes JS, Huls MH, Tewari P, Shpall EJ, et al. Automated cell enrichment of cytomegalovirus-specific T cells for clinical applications using the cytokine-capture system. *J Vis Exp* (2015) (104):e52808. doi:10.3791/52808
140. Lacey SF, Diamond DJ, Zaia JA. Assessment of cellular immunity to human cytomegalovirus in recipients of allogeneic stem cell transplants. *Biol Blood Marrow Transplant* (2004) 10(7):433–47. doi:10.1016/j.bbmt.2003.12.004
141. Tuthill M, Chen F, Paston S, De La Peña H, Rusakiewicz S, Madrigal A. The prevention and treatment of cytomegalovirus infection in haematopoietic stem cell transplantation. *Cancer Immunol Immunother* (2009) 58(9):1481–8. doi:10.1007/s00262-009-0722-7
142. Gratama JW, van Esser JWJ, Lamers CHJ, Tournay C, Löwenberg B, Bolhuis RLH, et al. Tetramer-based quantification of cytomegalovirus (CMV)-specific CD8+ T lymphocytes in T-cell-depleted stem cell grafts and after transplantation may identify patients at risk for progressive CMV infection. *Blood* (2001) 98(5):1358–64. doi:10.1182/blood.V98.5.1358
143. Lilleri D, Fornara C, Chiesa A, Caldera D, Alessandrino EP, Gerna G. Human cytomegalovirus-specific CD4+ and CD8+ T-cell reconstitution in adult allogeneic hematopoietic stem cell transplant recipients and immune control of viral infection. *Haematologica* (2008) 93(2):248–56. doi:10.3324/haematol.11912

144. Imai C, Iwamoto S, Campana D. Genetic modification of primary natural killer cells overcomes inhibitory signals and induces specific killing of leukemic cells. *Blood* (2005) 106(1):376–83. doi:10.1182/blood-2004-12-4797
145. Foster AE, Gottlieb DJ, Sartor M, Hertzberg MS, Bradstock KF. Cytomegalovirus-specific CD4+ and CD8+ T-cells follow a similar reconstitution pattern after allogeneic stem cell transplantation. *Biol Blood Marrow Transplant* (2002) 8(9):501–11. doi:10.1053/bbmt.2002.v8.pm12374455
146. Altman JD, Moss PA, Goulder PJ, Barouch DH, McHeyzer-Williams MG, Bell JI, et al. Phenotypic analysis of antigen-specific T lymphocytes. *Science* (1996) 274(5284):94–6. doi:10.1126/science.274.5284.94
147. Aubert G, Hassan-Walker AF, Madrigal JA, Emery VC, Morte C, Grace S, et al. Cytomegalovirus-specific cellular immune responses and viremia in recipients of allogeneic stem cell transplants. *J Infect Dis* (2001) 184(8): 955–63. doi:10.1086/323354
148. Chen FE, Aubert G, Travers P, Dodi IA, Madrigal JA. HLA tetramers and anti-CMV immune responses: from epitope to immunotherapy. *Cytotherapy* (2002) 4(1):41–8. doi:10.1080/146532402317251518
149. Lacey SF, Villacres MC, La Rosa C, Wang Z, Longmate J, Martinez J, et al. Relative dominance of HLA-B*07 restricted CD8+ T-lymphocyte immune responses to human cytomegalovirus pp65 in persons sharing HLA-A*02 and HLA-B*07 alleles. *Hum Immunol* (2003) 64(4):440–52. doi:10.1016/S0198-8859(03)00028-4
150. Akiyama Y, Kuzushima K, Tsurumi T, Yamaguchi K. Analysis of HLA-A24-restricted CMVpp65 peptide-specific CTL with HLA-A*2402-CMVpp65 tetramer. *Immunol Lett* (2004) 95(2):199–205. doi:10.1016/j.imlet.2004.07.010
151. Bunde T, Kirchner A, Hoffmeister B, Habedank D, Hetzer R, Cherepnev G, et al. Protection from cytomegalovirus after transplantation is correlated with immediate early 1-specific CD8 T cells. *J Exp Med* (2005) 201(7):1031–6. doi:10.1084/jem.20042384
152. Lilleri D, Gerna G, Fornara C, Lozza L, Maccario R, Locatelli F. Prospective simultaneous quantification of human cytomegalovirus-specific CD4+ and CD8+ T-cell reconstitution in young recipients of allogeneic hematopoietic stem cell transplants. *Blood* (2006) 108(4):1406–12. doi:10.1182/blood-2005-11-012864
153. Gratama JW, Brooimans RA, van der Holt B, Sintnicolaas K, van Doornum G, Niesters HG, et al. Monitoring cytomegalovirus IE-1 and pp65-specific CD4+ and CD8+ T-cell responses after allogeneic stem cell transplantation may identify patients at risk for recurrent CMV reactivations. *Cytometry B Clin Cytom* (2008) 74(4):211–20. doi:10.1002/cyto.b.20420
154. Koehl U, Dirkwinkel E, Koenig M, Erben S, Soerensen J, Bader P, et al. Reconstitution of cytomegalovirus specific T cells after pediatric allogeneic stem cell transplantation: results from a pilot study using a multi-allele CMV tetramer group. *Klin Padiatr* (2008) 220(6):348–52. doi:10.1055/s-0028-1086029
155. Giest S, Grace S, Senegaglia AC, Pasquini R, Gonzalo-Daganzo RM, Fernandez MN, et al. Cytomegalovirus-specific CD8(+) T cells targeting different HLA/peptide combinations correlate with protection but at different threshold frequencies. *Br J Haematol* (2010) 148(2):311–22. doi:10.1111/j.1365-2141.2009.07969.x
156. Gratama JW, Boeckh M, Nakamura R, Cornelissen JJ, Brooimans RA, Zaia JA, et al. Immune monitoring with iTAg MHC Tetramers for prediction of recurrent or persistent cytomegalovirus infection or disease in allogeneic hematopoietic stem cell transplant recipients: a prospective multicenter study. *Blood* (2010) 116(10):1655–62. doi:10.1182/blood-2010-03-273508
157. Lilleri D, Gerna G, Zelini P, Chiesa A, Rognoni V, Mastronuzzi A, et al. Monitoring of human cytomegalovirus and virus-specific T-cell response in young patients receiving allogeneic hematopoietic stem cell transplantation. *PLoS One* (2012) 7(7):e41648. doi:10.1371/journal.pone.0041648

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REVIEW
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B-Cell-Based and Soluble Biomarkers in Body Liquids for Predicting Acute/Chronic Graft-versus-Host Disease after Allogeneic Hematopoietic Stem Cell Transplantation

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Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is the main curative therapy for hematological malignancy such as leukemias, lymphomas, or multiple myelomas and some other hematological disorders. In this therapy, cure of hematological diseases relies on graft-versus-malignancy effects by allogenic immune cells. However, severe posttransplant treatment-associated complications such as acute graft-versus-host disease (aGvHD) and chronic graft-versus-host disease (cGvHD) limit this approach. Most research into GvHD has concentrated on the aGvHD, while the more complex and multifaceted chronic form has been largely poorly investigated. cGvHD is a multi-organ autoimmune disorder and is the major cause of non-relapse morbidity and mortality following allo-HSCT, occurring in about 50% of patients, or 13,000–15,000 patients per year worldwide. Therefore, there is a high medical need for an early prediction of these therapy-associated toxicities. Biomarkers have gained importance over the last decade in diagnosis, in prognosis, and in prediction of pending diseases or side effects. Biomarkers can be cells, factors isolated from target tissues, or soluble factors that can be detected in body fluids. In this review, we aim to summarize some of the recent developments of biomarkers in the field of allo-HSCT. We will focus on cell-based biomarkers (B-cell subsets) for cGvHD and soluble factors including microRNA (miRNA), which are excreted into serum/plasma and urine. We also discuss the potential role of cytosolic and extracellular 70 kDa heat shock proteins (HSP70) as potential biomarkers for aGvHD and their role in preclinical models. Proteomic biomarkers in the blood have been used as predictors of treatment responses in patients with aGvHD for many years. More recently, miRNAs have been found to serve as a biomarker to diagnose aGvHD in the plasma.

Another development relates to urine-based biomarkers that are usually detected by capillary electrophoresis and mass spectrometry. These biomarkers have the potential to predict the development of severe aGvHD (grades III–IV), overall mortality, and the pending development of cGvHD in patients posttransplant.

Keywords: biomarkers, graft-versus-host disease, proteomics, genomics, cellular, heat shock protein, B-cell subsets

COMPLICATIONS OF HEMATOPOIETIC STEM CELL TRANSPLANTATION (HSCT)

Acute and chronic graft-versus-host disease (a/cGvHD) are serious and frequent complications after allogeneic hematopoietic stem cell transplantation (allo-HSCT) that negatively impact on survival and quality of life of patients (1, 2). GvHD develops in approximately 40–60% of recipients after allo-HSCT. Donor-derived T-cells targeting alloantigens of the recipient play a key role in the induction of GvHD. Donor T-cells generally destroy host tissues by secreting pro-inflammatory cytokines such as interleukin-1 (IL-1), tumor necrosis factor alpha (TNF-α), and interferon gamma (IFN-γ) or by direct cytolytic activities of immune effector cells (1, 2). So far, GvHD is diagnosed based on unique diagnostic clinical signs and symptoms, as recommended by the National Institutes of Health (NIH) consensus development conferences (3, 4). In 2014, the NIH Consensus Conference updated requirements for the integration of assessment of potential biomarkers in prospective clinical studies of GvHD (4). Biomarkers that could be obtained by minimal invasive methods would be beneficial to predict GvHD and thus would increase safety and quality of life of patients. Biomarkers should fulfill certain requirements such as the confirmation of an aGvHD or cGvHD, objectively measure disease activity, allow a distinction of organ damage, should provide prognostic risk assessment, and predict responses to therapy (5). Detailed documentations on collection of specimens including excellent clinical data on patient characteristics and HSCT course, and standardized data analyses are crucial for further data processing (5). cGvHD is a multi-organ autoimmune disorder and is the major cause of non-relapse morbidity and mortality following allo-HSCT, occurring in about 50% of patients, or 13,000–15,000 patients per year worldwide that causes a plethora of comorbidities including cardiovascular, gastrointestinal (GI), liver, pulmonary, endocrine (diabetes, hyperlipidemia, thyroid and adrenal insufficiency, hypogonadism), neuropsychiatric (e.g., depression, chronic neurologic diseases), bone and joint (osteoarthritis, osteoporosis) disorders, infections (bacterial, viral, and fungal), and other more specific comorbidities (solid malignancy, obesity, and infertility) (1–5). Herein, we discuss B-cell-based, stress protein-, and microRNA (miRNA)-based biomarkers as predictors for a/cGvHD.

CELLULAR BIOMARKERS

The Role of T/B-Cells As Cellular Biomarkers in cGvHD

For a long time it has been known that cross-reactive allo-T cells that were immunized by environmental antigens derived from the donor are key players in the induction of GvHD. Therefore, the determination of these cells and the measurement of their cytolytic activities against host tissues have been used to determine GvHD (6). The outcome of haploidentical HSCT can be improved by the depletion of T cells from the donor graft. However, this procedure is often accompanied by graft failure and an increased incidence of GvHD, which could be overcome by megadose HSCT, injecting of pathogen-specific T cells to rebuild immunity or engineered T cells to induce suicide in case of allo-reactivity (7). The sequential infusion of regulatory T cells (Tregs) ($CD4^+/CD25^+$ and $CD4^+/CD25^-$) after HSCT, the selective *ex vivo* (photo) depletion of certain autoreactive T cell clones, the preservation of γ/δ T cells in the stem cell graft, and the selection of the best stem cells provide other options to improve GVL effects while GvHD is not increased (7). All these procedures contribute to fewer infection and toxicity rates and leukemia-related death cases.

Recent research has demonstrated that apart from T cells, B-cells also play key roles in the pathogenesis of cGvHD. Therefore, the presence of auto- and alloantibodies, elevated plasma levels of B-cell activation factor (BAFF), a cytokine of the tumor necrosis family, and an accumulation of $CD19^+CD21^{low}$ B-cells serve as biomarkers for GvHD. Apart from the depletion of T-cells by antibodies, the depletion of certain B-cell subpopulations might also provide a promising strategy to avoid GvHD (8–10). A delayed B-cell reconstitution with relative B-cell lymphopenia can result in downregulated B-cell counts in patients after HSCT (9–12). Low B-cell counts in the circulation may be explained in part by the insufficient production of B-cells in the bone marrow, as previously reported in patients with both, aGvHD and cGvHD (13). In contrast, a dysregulated B-cell homeostasis with persistent high BAFF levels can induce an upregulation of certain subpopulations of B-cells. In patients who do not develop cGvHD, elevated BAFF levels normalize after 6 months, whereas these remain highly elevated in patients developing cGvHD at later time points (11, 12). The observed high BAFF/B-cell ratio in patients with cGvHD suggests that during B-cell deficiency, autoreactive B-cell clones that would otherwise undergo negative selection could potentially survive due to an excess of BAFF, which in turn could possibly contribute to the pathophysiology of cGvHD (14–16). Furthermore, increased B-cell activation, aberrant B-cell signaling, and prolonged survival of activated B-cells have been found to be associated with cGvHD (17).

Perturbation of B-cell homeostasis can be associated with elevated or decreased numbers of different B-cell subpopulations during cGvHD (8, 11, 12, 16, 18, 19). Greinix and colleagues reported on elevated relative numbers of $CD19^+CD21^{low}$ B-cells in patients with active cGvHD compared to those without cGvHD in

a retrospective study on 70 patients (8). In addition, $CD19^+CD21^{low}$ B-cell counts higher than 15% in patients with active cGvHD were found to be significantly associated with the presence of severe opportunistic infections (8). Furthermore, the memory B-cell compartment showed significantly lower relative and absolute numbers of both, non-class-switched $CD19^+CD27^+IgD^+$ and class-switched $CD19^+CD27^+IgD^-$ memory B-cells. This observed perturbation of circulating B-cell subpopulations could be useful for assessing cGvHD activity and for identifying cGvHD patients at risk for severe infectious complications (8).

Kuzmina and colleagues investigated whether the number of $CD19^+CD21^{low}$ B-cells could predict the outcome of extracorporeal photopheresis (ECP), which is used as one option for an immunomodulatory treatment of cGvHD (19). ECP non-responders had significantly higher ($p = 0.02$) relative numbers of $CD19^+CD21^{low}$ B-cells (mean = 22%) in the peripheral blood prior to the start of ECP compared to patients achieving a complete response (CR) (mean = 8%) and partial response (mean = 16%) after 6 months of ECP therapy. These data suggest that $CD19^+CD21^{low}$ B-cell counts could serve as a predictive cellular biomarker. Moreover, CR patients had significantly lower relative numbers of $CD19^+CD21^{low}$ B-cells 6, 12, and 21 months after start of ECP compared to non-responders, confirming that $CD19^+CD21^{low}$ B-cells could be potential cellular biomarkers for objective response assessment in cGvHD (19).

$CD19^+CD21^{low}$ B-cells were further investigated in cGvHD patient cohorts in the context of impaired humoral immunity defined by increased or decreased serum immunoglobulin G (IgG) levels (11). cGvHD patients with hypogammaglobulinemia had significantly decreased absolute numbers of $CD19^+$ B-cells with elevated percentages of $CD19^+CD21^{low}$ B-cells and transitional $CD19^+CD21^{int\text{-}high}CD38^{high}IgM^{high}$ B-cells compared to cGvHD cohorts with normogammaglobulinemia or hypergammaglobulinemia, respectively. Furthermore, cGvHD patients with hypogammaglobulinemia also had a significant reduction of non-class-switched $CD19^+CD27^+IgD^+$ and class-switched $CD19^+CD27^+IgD^-$ memory B-cells compared to the other two cohorts. Of note, cGvHD patients with hypergammaglobulinemia presented with significantly higher BAFF/B-cell ratios and frequently had significantly more autoantibodies present compared to the hypogammaglobulinemia cohort (11). Taken together, these data suggested that B-cell subpopulations could indicate different pathogenic mechanisms involved in cGvHD and might allow a distinction between immunodeficiency and autoimmunity (11, 16).

Investigation of B-cell subpopulations in the context of specific organ involvement by cGvHD revealed significantly decreased absolute and relative numbers of $CD19^+$ B-cells in patients with newly diagnosed lung involvement seen as bronchiolitis obliterans syndrome (BOS) (4, 16). The prognosis of BOS is poor, and therefore, the identification of patients at an early disease stage when they may have an improved response to immunosuppressive therapy is a major clinical challenge. Kuzmina and colleagues observed that patients with newly diagnosed BOS had significantly increased relative numbers of $CD19^+CD21^{low}$ B-cells (25.5 versus 6.6%, $p < 0.0001$) and BAFF/$CD19^+$ ratio (0.18 versus 0.02 ng/10^3 $CD19^+$ B-cells, $p = 0.007$) compared with patients without cGvHD (16). Asymptomatic patients with a drop in pulmonary function tests and NIH-defined changes in high-resolution CT scan could be distinguished from patients without cGvHD due to significantly higher $CD19^+CD21^{low}$ B-cells and ratio of BAFF/$CD19^+$ B-cells. Interestingly, relative numbers of $CD19^+CD21^{low}$ B-cells were significantly elevated both at onset of BOS as well as in patients with long-lasting BOS not responding to immunosuppressive treatment confirming a role of $CD19^+CD21^{low}$ B-cells as cellular biomarkers for objective diagnosis of lung involvement as well as continued cGvHD activity during the course of therapy.

In a large prospective study with 227 patients, Greinix and colleagues performed serial analyses starting on day 100 after HSCT to investigate $CD19^+CD21^{low}$ B-cells as diagnostic and predictive cellular biomarkers in patients with newly diagnosed cGvHD (18). Higher relative numbers of $CD19^+CD21^{low}$ B-cells, analyzed on day 100 after HSCT and compared to time-matched controls without cGvHD, were significantly associated with later development of cGvHD independently of clinical parameters (23.5 versus 15.2%, $p = 0.004$). Furthermore, significantly higher percentages of $CD19^+CD21^{low}$ B-cells were also associated with first diagnosis of cGvHD (18.3 versus 9.9%, $p = 0.001$) (18). Although their exact biological functions still need to be elucidated, it is known that $CD19^+CD21^{low}$ B-cells express inflammatory tissue-homing receptors, such as CXCR3 reflecting an increased capacity to home to inflammatory sites (20). Suryani and colleagues reported the production of significantly higher amounts of anti-nuclear autoantibodies by $CD19^+CD21^{low}$ B-cells compared to other B-cell subpopulations such as naïve $CD19^+CD10^-CD21^{high}CD27^-$ and memory $CD19^+CD27^+IgD^-$ B-cells, respectively (21). However, recent findings suggest that $CD19^+CD21^{low}$ B-cells share the phenotype of anergic B-cells and do not proliferate in response to normal B-cell stimulation factors (22, 23).

Recently, regulatory B-cells (Bregs) have been shown to be involved in the pathogenesis of cGvHD (22, 24). Khoder and colleagues reported an enrichment of interleukin-10 (IL-10)-producing Bregs within memory $CD19^+IgM^+CD27^+$ and transitional $CD19^+CD24^{hi}CD38^{hi}$ B-cells in healthy individuals (25). In patients with cGvHD, Breg cells were found to be decreased and thus less likely to produce IL-10 compared to healthy donors and patients without cGvHD (25). Another study reported a decrease of $CD24^{hi}CD27^+$ B-cells and IL-10-producing $CD24^{hi}CD27^+$ B-cells in patients with active cGvHD (26). Moreover, de Masson and colleagues observed increased $CD24^-CD38^{hi}$ plasmablast frequencies, but decreased IL-10-producing plasmablasts in patients with active cGvHD compared to ones without cGvHD (22). Since $CD24^{hi}CD27^+$ B-cells and plasmablasts are among the most elevated cellular subsets within the Breg cell compartment, these observations could further support a possible role of Breg cells in the pathogenesis of cGvHD (22). Whether these novel cellular subpopulations could serve as biomarkers of active cGvHD, however, has to await further studies on well-defined patient cohorts including time-matched controls without cGvHD.

$CD4^+CD25^+Foxp3^+$ Tregs have an indispensable role in the maintenance of tolerance after allo-HSCT. Poor reconstitution

of Tregs after HSCT correlated with subsequent development of cGvHD (27–29). Furthermore, patients with long-lasting cGvHD are known to have a deficiency of Tregs in the circulation (26, 27). These findings led to therapeutic interventions aiming at enhancing Treg cell numbers by administration of low-dose interleukin-2 and thus, suppressing clinical manifestations of cGvHD (26). Based on these results, Tregs can be considered to be diagnostic and predictive cellular biomarkers of cGvHD.

Regarding other T-cell subpopulations, an increase in $CCR7^{-}CD45RA^{+}CD8$ T-cells that are effector memory T-cells and a decrease of $CCR7^{+}CD45RA^{+}$ naïve T-cells as well as $CCR7^{+}CD45RA^{-}$ central memory T-cells has been reported in patients with cGvHD (30). The authors speculated whether their findings were the consequence of prolonged alloantigen exposure or interleukin-15 (IL-15) stimulation since patients with cGvHD reportedly have elevated IL-15 levels and effector memory T-cells can be generated by IL-15 stimulation. Yamashita and colleagues also observed a significantly higher percentage of $CCR7^{-}CD62L^{low}CD4^{+}$ effector memory T-cells in patients with cGvHD compared to ones without cGvHD or healthy donors (31). Furthermore, a preponderance of effector memory $CD4^{+}$ T-cells relative to $CCR7^{+}CD45RA^{-}$ central memory T-cells was observed in severe cGvHD by these investigators. In view of the small patient numbers in these single-center studies, confirmation and validation of these findings is required prior to using these T-cell subpopulations as diagnostic cellular biomarkers of cGvHD.

Greinix and colleagues recently reported that $CD4^{+}CD45RA^{+}$ naïve T-cells and $CD4^{+}CD45RA^{+}CD31^{+}$ T-cells were significantly increased in patients with newly diagnosed cGvHD compared to a time-matched patient cohort without cGvHD (18). Furthermore, this T-cell subset when measured prospectively on day +100 after HSCT was also significantly elevated in patients subsequently developing cGvHD compared to patients never experiencing cGvHD. Besides these T-cell subpopulations, $CD3^{+}CD56^{+}$ NKT cells were significantly increased on day +100 after HSCT in patients subsequently developing cGvHD and thus, could also serve as a predictive cellular biomarker.

SOLUBLE BIOMARKERS

70-kDa Heat Shock Protein Family (HSP70) As a Biomarker for Tumors and GvHD

Members of the HSP70 family are known to play an important role in transport, folding, and unfolding of proteins and also in the induction of immune responses (32–38). The major stress-inducible Hsp70 has been found to be upregulated in many different diseases including inflammation, autoimmunity, and tumors (32, 39). Hsp70 is also found on the cell surface of many tumor types including leukemic cells via a tumor-specific lipid anchorage (40). Furthermore, it was shown that membrane Hsp70 positive, viable tumor cells are able to secrete Hsp70 into the circulation in lipid vesicles, termed exosomes (33, 41). Therefore, exosomal Hsp70 serves as a tumor-specific biomarker for viable tumor mass (42). Functionally, extracellular HSPs, either alone or in combination with antigenic peptides, play important roles in the induction of inflammatory immune responses (34, 35, 43, 44). Apart from lipid-bound Hsp70, Hsp70 can be released by necrotic and inflamed tissues as a free molecule. This means that the major stress-inducible Hsp70 is secreted into the circulation either by living tumor cells in lipid vesicles, presumably tumor exosomes (41), or as a free molecule by dying necrotic and/or inflamed tissues. In cGvHD, inflammatory responses can occur in skin (77%), lung (50%), mouth (63%), liver (58%), eye (54%), joints (32%), GI (20%), and genital tract (16%). Therefore, free Hsp70, antibodies directed against Hsp70, mRNA levels might provide potential novel molecular biomarkers to diagnose and predict the onset of inflammation in cGvHD. Apart from members of the HSP70 family other stress proteins are discussed as potential markers for GvHD.

In a rat skin explant model, Novota et al could demonstrate that the two major stress-inducible genes Hsp70-1 and Hsp70-2 were upregulated. Therefore, elevated mRNA levels were associated with the grade of graft-versus-host reactions (GvHR) (45). Moreover, in the study by Kim et al., it was shown that a polymorphism in Hsp70-hom plays an important role in the prognosis of patients who received a sibling HLA-matched allo-HSCT (46). Thus, Hsp70-hom gene polymorphism might also serve as a prognostic marker for GvHD (47). A subsequent study by Bogunia-Kubik et al showed that patients who were homozygous for the A allele of the Hsp70-hom +2,663 SNP presented more frequently with grade II and IV toxic lesions and aGvHD compared to patients with different genotypes (48).

Other studies clearly demonstrated an involvement of the Hsp70 protein levels in the pathogenesis of GvHD. In a rat GvHD model that was induced by the injection of DA parental lymphoid cells into irradiated (LEW × DA)F1 rats, it was shown that the expression levels of Hsp70 were significantly increased in spleen and lymph nodes 7 and 14 days after induction of the disease (49). At later time points of the disease, the Hsp70 levels dropped to levels that were comparable to that of untreated control animals.

Different results exist with respect to HSP70-specific antibodies in the circulation in a rat model and human patients. In a rat model, elevated levels of antibodies (IgM, IgG2a, and IgG2b) directed against HSP70 have been found to be associated with the onset of symptoms that were associated with the development of GvHD (50), whereas in pediatric patients, this association was not detected after allo-HSCT (51). In subsequent human studies however, elevated anti-HSP70 and anti-HSP90 antibody levels were found to be associated with the development of a/cGvHD (52).

An association of the severity of GvHD disease and the expression of Hsp70 might be explained by the immunomodulatory activity of Hsp70. To date, several studies reported an involvement of Hsp70 in the production of pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, and IL-12 and the secretion of nitric oxide (NO) and C-C chemokines by dendritic cells, monocytes, and macrophages (53, 54). Moreover, Hsp70 can activate intracellular signaling cascades that influence immunoregulatory functions of immune cells through binding of either free or lipid-bound Hsp70 to specific cell surface receptors such as Toll-like receptors 2 and 4 (TLR2/4), scavenger receptor CD36,

low-density lipoprotein receptor-related protein CD91, C-type lectin receptor LOX-1, scavenger receptor SR-A, and CD40 (35). Through the induction of pro-inflammatory cytokines, Hsp70 contributes to the pathogenesis of autoimmune and various chronic inflammatory diseases (55–58). Thus, Hsp70 can serve as a damage-associated molecular pattern that activates the host's adaptive and innate immune system by initiating alloimmunity (59). Another mechanism of the immunoregulatory activity of Hsp70 is based on its peptide-binding capacity. Exogenous Hsp70 can chaperone and cross-present antigens and cargo them to the antigen-presenting cells and thus elicit adaptive immune responses (44, 60). Therefore, it is not too surprising that Hsp70 is frequently upregulated in allografts, which in turn results in the progression of disease and allograft rejection (61–63).

Presumably, the modulation of Hsp70 levels might provide a therapeutic option for improving the outcome of GvHD. Previously, Oh et al showed, in a skin graft model, in which Hsp70 (Hsp70.1 gene) knock-out (KO) mice were used either as a donor or recipient, the importance of Hsp70 in acute allograft rejection (64). Allogeneic cells derived from Hsp70 KO mice were shown to induce lower rejection rates in recipients than those of Hsp70 wild-type animals. Therefore, the application of reagents that are able to silence Hsp70 expression may provide a promising strategy to reduce the risk for GvHD. The reduction of HSP70 levels by the administration of 15-deoxyspergualin (DSG), an immunosuppressive agent that binds to a constitutively expressed member of the 70-kDa heat shock protein family, has been shown to significantly reduce GvHD-associated mortality (65). DSG treatment reduced HSP70 levels in spleen and lymph nodes, inhibited the anti-HSP70 antibody production, and reduced the serum levels of IL-2, IFN-γ, TNF-α, and IL-10 (64).

In conclusion, monitoring levels of HSP70 protein and/or anti-HSP70 antibody levels in the serum after HSCT might serve as a diagnostic tool to predict the onset of GvHD. In addition, genetic or pharmacological modulation of the HSP70 expression may have a therapeutic potential in the treatment of GvHD.

Protein Markers in Body Fluids for Diagnosis of aGvHD

Serum, plasma (66–68), and saliva have been analyzed for prediction or diagnosis of aGvHD and cGvHD (69–71). A surface-enhanced laser desorption/ionization (SELDI) mass spectrometer was used to analyze plasma/serum in patients with and without GvHD post-HSCT by the group of Barrett in 2006 at the National Heart, Lung, and Blood Institute, National Institute of Health (NIH). They used SELDI to identify proteins/peptides differentially excreted into plasma (69–71). Another group from the NIH analyzed salvia samples using SELDI, no further data have been reported for either study. Therefore, plasma proteomics may be a more promising approach. The group of Ferrara and colleagues have intensively studied the serum/plasma proteomic approach using enzyme-linked immunosorbent assays to detect the presence of several proteins in the serum/plasma of patients post allo-HSCT (66–68). Among other markers, they have described plasma markers for GI aGvHD (68), regenerating islet-derived protein 3a (Reg-3a) and suppression of tumorigenicity (ST2) as diagnostic markers for aGvHD and survival after HSCT. Reg-3a was tested in samples from 1,014 HSCT patients from three transplantation centers and Reg3a concentrations were threefold higher in patients at onset of GI GvHD than in all other patients and correlated with lower GI GvHD. Reg3a concentrations at time of GvHD onset predicted response to therapy at 4 weeks, 1-year NRM, and 1-year survival ($p \leq 0.001$). In a multivariate analysis, advanced clinical stage, severe histologic damage, and high Reg3α concentrations at GvHD diagnosis independently predicted 1-year NRM. The combination of Reg3a with clinical stage and histologic grade of GvHD can be used to improve risk stratification of patients.

The described biomarkers have been tested in two different centers (67, 68). To fulfill the criteria of a reliable biomarker, the biomarkers have to be defined in a test set, confirmed in a first validation set and then validated in a multicenter validation trial (5), as biomarkers need to be robust under different clinical conditions and prophylaxis strategies.

The group of Levine and Ferrara has recently achieved this goal for the so-called Ann Arbor Score of aGvHD, which relies on three biomarkers (Reg3 alpha, ST2, and sTNFR1). If this score is high at the time of onset of GvHD, it strongly predicts day 28 treatment response and 6-month NRM irrespective of center-specific strategies (72).

An international Mount Sinai Acute GvHD International Consortium (MAGIC consortium) has been recently founded, which prospectively monitors clinical data and samples from more than 1,000 pts receiving allogeneic SCT/year and now allows to develop biomarker score-based treatment stratification at the time of onset of GvHD (73).

None of the serum/plasma markers described to date can predict aGvHD development, but they can help to diagnose aGvHD, especially GI aGvHD and to define prognosis at a very early time point where clinical presentation fails to allow exact prediction. Earlier biomarker scores (e.g., at day 7) after HSCT are currently developed and hopefully will allow stratification of preemptive treatment in the future. Taken together, the proteomic monitoring of patients holds promise for early diagnosis as well as risk stratification of patients.

Serum/plasma biomarker development for cGvHD is yet not at the state of prospective multicenter monitoring. Chemokine (C-X-C motif) ligand 9 (CXCL9), suppression of tumorigenicity 2 (ST2), osteopontin, and soluble BAFF levels are candidates among others (74–77). In particular, recent work by Yu et al has described a biomarker panel for cGvHD using quantitative proteomic profiling by high-resolution tandem mass spectrometry. Pooled plasma from patients with and without cGvHD at matched time points posttransplant were tested in a discovery set and two independent validation cohorts.

The matrix metalloproteinase 3 (MMP3) in the plasma correlates with BOS, a serious complication after allo-HSCT. An area under the receiver-operating characteristic (ROC) curve for MMP3 indicated a value of 0.77 (78).

Another biomarker that may be linked to cGvHD or to the maintenance of remission is the presence of H-Y specific antibodies that relate to a minor antigen mismatch. These antibodies appear approximately 4–12 months after HSCT predominantly

in male patients who were transplanted with female donor grafts. In the presence of alloantibodies, the cumulative incidence of cGvHD reached 89% at 5 years after HSCT compared with only 31% in the absence of H-Y antibodies. However, the cumulative incidence of relapse reached 48% at 5 years in the absence of H-Y antibodies compared with 0% in the presence of H-Y alloantibodies. Therefore, the authors concluded that antibody responses to H-Y antigens were associated with maintenance of disease remission in gender-mismatched HSCT. In a follow-up research project, the same group demonstrated that H-Y antigen-binding B-cells developed in male recipients of female hematopoietic cells that were associated with cGvHD (79). Of note, B-cells specific for the dominant H-Y epitope, DEAD box protein (DBY-2) appeared in significantly higher frequency in the circulation 6 months after HSCT in individuals who developed cGvHD later and thus, may predict cGvHD (79, 80). However, this single center study requires validation in larger prospective clinical studies to allow firm conclusions.

Biofluid miRNAs As Biomarkers for Graft-versus-Host Disease

Within the last decade, circulating short single-stranded miRNA has been identified in human plasma, serum, and also in urine (81, 82). Interestingly, these miRNAs were found to be resistant to RNase, boiling, changes in pH, extended storage, and freeze-thaw cycles (83). Functionally, miRNA molecules are considered as one of the major groups of translational regulators with the ability to regulate differentiation of blood cells and immune functions (84, 85). The stability of miRNA in the circulation can be attributed to three major RNase protection mechanisms: miRNAs can be bound to protective proteins, such as nucleophosmin 1 (NPM1) and/or Agonaute 2 (Ago2) (86–88), miRNA can form complexes with lipid or lipoproteins including high-density lipoprotein and low-density lipoprotein, and miRNA can be encapsulated in extracellular lipid vesicles, such as exosomes (89, 90). The complex of miRNA and these proteins and/or vesicles allows the selective export of miRNAs into the circulation and the protection of them within the extracellular environment (86). Circulating miRNAs have been shown to act as robust biomarkers for a various diseases, including autoimmune conditions, such as rheumatoid arthritis and systemic lupus erythematosus and tumors (91). A number of studies have also assessed the association between circulating miRNAs and the development of GvHD, with promising results (92–96).

Circulating miRNAs are robust and can be detected in biofluids by minimal invasive methods using relatively simple and accurate technologies. Furthermore, circulating miRNAs may offer advantages over protein-based biomarkers as they are lower in complexity, conserved among clinically relevant species, expressed specifically in different tissues or biological stages and easily measured using common laboratory techniques (97).

Although miRNA studies in relation to GvHD are still in their infancy, miRNA-155 was the first miRNA to be associated with aGvHD (98). miRNA-155 expression is upregulated in T cells of mice with severe aGvHD after allo-HSCT and a reduction of miRNA-155 results in a decreased severity of aGvHD and a prolonged survival of mice (99). miRNA-155 is encoded within the B cell integration cluster and is important for the regulation of acute inflammation and innate immunity (98). Expression of miRNA-155 can be activated by inflammatory mediators, such as IFN-α/γ and TNF-α (100), and Ceppi et al proposed that it functions as part of the negative feedback loop controlling the secretion of inflammatory cytokines by LPS-induced DC activation (101). Thus, miRNA-155 is pivotal in fine-tuning of the immune response (**Figure 1**). Physiologically, miRNA-155 is upregulated during T cell differentiation, plays a role in $CD4^+$ and $CD8^+$ T cell-mediated immunity and promotes the development of T cells, including Th17 and Treg subsets. Mice with a germ-line depletion of miRNA-155 have a normal lymphocyte development but defective T and B cell immunity (102, 103). Within Tregs, miRNA-155 targets forkhead box P3 (FoxP3), which regulates *in vivo* Treg survival (104). miRNA-155 also directly targets the IL-2 signaling protein suppressor of cytokine signaling 1 (SOCS1), whereby miRNA-155 deficiency in Tregs results in increased SOCS1 expression (96). This, in turn, leads to impaired activation of signal transducer and activator of transcription factor 5 (STAT5) phosphorylation and IL-2 receptor signaling (105). miRNA-155 is also a key regulator of $CD8^+$ T cell responses via SOCS1, where a deficiency of miRNA-155 results in defective STAT5-mediated cytokine signaling (106). In $CD4^+$ T cells, miRNA-155 targets phosphatidylinositol 3,4,5-triphosphate 5-phosphatase 1 (SHIP1) for downregulation, which normally functions to suppress Th1 responses and T cells by modulating IFN-γ production (102, 107).

In a seminal study in 2012, Ranganathan et al showed that miRNA-155 is upregulated in T-cells from mice developing GvHD following HSCT (98). Moreover, the use of miRNA-155 inhibitors decreased disease severity and prolonging survival (98). In addition, the group found increased miRNA-155 expression in small and large bowel biopsies compared to normal bowel tissue in patients with GI aGvHD (98). However, these studies focused on miRNA expression within tissue biopsies, and more recently, several groups have sought to exploit the biomarker potential of circulating miRNAs in the context of GvHD. Although the presence of miRNAs has been established in a number of biological fluids (103), studies to date have concentrated on serum and plasma fractions of the blood.

A comprehensive report published by Xiao et al (92) in 2013 used a high-throughput qRT-PCR-based array to profile the expression of 345 miRNAs in the plasma of patients with aGvHD compared to those with no GvHD. They employed a discovery and training cohort to identify a final signature of four miRNAs (miRNA-423, miRNA-199-3p, miRNA-93*, and miRNA-377). These four miRNAs were able to predict aGvHD 6 weeks post-HSCT, prior to the onset of symptoms. The model was significant in ROC analysis (AUC = 0.76, $p < 0.001$) and was also associated with disease severity and poor overall survival (92). The miRNAs were shown to be specific for aGvHD, as they were not detected in the plasma of lung transplant or non-transplant sepsis patients. This study highlighted the potential of biofluid miRNAs as independent markers for prediction, prognosis, and diagnosis of GvHD.

Although miRNA-155 has been previously associated with the development of GvHD, it was not included in the final model

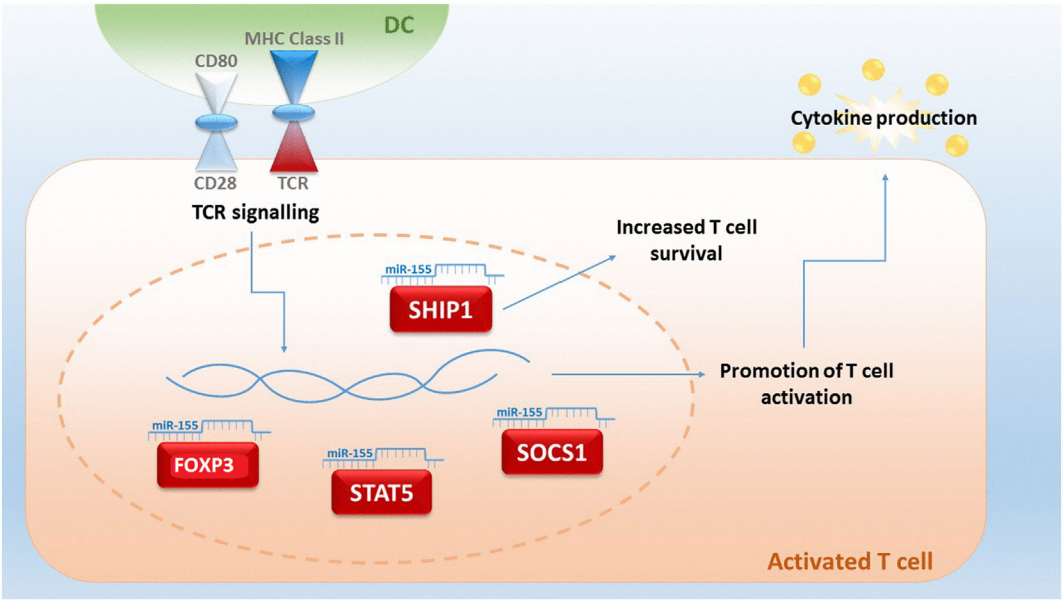

FIGURE 1 | An upregulated expression of microRNA (miRNA)-155 in T-cells is indicative for lethal acute graft-versus-host disease (aGvHD) in mice. miR-155 plays a central role in fine-tuning the immune response, and expression can be triggered by T cell receptor (TCR) signaling. miRNA-155 regulates FOXP3, which is important for the survival of T regulatory cells and influences the targets signal transducer and activator of transcription factor 5 (STAT5) and suppressor of cytokine signaling 1 (SOCS1). Targeting of 3,4,5-triphosphate 5-phosphatase 1 (SHIP1) by miRNA-155 results in an increased T-cell survival by an upregulated production of IFN-γ (93, 104–108).

proposed by Xiao et al. While expression was significantly upregulated in the plasma of aGvHD patients, levels were the lowest among the miRNA candidates identified (92). However, a recent study by Xie et al focusing on serum showed that miRNA-155 was significantly upregulated in aGvHD patients ($p = 0.003$) and also correlated with disease severity ($p < 0.001$) (93). Furthermore, expression was elevated in cGvHD patients compared to those who did not develop the disease ($p = 0.005$); however, levels were not sufficient to distinguish between aGvHD and cGvHD ($p = 0.96$) (93). Although this study showed promise for the inclusion of miRNA-155 as a GvHD biofluid biomarker, the study was restricted to one cohort of Han Chinese population ($n = 64$). Thus, the results need to be validated in independent prospective cohorts.

A small study by Sang et al confirmed elevated miRNA-155 plasma levels in patients with aGvHD, and additionally reported upregulation of miRNA-92b, while miRNA-150 and miRNA-181 were significantly downregulated (95). Interestingly, levels of the miRNAs were also altered prior to disease onset, highlighting their biomarker potential for predicting incidence (95). However, the difference in miRNA-181 was most pronounced, with no variation observed in control patients compared to reduced levels of expression in 19/22 (86%) patients prior to disease development (95). The group also demonstrated miRNA-181 to act as an effective predictor of aGvHD in a murine allo-HSCT model (95); however, larger cohorts are required in order to validate these findings.

More recently, Wang et al performed a study focusing on miRNA-586 expression in the plasma of HSCT patients (96). miRNA-586 was significantly upregulated in patients who developed aGvHD compared to no aGvHD as early as 7 days post-HSCT; however, expression was influenced by infection that reduced the significance of the association (96). As infections are common in early post-HSCT and can make distinguishing a diagnosis of aGvHD more challenging, it is important to validate the findings in larger studies as well as to elucidate the biological role of miRNA-586 in infections. Thus, although these results shown promise, miRNA-586 may play a greater role as a clinical biomarker to differentiate aGvHD from infectious complications.

In a separate study, Gimondi et al focused on lymphoma patients receiving MUD allo-transplants to profile the plasma using qRT-PCR for global miRNA expression (94). Assessing samples collected 28 days post-HSCT, 113 miRNAs were detected in all samples and of these, 27 could collectively discriminate between aGvHD versus no aGvHD and miRNA-194 and miRNA-518f were significantly upregulated in the patients who later developed the disease (94). The authors did not detect differential expression of miRNAs identified by Xiao et al (92), and there was no validation cohort included in the investigation. Thus, although results showed potential for the identification of aGvHD biomarkers, additional confirmatory studies are required.

Although circulating miRNAs as biomarkers for GvHD show great promise, these studies are still in their infancy and few overlapping targets between reports have been identified. A summary of presently known miRNAs for a/cGvHD have been presented in **Table 1**. Much work is needed to validate the findings in independent cohorts that reflect the heterogeneity in conditioning and prophylaxis regimens employed by different clinical

TABLE 1 | Role of different microRNAs (miRNAs) in acute and chronic graft-versus-host disease (a/cGvHD).

MicroRNA	Findings	Reference
miR-155	↑ in bowel tissue in aGvHD	(98)
miR-155	↑ in plasma in aGvHD and cGvHD	(93)
miR-155	↑ in plasma in aGvHD	(95)
miR-423	↑ in plasma in aGvHD	(92)
miR-199-3p	↑ in plasma in aGvHD	(92)
miR-93*	↑ in plasma in aGvHD	(92)
miR-377	↑ in plasma in aGvHD	(92)
miR-92b	↑ in plasma in aGvHD	(95)
miR-150	↓ in plasma in aGvHD	(95)
miR-181	↓ in plasma in aGvHD	(95)
miR-586	↑ in plasma in aGvHD	(96)
miR-194	↑ in plasma in aGvHD	(94)
miR-518f	↑ in plasma in aGvHD	(94)

centers. Moreover, collaboration between groups will allow for the standardization of protocols and technologies, which may greatly influence the reproducibility of findings and is a likely explanation for the lack of concordance in results to date. For blood studies, the choice of serum or plasma needs to be considered, with slightly more biomarker studies currently focusing on serum over plasma, while few whole blood or PBMC studies have been performed (108). Indeed, it has been proposed that profiles from isolated PBMC may yield information on the immune status of the disease, while the serum and plasma levels reflect the disease-dependent secretion and expression of miRNAs (108). The effect of hemolysis and platelet contamination as well as the choice of anti-coagulant should also be considered (109–111). Selection of the miRNA detection platform and normalization as well as the RNA isolation methodology employed has also been shown to affect results. Indeed, analytical variables have a huge potential to bias results, and this is particularly dependent on the normalization methods used (112). Some level of correction may be achieved by spiking in synthetic miRNAs (113); however, this approach can correct for technical variation such as the efficiency of RNA isolation and reverse transcription, but it will not account for intrinsic biological variation (114). Thus, the choice of normalization controls and/or global normalization becomes integral to the data analysis strategy. With regard to the miRNA detection platform, although qRT-PCR is a commonly used approach, variations on the fluorescent molecules including TaqMan and Sybr-Green, as well as the development of newer techniques including NanoString and Next-generation sequencing, have increased heterogeneity. This is important, as variation in results have been observed depending on the analysis platform, partly attributed to the difficulty in distinguishing small molecules at low abundance with high sequence homology (115).

Despite these considerations, it is expected that over the next few years, as the number of circulating miRNA biomarker studies increases, specific miRNA patterns for GvHD will be proposed and validated in the clinic. Although studies to date have focused on fractions of the blood, the potential for discovery of miRNA signatures in other fluids, including urine, is attractive. Indeed, initial data promise that miRNA signatures may be identified to predict the incidence of GvHD prior to onset, the severity of disease, to distinguish GvHD from other complications, and even to differentiate between aGvHD and cGvHD, particularly in relation to late onset aGvHD. Given the heterogeneity of transplantation protocols employed throughout different clinics, miRNA models appropriate to different transplant protocols may also be possible. Further investigation of validated miRNA signatures will allow the impact of miRNA dysregulation on the pathogenesis of GvHD to be studied, and conclusively, prospective investigations assessing the outcome of treatments selected by miRNA status will confirm their prognostic strength. Ultimately, the aim will be to diagnose GvHD and outcome before clinical symptoms manifest, allowing earlier introduction of therapy, tailored treatments, and reduced mortality and morbidity outcomes.

URINE PROTEOMICS BASED ON CAPILLARY ELECTROPHORESIS AND MASS SPECTROMETRY (CE-MS)

Proteomics for Prediction of aGvHD after HSCT

Proteomics of tissues or body fluids has gained significant importance over the last 10 years.

For example, urinary proteome-based classifiers, developed for aGvHD prediction, were first described in 2004 (116) and adapted in 2007 (117) and additional prospective evaluation was provided in 2014 (118). The urinary proteome profiling for aGvHD is done on prospectively collected urine samples from patients undergoing HSCT. The urine is collected prior to HSCT and conditioning, weekly after HSCT until day +35 and afterward biweekly until day +100. Urine can be frozen at −20°C until preparation of the sample for analyses using CE-MS (119–122). An extension to include patients with cGvHD was carried out in 2008; thus, samples were collected bimonthly after day +100 or at diagnosis of cGvHD (119, 123).

Acute and chronic GvHD are currently diagnosed on clinical parameters defined in NIH Consensus (2, 124, 125) and German Consensus Conferences (126–128). Prediction of aGvHD using the investigator-independent, unbiased proteomic classifier aGvHD_MS17 was done on more than 500 patients in at least 4 centers in Germany and UK and resulted in stable prediction of aGvHD about 7–10 days prior to clinical diagnosis or biopsy positivity (118, 119, 123, 129). The classifier aGvHD_MS17 consists of 17 peptides, 10 of which have been sequenced to date (117–119). The identified peptides are various fragments of collagen (indicating disturbances in metabolism of collagen and/or early organ damage) and fragments of fibronectin, beta-2-microglobulin (β2M), and CD99, an activation marker of T-cells. Multivariate regression analysis showed that aGvHD_MS17 positivity was the highest predictive parameter for aGvHD development ($p < 0.0001$, **Figure 2**).

FUTURE PERSPECTIVES

Numerous candidates for biomarkers are currently available, which include plasma and serum markers, cell-based markers such as B-cell subpopulations, and T follicular helper cells

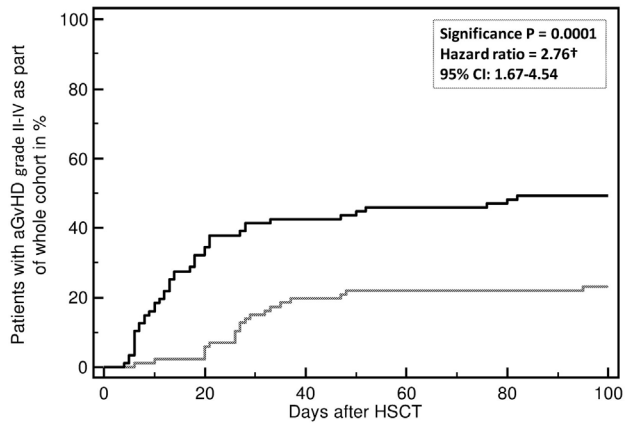

FIGURE 2 | Incidence of developing an acute graft-versus-host disease (aGvHD) grades II–IV. Incidence of aGvHD grades II–IV is predicted by aGvHD_MS17 positivity ($p = 0.0001$) when compared to patients without aGvHD_MS17 pattern positivity. Shown is the percentage (%) of patients with aGvHD II–IV with (black line) or without aGvHD_MS17 positivity over time [days after allogeneic hematopoietic stem cell transplantation (allo-HSCT)] (gray line).

as regulators of B-cell immunity in determining cGvHD in patients (130), miRNAs, different stress proteins, or proteomic approaches using plasma, serum, or urine. Proteomic mass spectrometry analysis coupled with computational biology approaches will further lead to the identification of novel biomarkers for GvHD such as BOS in the lung (131). Recently, the diversity of the intestinal microbiome as analyzed by 16 s rRNA sequencing of stool microbiota (131–134) and the assessment of bacterial metabolites (135) very early after transplantation has identified patients at high risk of lethal complications. On the other hand, biomarkers can also be used to tailor immunosuppressive therapies in transplanted patients and thus might predict the severity of GvHD.

The main challenge remains to integrate all these candidates and to validate them as biomarkers that are valid in multiple centers independent of the center's strategy of prophylaxis, and consortia on prospective testing of these markers together with clinical data collection as shown for the MAGIC consortium are urgently needed. The expectations have to be defined clearly, do we want to predict outcome in general or treatment response for aGvHD or cGvHD or do we want to make a diagnosis as precise as possible (e.g., for GI GvHD, for cGvHD) and the current standards and endpoints for each of these parameters have to be defined separately (e.g., d28 response for aGvHD, 2- or 3-month response for cGvHD, 6 months NRM for aGvHD, composite endpoints of GvHD-free and relapse-free survivals) (136).

New candidates have to be integrated in this process step by step, and bio-mathematical approaches are needed to define those markers that give true additive information. In addition, integration of older markers has not been performed (e.g., calprotectin in stool samples for GI GvHD (137), the poor man's biomarker albumin for GI GvHD) (138, 139), and more importantly, testing biomarkers against clinical predictors or markers of outcome is needed. For aGvHD, factors indicating poor prognosis are the extent of organ involvement and number of involved organs. For cGvHD, the type of onset and simultaneous occurrence of thrombocytopenia should be addressed. It might well be that some of the markers behave as highly sensitive and specific markers due to the correlation with extent and type of involved organ and the specific pathophysiology, which might be reflected by the correlation with clinical parameters (140).

AUTHOR CONTRIBUTIONS

MJ, MS, PM, and JO equally contributed to the manuscript. MJ, MS, PM, JO, and RC wrote the manuscript and prepared the figures. AD, HG, EH, EW, and GM outlined and proof-read the manuscript.

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REFERENCES

1. Socie G, Stone JV, Wingard JR, Weisdorf D, Henslee-Downey PJ, Bredeson C, et al. Long-term survival and late deaths after allogeneic bone marrow transplantation. Late Effects Working Committee of the International Bone Marrow Transplant Registry. *N Engl J Med* (1999) 341:14–21. doi:10.1056/NEJM199907013410103
2. Lee SJ, Vogelsang G, Flowers ME. Chronic graft-versus-host disease. *Biol Blood Marrow Transplant* (2003) 9:215–33. doi:10.1053/bbmt.2003.50026
3. Filipovich AH, Weisdorf D, Pavletic S, Socie G, Wingard JR, Lee SJ, et al. National Institutes of Health consensus development project on criteria for clinical trials in chronic graft-versus-host disease: I. Diagnosis and staging working group report. *Biol Blood Marrow Transplant* (2005) 11:945–56. doi:10.1016/j.bbmt.2005.09.004
4. Jagasia MH, Greinix HT, Arora M, Williams KM, Wolff D, Cowen EW, et al. National Institutes of Health consensus development project on criteria for clinical trials in chronic graft-versus-host disease: I. The 2014 diagnosis and staging working Group report. *Biol Blood Marrow Transplant* (2015) 21(389–401):e1. doi:10.1016/j.bbmt.2014.12.001
5. Paczesny S, Hakim FT, Pidala J, Cooke KR, Lathrop J, Griffith LM, et al. National Institutes of Health consensus development project on criteria for clinical trials in chronic graft-versus-host disease: III. The 2014 Biomarker Working Group Report. *Biol Blood Marrow Transplant* (2015) 21:780–92. doi:10.1016/j.bbmt.2015.01.003
6. Ferrara JL, Levine JE, Reddy P, Holler E. Graft-versus-host disease. *Lancet* (2009) 373:1550–61. doi:10.1016/S0140-6736(09)60237-3
7. Fuchs E. Haploidentical transplantation for hematologic malignancies: where do we stand? *Hematolopgy Am Soc Hematol Educ Program* (2012) 23(15):230–6. doi:10.1182/asheducation-2012.1.230

8. Greinix HT, Pohlreich D, Kouba M, Kormoczi U, Lohmann I, Feldmann K, et al. Elevated numbers of immature/transitional CD21- B lymphocytes and deficiency of memory CD27+ B cells identify patients with active chronic graft-versus-host disease. *Biol Blood Marrow Transplant* (2008) 14:208–19. doi:10.1016/j.bbmt.2007.10.009
9. Sarantopoulos S, Ritz J. Aberrant B-cell homeostasis in chronic GVHD. *Blood* (2015) 125:1703–7. doi:10.1182/blood-2014-12-567834
10. Socie G. Chronic GVHD: B cells come of age. *Blood* (2011) 117:2086–7. doi:10.1182/blood-2010-12-322297
11. Kuzmina Z, Greinix HT, Weigl R, Kormoczi U, Rottal A, Frantal S, et al. Significant differences in B-cell subpopulations characterize patients with chronic graft-versus-host disease-associated dysgammaglobulinemia. *Blood* (2011) 117:2265–74. doi:10.1182/blood-2010-07-295766
12. Sarantopoulos S, Stevenson KE, Kim HT, Cutler CS, Bhuiya NS, Schowalter M, et al. Altered B-cell homeostasis and excess BAFF in human chronic graft-versus-host disease. *Blood* (2009) 113:3865–74. doi:10.1182/blood-2008-09-177840
13. Storek J, Witherspoon RP, Webb D, Storb R. Lack of B cell precursors in marrow transplant recipients with chronic graft-versus-host disease. *Am J Hematol* (1996) 52:82–9. doi:10.1002/(SICI)1096-8652(199606)52:2<82::AID-AJH3>3.0.CO;2-1
14. Rolink AG, Melchers F. BAFFled B cells survive and thrive: roles of BAFF in B-cell development. *Curr Opin Immunol* (2002) 14:266–75. doi:10.1016/S0952-7915(02)00332-1
15. Sarantopoulos S, Stevenson KE, Kim HT, Bhuiya NS, Cutler CS, Soiffer RJ, et al. High levels of B-cell activating factor in patients with active chronic graft-versus-host disease. *Clin Cancer Res* (2007) 13:6107–14. doi:10.1158/1078-0432.CCR-07-1290
16. Kuzmina Z, Krenn K, Petkov V, Kormoczi U, Weigl R, Rottal A, et al. CD19(+)CD21(low) B cells and patients at risk for NIH-defined chronic graft-versus-host disease with bronchiolitis obliterans syndrome. *Blood* (2013) 121:1886–95. doi:10.1182/blood-2012-06-435008
17. Allen JL, Fore MS, Wooten J, Roehrs PA, Bhuiya NS, Hoffert T, et al. B cells from patients with chronic GVHD are activated and primed for survival via BAFF-mediated pathways. *Blood* (2012) 120:2529–36. doi:10.1182/blood-2012-06-438911
18. Greinix HT, Kuzmina Z, Weigl R, Kormoczi U, Rottal A, Wolff D, et al. CD19+CD21low B cells and CD4+CD45RA+CD31+ T cells correlate with first diagnosis of chronic graft-versus-host disease. *Biol Blood Marrow Transplant* (2015) 21:250–8. doi:10.1016/j.bbmt.2014.11.010
19. Kuzmina Z, Greinix HT, Knobler R, Worel N, Kouba M, Weigl R, et al. Proportions of immature CD19+CD21- B lymphocytes predict the response to extracorporeal photopheresis in patients with chronic graft-versus-host disease. *Blood* (2009) 114:744–6. doi:10.1182/blood-2009-05-221028
20. Rakhmanov M, Keller B, Gutenberger S, Foerster C, Hoenig M, Driessen G, et al. Circulating CD21low B cells in common variable immunodeficiency resemble tissue homing, innate-like B cells. *Proc Natl Acad Sci U S A* (2009) 106:13451–6. doi:10.1073/pnas.0901984106
21. Suryani S, Fulcher DA, Santner-Nanan B, Nanan R, Wong M, Shaw PJ, et al. Differential expression of CD21 identifies developmentally and functionally distinct subsets of human transitional B cells. *Blood* (2010) 115:519–29. doi:10.1182/blood-2009-07-234799
22. de Masson A, Bouaziz JD, Le Buanec H, Robin M, O'Meara A, Parquet N, et al. CD24(hi)CD27(+) and plasmablast-like regulatory B cells in human chronic graft-versus-host disease. *Blood* (2015) 125:1830–9. doi:10.1182/blood-2014-09-599159
23. Cambier JC, Gauld SB, Merrell KT, Vilen BJ. B-cell anergy: from transgenic models to naturally occurring anergic B cells? *Nat Rev Immunol* (2007) 7:633–43. doi:10.1038/nri2133
24. de Masson A, Socie G, Bagot M, Bensussan A, Bouaziz JD. Deficient regulatory B cells in human chronic graft-versus-host disease. *Oncoimmunology* (2015) 4:e1016707. doi:10.1080/2162402X.2015.1016707
25. Khoder A, Sarvaria A, Alsuliman A, Chew C, Sekine T, Cooper N, et al. Regulatory B cells are enriched within the IgM memory and transitional subsets in healthy donors but are deficient in chronic GVHD. *Blood* (2014) 124:2034–45. doi:10.1182/blood-2014-04-571125
26. Koreth J, Matsuoka K, Kim HT, McDonough SM, Bindra B, Alyea EP III, et al. IL-2 and regulatory T cells in graft-versus-host disease. *N Engl J Med* (2011) 365(22):2055–66. doi:10.1056/NEJMoa1108188
27. Miura Y, Thoburn CJ, Bright EC, Phleps ML, Shin T, Matsui WH, et al. Association of Foxp3 regulatory gene expression with graft-versus-hist disease. *Blood* (2004) 104(7):2187–93. doi:10.1182/blood-2004-03-1040
28. Zorn E, Kim HT, Lee SJ, Floyd BH, Litsa D, Arumugarajah S, et al. Reduced frequency of FOXP3+CD4+CD25+ regulatory T cells in patients with chronic graft-versus-host disease. *Blood* (2005) 106(8):2903–11. doi:10.1182/blood-2005-03-1257
29. Matsuoka KI, Kim HT, McDonough S, Bascug G, Warshauer B, Koreth J, et al. Altered regulatory T cell homeostasis in patients with CD4+ lymphopenia following allogeneic hematopoietic stem cell transplantation. *J Clin Invest* (2010) 120(5):1479–93. doi:10.1172/JCI41072
30. D`Asaro M, Dieli F, Caccamo N, Mussa M, Porretto F, Salerno A. Increase of CCR7-CD45RA+CD8 T cells (T(EMRA)) in chronic graft-versus-host diesease. *Leukemia* (2006) 20(3):545–7. doi:10.1038/sj.leu.2404079
31. Yamashita K, Choi U, Woltz PC, Foster SF, Sneller MC, Hakim FT, et al. Severe chronic graft-versus-host disease is characterized by a preponderance of CD4(+) effector memory cells relative to central memory cells. *Blood* (2004) 103(10):3986–8. doi:10.1182/blood-2003-09-3286
32. Gehrmann M, Schmetzer H, Eissner G, Haferlach T, Hiddemann W, Multhoff G. Membrane-bound heat shock protein 70 (Hsp70) in acute myeloid leukemia: a tumor specific recognition structure for the cytolytic activity of autologous NK cells. *Haematologica* (2003) 88:474–6.
33. Guzhova IV, Shevtsov MA, Abkin SV, Pankratova KM, Margulis BA. Intracellular and extracellular Hsp70 chaperone as a target for cancer therapy. *Int J Hyperthermia* (2013) 29:399–408. doi:10.3109/02656736.2013.807439
34. Chen T, Cao X. Stress for maintaining memory: HSP70 as a mobile messenger for innate and adaptive immunity. *Eur J Immunol* (2010) 40:1541–4. doi:10.1002/eji.201040616
35. Tsan MF, Gao B. Heat shock proteins and immune system. *J Leukoc Biol* (2009) 85:905–10. doi:10.1189/jlb.0109005
36. Finka A, Sharma SK, Goloubinoff P. Multi-layered molecular mechanisms of polypeptide holding, unfolding and disaggregation by HSP70/HSP110 chaperones. *Front Mol Biosci* (2015) 2:29. doi:10.3389/fmolb.2015.00029
37. Clerico EM, Tilitsky JM, Meng W, Gierasch LM. How hsp70 molecular machines interact with their substrates to mediate diverse physiological functions. *J Mol Biol* (2015) 427:1575–88. doi:10.1016/j.jmb.2015.02.004
38. Duncan EJ, Cheetham ME, Chapple JP, van der Spuy J. The role of HSP70 and its co-chaperones in protein misfolding, aggregation and disease. *Subcell Biochem* (2015) 78:243–73. doi:10.1007/978-3-319-11731-7_12
39. Gross C, Holler E, Stangl S, Dickinson A, Pockley AG, Asea AA, et al. An Hsp70 peptide initiates NK cell killing of leukemic blasts after stem cell transplantation. *Leuk Res* (2008) 32:527–34. doi:10.1016/j.leukres.2007.03.027
40. Gehrmann M, Liebisch G, Schmitz G, Anderson R, Steinem C, De MA, et al. Tumor-specific Hsp70 plasma membrane localization is enabled by the glycosphingolipid Gb3. *PLoS One* (2008) 3:e1925. doi:10.1371/journal.pone.0001925
41. Gastpar R, Gehrmann M, Bausero MA, Asea A, Gross C, Schroeder JA, et al. Heat shock protein 70 surface-positive tumor exosomes stimulate migratory and cytolytic activity of natural killer cells. *Cancer Res* (2005) 65:5238–47. doi:10.1158/0008-5472.CAN-04-3804
42. Breuninger S, Erl J, Knape C, Gunther S, Regel I, Rodel F, et al. Quantitative analysis of liposomal heat shock protein 70 (Hsp70) in the blood of tumor patients using an novel lipHsp70 ELISA. *Clin Cell Immunol* (2014) 5:2–10. doi:10.4172/2155-9899.1000264
43. Asea A. Stress proteins and initiation of immune response: chaperokine activity of Hsp72. *Exerc Immunol Rev* (2005) 11:34–45.
44. Shevtsov M, Multhoff G. Heat shock protein-peptide and HSP-based immunotherapies for the treatment of Cancer. *Front Immunol* (2016) 7:171. doi:10.3389/fimmu.2016.00171
45. Novota P, Sviland L, Zinocker S, Stocki P, Balavarca Y, Bickeboller H, et al. Correlation of Hsp70-1 and Hsp70-2 gene expression with the degree of graft-versus-host reaction in a rat skin explant model. *Transplantation* (2008) 85:1809–16. doi:10.1097/TP.0b013e31817753f7
46. Kim I, Kim JH, Rhee JY, Kim JW, Cho HJ, Cho EY, et al. Patient HSP70-hom TG haplotype is associated with decreased transplant-related mortality and improved survival after sibling HLA-matched

hematopoietic stem cell transplantation. *Clin Transplant* (2010) 24:459–66. doi:10.1111/j.1399-0012.2009.01094.x
47. Bogunia-Kubik K, Wysoczanska B, Lange A. Non-HLA gene polymorphisms and the outcome of allogeneic hematopoietic stem cell transplantation. *Curr Stem Cell Res Ther* (2006) 1:239–53. doi:10.2174/157488806776956850
48. Bogunia-Kubik K, Lange A. HSP70-hom gene polymorphism in allogeneic hematopoietic stem-cell transplant recipients correlates with the development of acute graft-versus-host disease. *Transplantation* (2005) 79:815–20. doi:10.1097/01.TP.0000153157.97736.2C
49. Goral J, Mathews HL, Clancy J Jr. Expression of 70-kDa heat-shock protein during acute graft-versus-host disease. *Clin Immunol Immunopathol* (1998) 86:252–8. doi:10.1006/clin.1997.4473
50. Goral J, Mathews HL, Clancy J Jr. Antibodies specific for the 70-kDa heat-shock protein parallel the development of acute graft-versus-host disease in (DA x LEW)F1 rats. *Clin Immunol Immunopathol* (1995) 75:147–53. doi:10.1006/clin.1995.1064
51. Zlacka D, Sedlacek P, Prucha M, Hromadnikova I. Antibodies to 60, 65 and 70 kDa heat shock proteins in pediatric allogeneic stem cell transplant recipients. *Pediatr Transplant* (2006) 10:794–804. doi:10.1111/j.1399-3046.2006.00566.x
52. Goral J, Shenoy S, Mohanakumar T, Clancy J Jr. Antibodies to 70 kD and 90 kD heat shock proteins are associated with graft-versus-host disease in peripheral blood stem cell transplant recipients. *Clin Exp Immunol* (2002) 127:553–9. doi:10.1046/j.1365-2249.2002.01770.x
53. Panjwani NN, Popova L, Srivastava PK. Heat shock proteins gp96 and hsp70 activate the release of nitric oxide by APCs. *J Immunol* (2002) 168:2997–3003. doi:10.4049/jimmunol.168.6.2997
54. Wang Y, Kelly CG, Singh M, McGowan EG, Carrara AS, Bergmeier LA, et al. Stimulation of Th1-polarizing cytokines, C-C chemokines, maturation of dendritic cells, and adjuvant function by the peptide binding fragment of heat shock protein 70. *J Immunol* (2002) 169:2422–9. doi:10.4049/jimmunol.169.5.2422
55. Mansilla MJ, Costa C, Eixarch H, Tepavcevic V, Castillo M, Martin R, et al. Hsp70 regulates immune response in experimental autoimmune encephalomyelitis. *PLoS One* (2014) 9:e105737. doi:10.1371/journal.pone.0105737
56. Mosenson JA, Eby JM, Hernandez C, Le Poole IC. A central role for inducible heat-shock protein 70 in autoimmune vitiligo. *Exp Dermatol* (2013) 22:566–9. doi:10.1111/exd.12183
57. Alam MU, Harken JA, Knorn AM, Elford AR, Wigmore K, Ohashi PS, et al. Transgenic expression of Hsc70 in pancreatic islets enhances autoimmune diabetes in response to beta cell damage. *J Immunol* (2009) 183:5728–37. doi:10.4049/jimmunol.0901288
58. Pockley AG. *Heat Shock Proteins in Health and Disease: Therapeutic Targets or Therapeutic Agents? Expert Reviews in Molecular Medicine*. Cambridge, UK: Cambridge University Press (2001). p. 1–21.
59. Williams JH, Ireland HE. Sensing danger – Hsp72 and HMGB1 as candidate signals. *J Leukoc Biol* (2008) 83:489–92. doi:10.1189/jlb.0607356
60. Calderwood SK, Theriault JR, Gong J. Message in a bottle: role of the 70-kDa heat shock protein family in anti-tumor immunity. *Eur J Immunol* (2005) 35:2518–27. doi:10.1002/eji.200535002
61. O'Neill S, Ingman TG, Wigmore SJ, Harrison EM, Bellamy CO. Differential expression of heat shock proteins in healthy and diseased human renal allografts. *Ann Transplant* (2013) 18:550–7. doi:10.12659/AOT.889599
62. Land WG. Role of heat shock protein 70 in innate alloimmunity. *Front Immunol* (2011) 2:89.
63. Lee YS, Sakurai M, Imai Y, Horinouchi T, Goto T, Yamada A. Induction of heat shock proteins in a rat composite tissue allotransplantation model of acute rejection. *Plast Reconstr Surg* (2006) 118:29–40. doi:10.1097/01.prs.0000220482.94982.01
64. Oh KH, Kim JY, Kim D, Lee EM, Oh HY, Seo JS, et al. Targeted gene disruption of the heat shock protein 72 gene (hsp70.1) in the donor tissue is associated with a prolonged rejection-free survival in the murine skin allograft model. *Transpl Immunol* (2004) 13:273–81. doi:10.1016/j.trim.2004.08.003
65. Goral J, Mathews HL, Nadler SG, Clancy J. Reduced levels of Hsp70 result in a therapeutic effect of 15-deoxyspergualin on acute graft-versus-host disease in (DA x LEW)F1 rats. *Immunobiology* (2000) 202:254–66. doi:10.1016/S0171-2985(00)80032-7
66. Harris AC, Ferrara JL, Braun TM, Holler E, Teshima T, Levine JE, et al. Plasma biomarkers of lower gastrointestinal and liver acute GVHD. *Blood* (2012) 119:2960–3. doi:10.1182/blood-2011-10-387357
67. Levine JE, Logan BR, Wu J, Alousi AM, Bolanos-Meade J, Ferrara JL, et al. Acute graft-versus-host disease biomarkers measured during therapy can predict treatment outcomes: a Blood and Marrow Transplant Clinical Trials Network study. *Blood* (2012) 119:3854–60. doi:10.1182/blood-2012-01-403063
68. Ferrara JL, Harris AC, Greenson JK, Braun TM, Holler E, Teshima T, et al. Regenerating islet-derived 3-alpha is a biomarker of gastrointestinal graft-versus-host disease. *Blood* (2011) 118:6702–8. doi:10.1182/blood-2011-08-375006
69. Paczesny S, Krijanovski OI, Braun TM, Choi SW, Clouthier SG, Kuick R, et al. A biomarker panel for acute graft-versus-host disease. *Blood* (2009) 113:273–8. doi:10.1182/blood-2008-07-167098
70. Srinivasan R, Daniels J, Fusaro V, Lundqvist A, Killian JK, Geho D, et al. Accurate diagnosis of acute graft-versus-host disease using serum proteomic pattern analysis. *Exp Hematol* (2006) 34:796–801. doi:10.1016/j.exphem.2006.02.013
71. Imanguli MM, Atkinson JC, Harvey KE, Hoehn GT, Ryu OH, Wu T, et al. Changes in salivary proteome following allogeneic hematopoietic stem cell transplantation. *Exp Hematol* (2007) 35:184–92. doi:10.1016/j.exphem.2006.10.009
72. Levine JE, Braun TM, Harris AC, Holler E, Taylor A, Miller H, et al. A prognostic score for acute graft-versus-host disease based on biomarkers: a multicentre study. *Lancet Haematol* (2015) 2:e21–9. doi:10.1016/S2352-3026(14)00035-0
73. Harris AC, Young R, Devine S, Hogan WJ, Ayuk F, Bunworasate U, et al. International, multicenter standardization of acute graft-versus-host disease clinical data collection: a report from the Mount Sinai acute GVHD international consortium. *Biol Blood Marrow Transplant* (2016) 22:4–10. doi:10.1016/j.bbmt.2015.09.001
74. Kitko CL, Levine JE, Storer BE, Chai X, Fox DA, Braun TM, et al. Plasma CXCL9 elevations correlate with chronic GVHD diagnosis. *Blood* (2014) 123:786–93. doi:10.1182/blood-2013-08-520072
75. Yu J, Storer BE, Kushekhar K, Abu Zaid M, Zhang Q, Gafken PR, et al. Biomarker panel for chronic graft-versus-host disease. *J Clin Oncol* (2016) 34:2583–90. doi:10.1200/JCO.2015.65.9615
76. Ahmed SS, Wang XN, Norden J, Pearce K, El-Gezawy E, Atarod S, et al. Identification and validation of biomarkers associated with acute and chronic graft versus host disease. *Bone Marrow Transplant* (2016) 51:890. doi:10.1038/bmt.2016.125
77. Pidala J, Sarwal M, Roedder S, Lee SJ. Biologic markers of chronic GVHD. *Bone Marrow Transplant* (2014) 49:324–31. doi:10.1038/bmt.2013.97
78. Liu X, Yue Z, Yu J, Daguindau E, Kushekhar K, Zhang Q, et al. Proteomic characterization reveals that MMP-3 correlates with bronchiolitis obliterans syndrome following allogeneic hematopoietic cell and lung transplantation. *Am J Transplant* (2016) 16:2342–51. doi:10.1111/ajt.13750
79. Sahaf B, Yang Y, Arai S, Herzenberg LA, Herzenberg LA, Miklos DB. H-Y antigen-binding B cells develop in male recipients of female hematopoietic cells and associate with chronic graft vs host disease. *Proc Natl Acad Sci U S A* (2013) 110:3005–10. doi:10.1073/pnas.1222900110
80. Miklos DB, Kim HT, Miller KH, Guo L, Zorn E, Lee SJ, et al. Antibody responses to H-Y minor histocompatibility antigens correlate with chronic graft-versus-host disease and disease remission. *Blood* (2005) 105:2973–8. doi:10.1182/blood-2004-09-3660
81. Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, et al. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res* (2008) 18:997–1006. doi:10.1038/cr.2008.282
82. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A* (2008) 105:10513–8. doi:10.1073/pnas.0804549105
83. Serody J. GvHD and miR: good things in small packages. *Blood* (2015) 126(11):1265–7. doi:10.1182/blood-2015-07-657114
84. Ameres SL, Zamore PD. Diversifying microRNA sequence and function. *Nat Rev Mol Cell Biol* (2013) 14:475–88. doi:10.1038/nrm3611

85. Hunter MP, Ismail N, Zhang X, Aguda BD, Lee EJ, Yu L, et al. Detection of microRNA expression in human peripheral blood microvesicles. *PLoS One* (2008) 3:e3694. doi:10.1371/journal.pone.0003694
86. Etheridge A, Lee I, Hood L, Galas D, Wang K. Extracellular microRNA: a new source of biomarkers. *Mutat Res* (2011) 717:85–90. doi:10.1016/j.mrfmmm.2011.03.004
87. Vickers KC, Palmisano BT, Shoucri BM, Shamburek RD, Remaley AT. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. *Nat Cell Biol* (2011) 13:423–33. doi:10.1038/ncb2210
88. Turchinovich A, Weiz L, Langheinz A, Burwinkel B. Characterization of extracellular circulating microRNA. *Nucleic Acids Res* (2011) 39:7223–33. doi:10.1093/nar/gkr254
89. Gallo A, Tandon M, Alevizos I, Illei GG. The majority of microRNAs detectable in serum and saliva is concentrated in exosomes. *PLoS One* (2012) 7:e30679. doi:10.1371/journal.pone.0030679
90. Taylor DD, Gercel-Taylor C. MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. *Gynecol Oncol* (2008) 110:13–21. doi:10.1016/j.ygyno.2008.04.033
91. Pauley KM, Cha S, Chan EK. MicroRNA in autoimmunity and autoimmune diseases. *J Autoimmun* (2009) 32:189–94. doi:10.1016/j.jaut.2009.02.012
92. Xiao B, Wang Y, Li W, Baker M, Guo J, Corbet K, et al. Plasma microRNA signature as a noninvasive biomarker for acute graft-versus-host disease. *Blood* (2013) 122:3365–75. doi:10.1182/blood-2013-06-510586
93. Xie LN, Zhou F, Liu XM, Fang Y, Yu Z, Song NX, et al. Serum microRNA155 is increased in patients with acute graft-versus-host disease. *Clin Transplant* (2014) 28:314–23. doi:10.1111/ctr.12314
94. Gimondi S, Dugo M, Vendramin A, Bermema A, Biancon G, Cavane A, et al. Circulating miRNA panel for prediction of acute graft-versus-host disease in lymphoma patients undergoing matched unrelated hematopoietic stem cell transplantation. *Exp Hematol* (2016) 44(624–34):e1. doi:10.1016/j.exphem.2016.03.005
95. Sang W, Zhang C, Zhang D, Wang Y, Sun C, Niu M, et al. MicroRNA-181a, a potential diagnosis marker, alleviates acute graft versus host disease by regulating IFN-gamma production. *Am J Hematol* (2015) 90:998–1007. doi:10.1002/ajh.24136
96. Wang Y, Zhao X, Ye X, Luo H, Zhao T, Diao Y, et al. Plasma microRNA-586 is a new biomarker for acute graft-versus-host disease. *Ann Hematol* (2015) 94:1505–14. doi:10.1007/s00277-015-2414-z
97. Wang K, Yuan Y, Cho JH, McClarty S, Baxter D, Galas DJ. Comparing the microRNA spectrum between serum and plasma. *PLoS One* (2012) 7:e41561. doi:10.1371/journal.pone.0041561
98. Ranganathan P, Heaphy CE, Costinean S, Stauffer N, Na C, Hamadani M, et al. Regulation of acute graft-versus-host disease by microRNA-155. *Blood* (2012) 119:4786–97. doi:10.1182/blood-2011-10-387522
99. Rodriguez A, Vigorito E, Clare S, Warren MV, Couttet P, Soond DR, et al. Requirement of bic/microRNA-155 for normal immune function. *Science* (2007) 316:608–11. doi:10.1126/science.1139253
100. O'Connell RM, Taganov KD, Boldin MP, Cheng G, Baltimore D. MicroRNA-155 is induced during the macrophage inflammatory response. *Proc Natl Acad Sci U S A* (2007) 104:1604–9. doi:10.1073/pnas.0610731104
101. Ceppi M, Pereira PM, Dunand-Sauthier I, Barras E, Reith W, Santos MA, et al. MicroRNA-155 modulates the interleukin-1 signaling pathway in activated human monocyte-derived dendritic cells. *Proc Natl Acad Sci U S A* (2009) 106:2735–40. doi:10.1073/pnas.0811073106
102. Huffaker TB, Hu R, Runtsch MC, Bake E, Chen X, Zhao J, et al. Epistasis between microRNAs 155 and 146a during T cell-mediated antitumor immunity. *Cell Rep* (2012) 2:1697–709. doi:10.1016/j.celrep.2012.10.025
103. Weber JA, Baxter DH, Zhang S, Huang DY, Huang KH, Lee MJ, et al. The microRNA spectrum in 12 body fluids. *Clin Chem* (2010) 56:1733–41. doi:10.1373/clinchem.2010.147405
104. Kohlhaas S, Garden OA, Scudamore C, Turner M, Okkenhaug K, Vigorito E. Cutting edge: the Foxp3 target miR-155 contributes to the development of regulatory T cells. *J Immunol* (2009) 182:2578–82. doi:10.4049/jimmunol.0803162
105. Lu LF, Thai TH, Calado DP, Chaudhry A, Kubo M, Tanaka K, et al. Foxp3-dependent microRNA155 confers competitive fitness to regulatory T cells by targeting SOCS1 protein. *Immunity* (2009) 30:80–91. doi:10.1016/j.immuni.2008.11.010
106. Dudda JC, Salaun B, Ji Y, Palmer DC, Monnot GC, Merck E, et al. MicroRNA-155 is required for effector CD8+ T cell responses to virus infection and cancer. *Immunity* (2013) 38:742–53. doi:10.1016/j.immuni.2012.12.006
107. Tarasenko T, Kole HK, Chi AW, Mentink-Kane MM, Wynn TA, Bolland S. T cell-specific deletion of the inositol phosphatase SHIP reveals its role in regulating Th1/Th2 and cytotoxic responses. *Proc Natl Acad Sci U S A* (2007) 104:11382–7. doi:10.1073/pnas.0704853104
108. De Guire V, Robitaille R, Tetreault N, Guerin R, Menard C, Bambace N, et al. Circulating miRNAs as sensitive and specific biomarkers for the diagnosis and monitoring of human diseases: promises and challenges. *Clin Biochem* (2013) 46:846–60. doi:10.1016/j.clinbiochem.2013.03.015
109. Kim DJ, Linnstaedt S, Palma J, Park JC, Ntrivalas E, Kwak-Kim JY, et al. Plasma components affect accuracy of circulating cancer-related microRNA quantitation. *J Mol Diagn* (2012) 14:71–80. doi:10.1016/j.jmoldx.2011.09.002
110. McDonald JS, Milosevic D, Reddi HV, Grebe SK, Algeciras-Schimnich A. Analysis of circulating microRNA: preanalytical and analytical challenges. *Clin Chem* (2011) 57:833–40. doi:10.1373/clinchem.2010.157198
111. Pritchard CC, Kroh E, Wood B, Arroyo JD, Dougherty KJ, Miyaji MM, et al. Blood cell origin of circulating microRNAs: a cautionary note for cancer biomarker studies. *Cancer Prev Res (Phila)* (2012) 5:492–7. doi:10.1158/1940-6207.CAPR-11-0370
112. Leidner RS, Li L, Thompson CL. Dampening enthusiasm for circulating microRNA in breast cancer. *PLoS One* (2013) 8:e57841. doi:10.1371/journal.pone.0057841
113. Sapre N, Selth LA. Circulating MicroRNAs as biomarkers of prostate cancer: the state of play. *Prostate Cancer* (2013) 2013:539680. doi:10.1155/2013/539680
114. Huang X, Liang M, Dittmar R, Wang L. Extracellular microRNAs in urologic malignancies: chances and challenges. *Int J Mol Sci* (2013) 14:14785–99. doi:10.3390/ijms140714785
115. Mestdagh P, Hartmann N, Baeriswyl L, Andreasen D, Bernard N, Chen C, et al. Evaluation of quantitative miRNA expression platforms in the microRNA quality control (miRQC) study. *Nat Methods* (2014) 11:809–15. doi:10.1038/nmeth.3014
116. Kaiser T, Kamal H, Rank A, Kolb HJ, Holler E, Ganser A, et al. Proteomics applied to the clinical follow-up of patients after allogeneic hematopoietic stem cell transplantation. *Blood* (2004) 104:340–9. doi:10.1182/blood-2004-02-0518
117. Weissinger EM, Schiffer E, Hertenstein B, Ferrara JL, Holler E, Stadler M, et al. Proteomic patterns predict acute graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. *Blood* (2007) 109:5511–9. doi:10.1182/blood-2007-01-069757
118. Weissinger EM, Metzger J, Dobbelstein C, Wolff D, Schleuning M, Kuzmina Z, et al. Proteomic peptide profiling for preemptive diagnosis of acute graft-versus-host disease after allogeneic stem cell transplantation. *Leukemia* (2014) 28:842–52. doi:10.1038/leu.2013.210
119. Weissinger EM, Mischak H, Kontsendorn J, Hahn A, Hahn N, Morgan M, et al. Proteome analysis in hematology using capillary electrophoresis coupled on-line to mass spectrometry. *Mini Rev Med Chem* (2009) 9:627–623. doi:10.2174/138955709788167583
120. Weissinger EM, Mischak H. Application of proteomics to post-transplantational follow-up. *Methods Mol Med* (2007) 134:217–28. doi:10.1007/978-1-59745-223-6_15
121. Weissinger EM, Hertenstein B, Mischak H, Ganser A. Online coupling of capillary electrophoresis with mass spectrometry for the identification of biomarkers for clinical diagnosis. *Expert Rev Proteomics* (2005) 2:639–47. doi:10.1586/14789450.2.5.639
122. Weissinger EM, Mischak H, Ganser A, Hertenstein B. Value of proteomics applied to the follow-up in stem cell transplantation. *Ann Hematol* (2006) 85:205–11. doi:10.1007/s00277-005-0057-1
123. Weissinger EM, Dickinson AM. Immunogenomics and proteomics in hematopoietic stem cell transplantation: predicting post-hematopoietic stem cell transplant complications. *Cancer Treat Res* (2009) 144:95–129. doi:10.1007/978-0-387-78580-6_5
124. Pavletic SZ, Martin P, Lee SJ, Mitchell S, Jacobsohn D, Cowen EW, et al. Measuring therapeutic response in chronic graft-versus-host disease: National Institutes of Health consensus development project on criteria

for clinical trials in chronic graft-versus-host disease: IV. Response criteria working group report. *Biol Blood Marrow Transplant* (2006) 12:252–66. doi:10.1016/j.bbmt.2006.01.008

125. Jacobsohn DA, Kurland BF, Pidala J, Inamoto Y, Chai X, Palmer JM, et al. Correlation between NIH composite skin score, patient-reported skin score, and outcome: results from the Chronic GVHD Consortium. *Blood* (2012) 120:2545–52;quiz774. doi:10.1182/blood-2012-04-424135
126. Landfried K, Wolff D, Holler E. Pathophysiology and management of graft-versus-host disease in the era of reduced-intensity conditioning. *Curr Opin Oncol* (2009) 21(Suppl 1):S39–41. doi:10.1097/01.cco.0000357475.66035.d2
127. Wolff D, Steiner B, Hildebrandt G, Edinger M, Holler E. Pharmaceutical and cellular strategies in prophylaxis and treatment of graft-versus-host disease. *Curr Pharm Des* (2009) 15:1974–97. doi:10.2174/138161209788453158
128. Wolff D, Gerbitz A, Ayuk F, Kiani A, Hildebrandt GC, Vogelsang GB, et al. Consensus conference on clinical practice in chronic graft-versus-host disease (GVHD): first-line and topical treatment of chronic GVHD. *Biol Blood Marrow Transplant* (2010) 16:1611–28. doi:10.1016/j.bbmt.2010.06.015
129. Weissinger EM, Zurbig P, Ganser A. Proteomics studies after hematopoietic stem cell transplantation. *Methods Mol Biol* (2009) 506:437–52. doi:10.1007/978-1-59745-409-4_29
130. Forcade E, Kim HT, Cutler C, Wang K, Alho AC, Nikiforow S, et al. Circulating T follicular helper cells with increased function during chronic graft-versus-host disease. *Blood* (2016) 127:2489–97. doi:10.1182/blood-2015-12-688895
131. Jenq RR, Taur Y, Devlin SM, Ponce DM, Goldberg JD, Ahr KF, et al. Intestinal *Blautia* is associated with reduced death from graft-versus-host disease. *Biol Blood Marrow Transplant* (2015) 21:1373–83. doi:10.1016/j.bbmt.2015.04.016
132. Taur Y, Jenq RR, Ubeda C, van den Brink M, Pamer EG. Role of intestinal microbiota in transplantation outcomes. *Best Pract Res Clin Haematol* (2015) 28:155–61. doi:10.1016/j.beha.2015.10.013
133. Holler E, Butzhammer P, Schmid K, Hundsrucker C, Koestler J, Peter K, et al. Metagenomic analysis of the stool microbiome in patients receiving allogeneic stem cell transplantation: loss of diversity is associated with use of systemic antibiotics and more pronounced in gastrointestinal graft-versus-host disease. *Biol Blood Marrow Transplant* (2014) 20:640–5. doi:10.1016/j.bbmt.2014.01.030
134. Taur Y, Jenq RR, Perales MA, Littmann ER, Morjaria S, Ling L, et al. The effects of intestinal tract bacterial diversity on mortality following allogeneic hematopoietic stem cell transplantation. *Blood* (2014) 124:1174–82. doi:10.1182/blood-2014-02-554725
135. Weber D, Oefner PJ, Hiergeist A, Koestler J, Gessner A, Weber M, et al. Low urinary indoxyl sulfate levels early after transplantation reflect a disrupted microbiome and are associated with poor outcome. *Blood* (2015) 126:1723–8. doi:10.1182/blood-2015-04-638858
136. Holtan SG, DeFor TE, Lazaryan A, Bejanyan N, Arora M, Brunstein CG, et al. Composite end point of graft-versus-host disease-free, relapse-free survival after allogeneic hematopoietic cell transplantation. *Blood* (2015) 125:1333–8. doi:10.1182/blood-2014-10-609032
137. Rodriguez-Otero P, Porcher R, Peffault de Latour R, Contreras M, Bouhnik Y, Xhaard A, et al. Fecal calprotectin and alpha-1 antitrypsin predict severity and response to corticosteroids in gastrointestinal graft-versus-host disease. *Blood* (2012) 119:5909–17. doi:10.1182/blood-2011-12-397968
138. Ayuk F, Bussmann L, Zabelina T, Veit R, Alchalby H, Wolschke C, et al. Serum albumin level predicts survival of patients with gastrointestinal acute graft-versus-host disease after allogeneic stem cell transplantation. *Ann Hematol* (2014) 93:855–61. doi:10.1007/s00277-013-1957-0
139. Rezvani AR, Storer BE, Storb RF, Mielcarek M, Maloney DG, Sandmaier BM, et al. Decreased serum albumin as a biomarker for severe acute graft-versus-host disease after reduced-intensity allogeneic hematopoietic cell transplantation. *Biol Blood Marrow Transplant* (2011) 17:1594–601. doi:10.1016/j.bbmt.2011.07.021
140. MacMillan ML, Robin M, Harris AC, DeFor TE, Martin PJ, Alousi A, et al. A refined risk score for acute graft-versus-host disease that predicts response to initial therapy, survival, and transplant-related mortality. *Biol Blood Marrow Transplant* (2015) 21:761–7. doi:10.1016/j.bbmt.2015.01.001

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REVIEW
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Genetic Association of Hematopoietic Stem Cell Transplantation Outcome beyond Histocompatibility Genes

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The outcome of hematopoietic stem cell transplantation (HSCT) is controlled by genetic factors among which the leukocyte antigen human leukocyte antigen (HLA) matching is most important. In addition, minor histocompatibility antigens and non-HLA gene polymorphisms in genes controlling immune responses are known to contribute to the risks associated with HSCT. Besides single-nucleotide polymorphisms (SNPs) in protein coding genes, SNPs in regulatory elements such as microRNAs (miRNAs) contribute to these genetic risks. However, genetic risks require for their realization the expression of the respective gene or miRNA. Thus, gene and miRNA expression studies may help to identify genes and SNPs that indeed affect the outcome of HSCT. In this review, we summarize gene expression profiling studies that were performed in recent years in both patients and animal models to identify genes regulated during HSCT. We discuss SNP–mRNA–miRNA regulatory networks and their contribution to the risks associated with HSCT in specific examples, including forkheadbox protein 3 and regulatory T cells, the role of the miR-155 and miR-146a regulatory network for graft-versus-host disease, and the function of MICA and its receptor NKG2D for the outcome of HSCT. These examples demonstrate how SNPs affect expression or function of proteins that modulate the allo-immune response and influence the outcome of HSCT. Specific miRNAs targeting these genes and directly affecting expression of mRNAs are identified. It might be valuable in the future to determine SNPs and to analyze miRNA and mRNA expression in parallel in cohorts of HSCT patients to further elucidate genetic risks of HSCT.

Keywords: gene expression profiling, regulatory networks, non-human leukocyte antigen single-nucleotide polymorphisms, microRNAs, forkheadbox protein 3, miR-155, miR-146a, MICA

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INTRODUCTION

A considerable proportion of the risk of adverse outcome after hematopoietic stem cell transplantation (HSCT) is genetically determined and can be attributed to various factors including human leukocyte antigen (HLA) matching, killer-immunoglobulin-like receptor matching, minor histocompatibility antigens (miHAg), and non-HLA gene polymorphisms. Outcomes such as acute and chronic graft-versus-host disease (aGvHD and cGvHD), relapse, and survival have been shown to be modified by

functionally relevant polymorphisms in non-HLA genes that are involved in immune responses (1, 2). Such functional polymorphisms are complicated to pinpoint among other polymorphisms localized near these genes that have no direct effects on gene function. Reliable identification of polymorphisms that result in differences in gene expression or protein function and affect the outcome of HSCT is challenging in view of the complexity of the human genome (3).

The most frequent genetic variations of the human genome are single-nucleotide polymorphisms (SNPs), which occur on average in 1 out of 300 bp throughout the genome (4–6). The majority of the SNPs arise in non-coding regions including intronic, intergenic, and untranslated regions (UTRs) (7). Those which are within genes, including genes affecting the immune response, may alter the expression of the gene or the structure of the encoded proteins (8). In microRNAs (miRNAs), SNPs can alter regulatory properties, but elucidation of the functions of these SNPs is not straight forward (9). Understanding the biogenesis of miRNAs is key to comprehending the impact of SNPs on these molecules (**Figure 1**). The miRNAs are a class of small endogenous non-coding RNAs of 21–25 nucleotides in length that originate as primary transcripts (pri-miRNAs) from miRNA genes. After transcription of pri-miRNAs by RNA polymerase II, they are processed by DROSHA, a RNA specific ribonuclease enzyme complex, producing short precursor-miRNAs (pre-miRNAs) of approximately 70 nucleotides length (10). The pre-miRNAs are then transported from the nucleus to the cytoplasm by exportin 5 (10). In the cytoplasm, they undergo further cleavage by an endonuclease enzyme (DICER), resulting in the generation of mature miRNA (11, 12). Accordingly, functionally relevant SNPs can be present in miRNA biogenesis-related genes, in specific miRNA-encoding genomic loci or in the seed match sequence of target mRNA 3′ UTRs. SNPs may lead to either an alteration in miRNA expression level, a decreased or increased miRNA-target interaction, or a new miRNA-target interaction (13). Atarod and Dickinson (14) described the driving gears of GvHD as miRNA's regulating gene expression, chemokine and cytokine secretion, while their expression in turn is affected by SNPs in mRNA genes (**Figure 2**).

In this review, we will pinpoint SNP–mRNA–miRNA regulatory network alterations and their contribution to the risks associated with HSCT in specific examples, elucidating the consequences of the interaction between these three genetic elements. Moreover, we will summarize mRNA and miRNA profiling studies aiming to decipher genetic risks of HSCT.

EXAMPLES OF SNP–mRNA–miRNA REGULATORY NETWORKS CONTROLLING OUTCOME OF HSCT

Forkheadbox Protein 3 (FOXP3) Polymorphisms, mRNA Expression, and FOXP3-Regulating miRNAs

Regulatory T cells (T_{regs}) have been the focus of several HSCT studies due to their ability to suppress alloreactivity during GvHD (16). T_{regs}, defined as $CD4^+CD25^+$ $FOXP3^+$ T cells, are involved in the maintenance of immunological tolerance (17). They reduce the invasion of $CD8^+$ effector T cells (T_{eff}) in target tissue and ameliorate GvH tissue damage (18). Cuzzola and colleagues found an increased expression of *FOXP3* mRNA in patients who were responsive to anti-GvHD therapies (19). These results are in concordance with previous data reporting an inverse correlation between the amount of T_{regs} and progression of aGvHD (20). Other studies showed correlation between a lower incidence of aGvHD and improved survival in HSCT recipients with an increased number of donor T_{regs} (21, 22). Low numbers of T_{regs} have also been associated with higher cGvHD incidence (23). Similarly, the severity of aGvHD and extent of cGvHD in patients were found to be associated with T_{reg} numbers (24). Furthermore, inducing selective expansion of T_{regs} by the daily administration of low doses of interleukin (IL)-2 showed an improvement in clinical cGvHD symptoms in patients (25). Notably, not only $CD4^+$ T_{regs} can mitigate GvHD but also $CD8^+FOXP3^+$ T_{regs} become induced during GvHD and can suppress the disease in mouse models (26, 27). $CD8^+$ T_{regs} might have even advantageous over $CD4^+$ T_{regs} since they have been reported not to abrogate graft-versus-leukemia (GvL) effects (28). The potency of $CD8^+$ T_{regs} cells is further emphasized by their ability to prevent the rejection of heart allografts in rats (29).

Currently, 90 SNPs have been identified in the *FOXP3* gene region, and several have been identified as risk factors for a number of malignant and autoimmune diseases (30). An SNP (rs3761548) in the promoter region of *FOXP3* (**Figure 3**) resulting in an *A*/*C* base exchange causes loss of binding to the E47 and c-Myb factors and leads to defective transcription of the *FOXP3* gene (31). In patients undergoing HSCT, this SNP has been associated with a higher incidence of hepatic veno-occlusive disease and cytomegalovirus (CMV) infection but a lower treatment-related mortality, resulting in a difference in the overall survival of patients with the *CC* genotype (32). However, the authors found no difference in the incidence of GvHD, relapse, or blood stream infection to be associated with this polymorphism (32).

Posttransplant chimerism analysis in clinical HSCT largely uses polymorphisms of short tandem repeats of <10 nucleotides, or microsatellites (Msat). They have a higher degree of allelic polymorphism compared to SNPs, and therefore, a larger degree of information (33). An Msat studied in *FOXP3* is the (*GT*)n polymorphism in the promoter/enhancer region of the *FOXP3* gene (34). This polymorphism was shown to be associated with the development of auto- or alloimmune conditions, including type I diabetes, and graft rejection in renal transplant recipients (35). Moreover, this polymorphism has been associated with a lower incidence of grade III–IV GvHD in patients transplanted from donors carrying short alleles [≤(*GT*)15] (35). However, this polymorphism did not affect relapse, event free survival or overall survival in patients with aGvHD and cGvHD (35).

Recently, several miRNAs including miR-155 and miR-10a have been identified that impact T cell differentiation and function (36, 37). MiR-155 is required for T_{reg} development (38) and for maintaining T_{reg} homeostasis and survival by targeting *SOCS1* (39). MiR-155 is an important positive regulator of natural T_{reg} (nT_{reg}) development and miR-155 gene transcription is driven by FOXP3 (**Figure 3**) (38). In mice, miR-155 is upregulated in

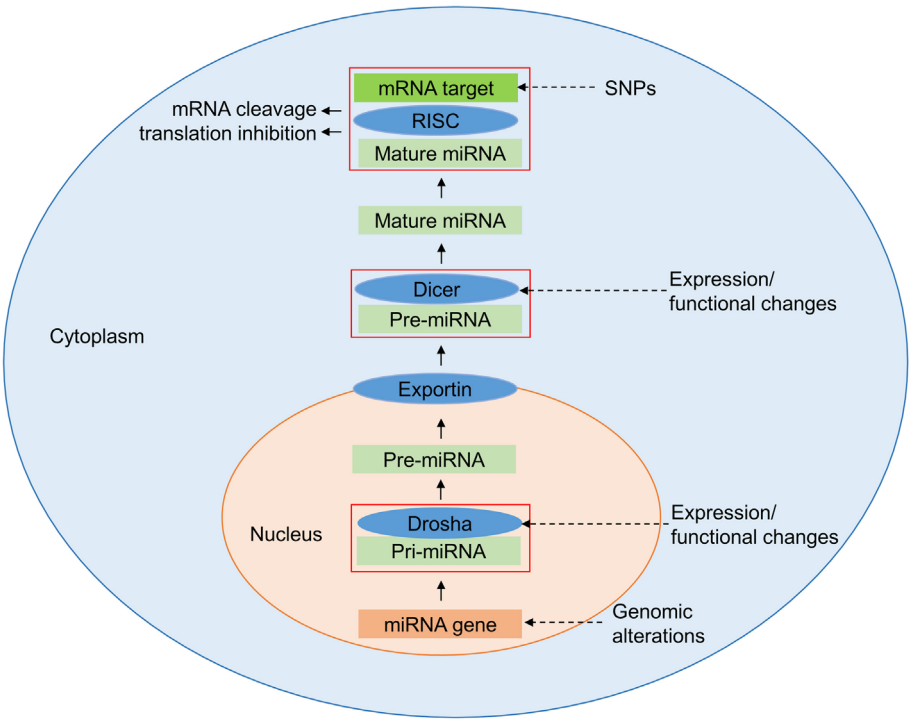

FIGURE 1 | Regulation of microRNAs (miRNAs). Expression of miRNAs can be altered at various stages of its biogenesis by genomic [single-nucleotide polymorphisms (SNPs) and mutations] and epigenetic alterations. Changes in the expression and function of Drosha and Dicer, part of the miRNA processing machinery, lead to the deregulation of mature miRNAs. The figure has been adapted from Ref. (14, 15).

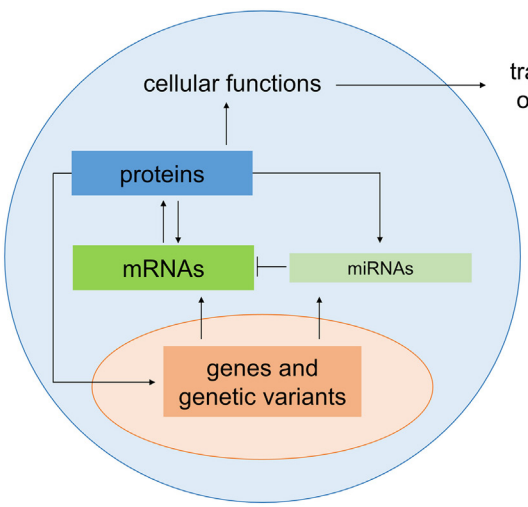

FIGURE 2 | Genetic regulation of transplant outcome. Genetic variants in protein encoding genes and microRNAs (miRNAs) alter gene expression as well as protein and cellular functions which in turn contribute to the regulating the outcome of transplantation. The color code introduced here is used for genes, miRNAs, mRNAs, and proteins in **Figures 3–5**.

mature T_{regs} ($CD4^+CD25^+FOXP3^+$) relative to conventional T cells ($CD4^+CD25^-FOXP3^-$) as well as in $FOXP3^+$ double positive and single positive thymocytes (37, 40). MiR-155 knockout mice have reduced T_{reg} numbers in both the thymus and periphery, and miR-155-deficient T_{regs} have a reduced proliferative potential and impaired IL-2 signaling (39). In this context, miR-155 promotes T_{reg} survival and proliferation in the thymus and periphery by enhancing their sensitivity to IL-2 (39). As shown in **Figure 3**, miR-155 achieves this by targeting and downregulating *SOCS1*, an inhibitor of IL-2 signaling, thus increasing levels of activated STAT5 and enhancing IL-2 signaling (40). MiR-10a is functionally linked to stabilization of FOXP3 in T_{regs} (41) (**Figure 3**) and interestingly, although miR-10a has not been specifically investigated in relation to HSCT, an inverse correlation between miR-10a and susceptibility to autoimmune disease has been identified (41). With regard to miR-10a, it is uniquely expressed in T_{regs}, but not other T cells, where it is crucial for long-term maintenance of their stability and function (41). *FOXP3* itself can be regulated by other miRNAs, including miR-21 and miR-31, which positively and negatively regulate *FOXP3*, respectively, thus having opposing effects on its expression (36) (**Figure 3**). MiR-31 can directly target *FOXP3* by binding to a specific recognition site in the 3′ UTR region, while miR-21 regulation is believed to be indirect as no potential target sequence in *FOXP3* was identified (36). The specific function of miR-21 and miR-31 in T_{regs} in the setting of HSCT is still to be explored.

The miR-155 and miR-146a Regulatory Network

Notably, miR-155 is involved in a larger regulatory network that affects the outcome of HSCT (**Figure 4**). Although miR-155

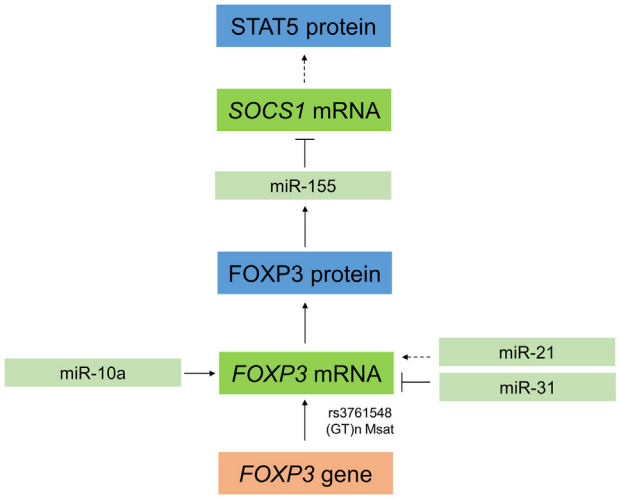

FIGURE 3 | Interaction between microRNAs and forkheadbox protein 3 (FOXP3) in regulatory T cells (T_{regs}). In T_{regs}, miR-10a stabilizes *FOXP3*, and FOXP3 can positively regulate expression of miR-155. This leads to a downregulation of the target *SOCS1* (T), which in turn results indirectly in increased expression of STAT5. *FOXP3* can also be regulated by miR-21, which indirectly positively regulates *FOXP3* in a process that is not yet completely understood. Moreover, *FOXP3* is downregulated by miR-31 by direct targeting of the 3′ untranslated region. To further complicate this regulatory network, single-nucleotide polymorphisms (SNPs) in *FOXP3* also affect its expression in T_{regs}, such as the SNP rs3761548 or a GT(n) microsatellites (Msat).

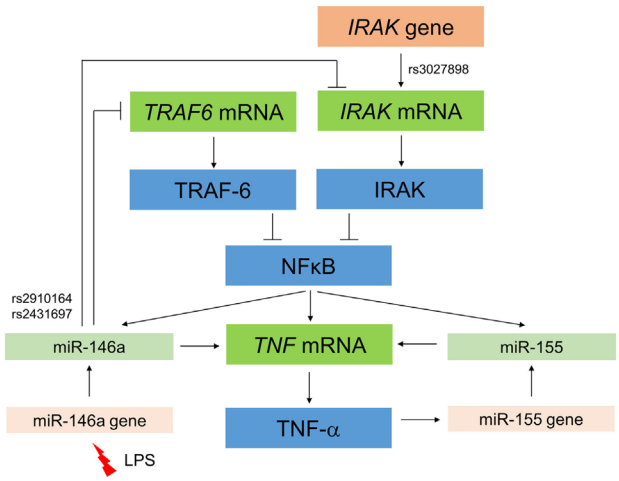

FIGURE 4 | Interaction between miR-146 and miR-155, their effects on the nuclear factor (NF)-kB pathway, and the expression of IRAK1 and tumor necrosis factor (TNF)-α. Activation of the NF-kB pathway represents a hallmark of the pathophysiology of GvHD. NF-kB activation induces expression of miR-146a and in turn, miR-146a inhibits these pathways through targeting key adapter proteins, IRAK1(⊥) and TRAF6 (⊥). The presence of single-nucleotide polymorphisms in coding regions of these genes, such as rs3027898 in *IRAK1*, further influences expression within the network. MiR-146a expression can also be stimulated by lipopolysaccharide (LPS) release during GvHD conditioning. The miR-146a and miR-155 mediate an increase in TNF-α, which in turn can positively regulate miR-155 in a feedback loop.

promotes the development of T_{regs} as explained above, it may also have pro-inflammatory functions. MiR-155 and miR-146a were found to be upregulated in the skin of rats suffering from aGVHD (42). Atarod and colleagues showed that low expression levels of both miR-155 and miR-146a were associated with higher incidence of aGVHD at day 28 post-allo-HSCT in patients and that both regulate expression of the transcription factor SPI1 (43). Pontoppidan and colleagues showed that miR-155 was increased in patient plasma at the time of maximal toxicity of preconditioning at day 7 posttransplantation and remained increased until day 21. This was inversely mirrored by miR-146a, which was significantly reduced from day 7 to day 21 after transplantation (44). Together, this suggests that miR-155 and miR-146a play opposite roles having pro-inflammatory and anti-inflammatory properties, respectively, and thus play a role in regulating the systemic inflammatory response during maximum toxicity of preconditioning in HSCT patients (44). Relevantly, miR-155 expression was upregulated in donor T cells in mice during aGvHD and mice receiving miR-155-deficient splenocytes developed less severe aGvHD and had increased survival rates compared to mice receiving wild type splenocytes (45). Specific targeting of miR-155 using antagomirs effectively mitigated aGvHD in mice and increased survival rates (45). MiR-155-deficiency in the dendritic cell (DC) compartment also protected mice from aGvHD since miR-155 appears to promote the migration of DC toward sites of tissue damage (46). MiR-155 expression was increased in mouse T cells as well as in intestinal patient biopsies during aGvHD (45). Expression of miR-155 can be stimulated by tumor necrosis factor (TNF) α (47), and similarly, miR-155 can promote TNF-α production in a positive feedback loop (48), thus exacerbating the inflammatory cascade (**Figure 4**). Altogether, these data indicate a role for miR-155 in the modulation of aGvHD.

MiR-146a is distinctly increased in response to lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria. LPS is released in response to GvHD conditioning regimens (**Figure 4**) and acts as a potent enhancer of cytokine secretion (49). Using a genetically engineered mouse model, it was demonstrated that a deletion of miR-146a results in several immune pathologies (50). Specifically, lack of miR-146a expression increased responsiveness of macrophages to LPS and exacerbated the inflammatory response in LPS-challenged mice. TNF receptor-associated factor 6 (*Traf6*) and IL-1 receptor-associated kinase 1 (*Irak1*) genes have also been identified as targets of miR-146a (**Figure 4**), contributing to the phenotype of miR-146a-deficient mice (50). Both TRAF6 and IRAK1 act as adapter proteins in the nuclear factor (NF)-κB activation pathway and in addition to innate immune cells, miR-146a has also been shown to target these genes in T cells resulting in their downregulation (**Figure 4**). T cells that are lacking miR-146a are hyperactive in both acute antigenic responses and chronic inflammatory autoimmune responses (51). However, the presence of SNPs in these genes that may affect miR-146a binding, such as rs3027898 in the *IRAK1* 3′UTR may further complicate this already complex network of interactions. Furthermore, activation of NF-κB has been described to upregulate miR-146a expression, which in turn downregulated NF-κB *via TRAF6* and

IRAK1 repression in a negative feedback loop (52, 53) (**Figure 4**). More recently, miR-146a regulation of *TRAF6* and *IRAK1* was specifically associated with aGvHD, since upregulation of miR-146a expression was observed in T cells of mice developing aGvHD compared to untreated mice (52). When transplanted with miR-146a-deficient T cells, recipient mice developed GvHD of increased severity resulting in reduced survival, and they also had elevated TNF-α serum levels (52). The protein levels of TRAF6 were upregulated in miR-146a-deficient T cells following alloantigen stimulation, and this translated into increased NF-κB activity. Conversely, miR-146a overexpression reduced aGvHD severity. In an autoimmune setting, miR-146a expression can be induced by TNF-α (47), which in turn is stimulated by NF-κB, thus further confounding this complex regulatory network (**Figure 4**). Interestingly, another member of the miR-146 family, i.e., miR-146b, is highly expressed in $CD4^+CD25^+FOXP3^+$ thymic-derived T_{regs} and has been reported to promote survival, proliferation, and suppressor function of these cells by targeting *TRAF6* and subsequently increasing NF-κB activity (54).

Single-nucleotide polymorphisms within miRNA coding regions as well as those within their target mRNA seed regions can directly influence miRNA–mRNA interactions. Indeed, with regard to miR-146a, two SNPs rs2431697 and rs2910164 have been reported that cause single base changes and altered expression of the mature microRNA (**Figure 4**). The SNP rs2910164 specifically results in a change from a G:U pair to a C:U mismatch in the stem structure of the miR-146a precursor. This results in processing variation and lower expression of the mature miRNA, which has been associated with the development of a range of cancers (55). Stickel and colleagues reported that the minor *CC* genotype caused a decrease in miR-146a production (52). The same team also provided evidence that miR-146a acts as an important negative regulator in murine and human GVHD, consistent with an anti-inflammatory role for miR-146a, and suggested the exogenous increase of miR-146a as a potential novel strategy for therapeutic intervention in this disease (52). Further interactions that have been described between miRNAs and the induction of GvHD have been recently reviewed by Atarod and Dickinson (14).

MICA Polymorphisms, mRNA Expression, and MICA-Regulating miRNAs

The major histocompatibility complex (MHC) class I chain-related molecule A (MICA) is a highly polymorphic ligand for the activating natural killer (NK) cell receptor NKG2D (**Figure 5**). An SNP within this gene, rs1051792, which leads to an amino acid exchange from valine to methionine at position 129 (56), was investigated for its association with the outcome of HSCT. We found that the MICA-129Met variant was associated with an increased overall survival and a reduced risk to die from aGvHD, despite homozygous carriers of the *MICA-129Val* allele having an increased risk of developing aGvHD (57). The NKG2D pathway was expected to be directly related to the outcome of HSCT, since it is an activating receptor on NK cells (58) and a costimulatory receptor on $CD8^+$ T cells (59). On the functional level, we found the MICA-129Met isoform triggered more cytotoxicity and interferon (IFN)-γ release by NK cells and it activated alloreactive cytotoxic T cells faster. This variant also induced more rapid and severe downregulation of NKG2D on NK and cytotoxic T cells (57). Normally, most cell types do not express MICA, but it becomes induced by cellular and genotoxic stress, including virus infection and malignant transformation. Therefore, it renders stressed cells susceptible to killing by NK cells and allows them, despite being non-professional antigen presenting cells (APCs), to directly activate cytotoxic T cells specific for antigens presented by these cells. Notably, MICA expression was found to be increased in GvHD-affected tissue samples from patients (60). The MICA-129Met variant can therefore initially confer a higher risk of aGvHD due to a faster activation of alloreactive cytotoxic T cells (57). However, in the longer perspective, the strong-counter regulation of NKG2D by this variant appears to be associated with a decreased risk of cGvHD and an increased risk of relapse due to lesser GvL effects by cytotoxic T cells and NK cells (61).

Interestingly, the biological effects of the MICA-129 variants were strongly influenced by MICA expression intensity (57). The MICA-129Met variant triggered increased NKG2D signals at low expression intensities, whereas the MICA-129Val variant elicited more NKG2D-mediated effects at high expression intensities. At high expression intensity, the functional effects of the MICA-129Met variant were impaired due to a rapid downregulation of NKG2D (57). Thus, MICA expression intensity could change the biological effect of this SNP, giving an interesting example of the

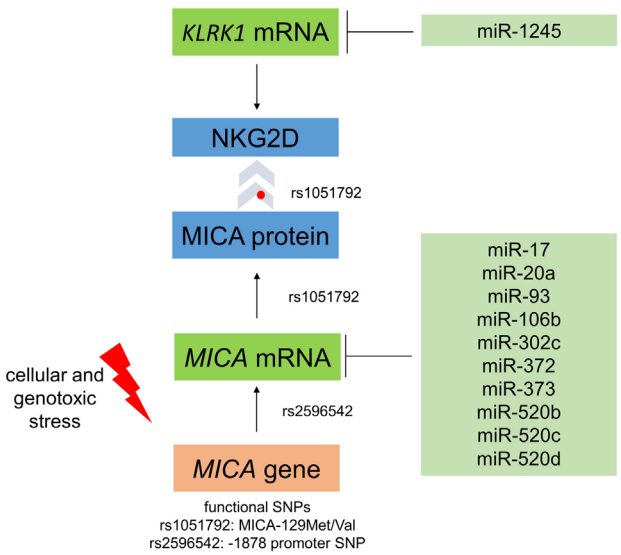

FIGURE 5 | Regulation of MICA expression and interaction with NKG2D. The single-nucleotide polymorphism (SNP) rs1051792 results in a valine to a methionine exchange at position 129 of MICA and distinguishes MICA variants into those binding the receptor NKG2D with high (MICA-129Met) or low (MICA-129Val) avidity. This polymorphism also affects the cell surface expression of MICA protein. The SNP rs2596542 in the promoter of *MICA* affects mRNA expression. Moreover, several microRNAs target *MICA* and downregulate its expression. Moreover, cellular and genotoxic stress induces the expression of MICA. The MICA receptor NKG2D is encoded by the *KLRK1* gene and can be targeted by miR-1245.

complex functional interactions between SNPs and gene expression (**Figure 5**). Moreover, the SNP might interact with other SNPs in the NKG2D signaling pathway (62) including *KLRK1*, encoding NKG2D. Polymorphisms in the *KLRK1* locus have been described also to affect the outcome of HSCT (63).

Notably, MICA expression intensities can vary for certain *MICA* alleles (64). The SNP at −1878 (rs2596542) in the promoter region of the *MICA* gene was described to affect the transcriptional activity (65). A polymorphic microsatellite in exon 5 encoding the transmembrane region of MICA modifies its plasma membrane expression (66). We have recently shown that the MICA-129Met/Val dimorphism also affects plasma membrane expression. Increased levels of the MICA-129Met variant were retained intracellularly and if expressed at the cell surface, the MICA-129Met variant was more prone to shedding than the MICA-129Val isoform (67).

Matching of donor and recipient for *MICA* alleles (68–71) and specifically for the *MICA-129*, polymorphism (72) has been found to be beneficial in HSCT, although not in all studies (73, 74). The effect of *MICA* matching appears hardly explainable solely by the avoidance of potential miHAg and further points toward an important biological function of MICA after HSCT.

Several stress pathways regulate the transcription of the *MICA* gene (75), and several miRNAs have been implicated in controlling MICA expression *via* posttranscriptional mechanisms (**Figure 5**). Stern-Ginossar and colleagues described that the expression of *MICA* was decreased by miR-17, miR-20a, miR-93, miR-106b, miR-372, miR-373, and miR-520d (76). Effects of miR-17 (77), miR-20a (77–80), miR-93 (77, 80–82), and miR-106b (80, 81) on *MICA* expression have also been confirmed in subsequent studies. Moreover, the IFN-γ-induced miR-520b can lead to a reduction in MICA plasma membrane expression intensity (83). Interestingly, miR-520d acts on both the *MICA* 3′-UTR and the promoter region to decrease *MICA* transcript levels (83). MiR-302c and miR-520c are two further miRNAs that can target *MICA* (84). Human CMV can also target MICA by US18 and US20, which promote the degradation of MICA in lysosomes (85). The regulation of NKG2D ligands by miRNAs has been recently reviewed in more detail (86, 87). Notably, also the expression of NKG2D has been found to be attenuated by miRNAs, specifically, miR-1245 (88).

miRNA AND mRNA EXPRESSION PROFILING IN HSCT

A number of large-scale gene expression profiling studies have been performed in both patients and animal models to identify genes regulated during HSCT. In animals, both acute and chronic GvHD models were investigated. Studies performed on human samples mostly used blood or in cases of cGvHD, conjunctiva from patients. The advantage of using animal models for gene expression is the broad availability of specific target tissues of GvHD, such as liver and skin (89). A selection of the most relevant gene expression profiling studies is listed in **Table 1**. MiRNAs have also been studied in relation to HSCT, and there is increasing evidence to show that miRNAs are present in plasma, serum, saliva, urine, and other body fluids in a remarkably stable form that is protected from endogenous RNase activity (90). Circulating miRNAs have the potential to serve as novel and non-invasive biomarkers for various diseases such as cancer, cardiovascular disease, and organ transplant rejection and infection (91).

TABLE 1 | Summary of large-scale mRNA expression profiling studies during GvHD.

Species	Tissue	Disease	Upregulated	Downregulated	Technique	Reference
Human	Peripheral blood mononuclear cells (PBMCs)	Acute graft-versus-host disease (aGvHD)	*CXCL8*, *GOS2*, *ANXA3*, *NR4A2*	*CDKN1C*	qRT-PCR	(92)
Human	PBMC	aGvHD	*PCDHB5*, *IL22RA2*, *PDCDILG2*, *IL2*, *PKD1*	*PCDHB16*, *IL27*, *IGHD*, *CCL1*, *CF1*	Microarray	(93)
Human	$CD4^+$ and $CD8^+$ T cells	Chronic graft-versus-host disease (cGvHD)	*EP300*, *FURIN*, *FNBP3*, *SMAD3*, *TGFBI*, *TGIF*	*PRF1*	qRT-PCR	(94)
Human	PBMC	aGvHD	*TNFSF10/TRAIL*, *IL1RN*, *IFI27*, *GZMB*, *CCR5*	*CLK1*, *TNFAIP3*, and *BTG1*	Microarray	(95)
Human	PBMC	cGvHD	*IL21R IL18*, *CD28*, *IL17A*, *IL6R*, *PI3K*	*IFNG*, *CCl1*, *IRAK3*, *IL12B*, *SOC2*	Microarray	(96)
Human	Conjunctiva	cGvHD-DE	*CXCL9*, *CXCL10*, *CXCL11*, *CXCR3*		qRT-PCR	(97)
Human	Conjunctiva	GvHD-DE	*IL6*, *IL9*, *IL10*, *CCL24*, *CCL28*, *CCL2*	*EGFR*	qRT-PCR	(98)
Mouse	Ear skin day 7	aGvHD	*Saa3*, *Cxcl9*, *Cxcl10*, *Ccl5*, *Ubd*	*Trp1*, *Col1a1*	Microarray	(99)
	Ear skin day 40		*Ccl5*, *Saa3*, *Il1beta*, *Cxcl9*	*Ces3*		
Mouse	Liver day 7	aGvHD	*Vcam1*, *Cxcl9*, *Gbp2*, *Tgtp*, *Tap1*	*Lck*, *Ltf*	Microarray	(100)
	Liver day 35		*Ccl5*, *Icam1*	*Lck*, *Ltf*		
Mouse	Liver versus kidney	aGvHD	*Cxcl9*, *Cxcl10*, *Vcam1*, *Stat1*, *Icam1*		Microarray	(101)
Mouse	Scl[a] skin	GvHD	*CXCL5*, *CXCL11*, *CXCL9*, *CXCL10*, *IL6*, *TGF-BR1*	*Cdh5*, *Cdh13*	Microarray	(102)
Rat	Skin explant model	aGvHD	*RT1-CE10 Lst1*, *Ubd*, *Aif1*, *RT-DMb*	*Ly6g6e*, *Bat5*	Microarray	(103)

The listed mRNA profiling studies were carried out with human or rodent tissues obtained during both aGvHD and cGvHD. The genes regulated during GvHD with the highest fold changes and significant P-values are indicated.

[a]*Scl—sclerodermatous skin model of GvHD.*

miRNAs Targeting Immune-Related Genes and Their Application on Prediction of HSCT Outcome

In a recent clinical study, a micro-RNA-based model was developed to predict the probability of aGvHD comprising miR-423, miR-199a-3p, miR-93 and miR-377. Elevated levels of these miRNAs were detected in plasma before the onset of aGvHD (average 16 days before diagnosis), and their expression was associated with the severity of aGvHD as well as with a lower overall survival (91). In another profiling study of 48 miRNAs in the plasma of aGvHD patients, miR-586 expression was decreased upon the occurrence of aGvHD (104).

Interestingly, a recent publication by Wu and colleagues (105), which has been reviewed by Serody (106), has been shown that the miR-17–92 cluster, which is conserved among vertebrates, is important for the development of aGvHD. The miR-17–92 cluster is critical for the proliferation, survival and function of Th_1 and Th_{17} effector T cells and inhibits Th_2 and T_{reg} differentiation (107). It has now also been shown that this cluster of miRNAs promotes the migration of $CD8^+$ T cells to GvHD target organs and confers GvL effects (105). Donor T cells lacking the miR-17–92 complex gave rise to diminished GvHD in a mouse model of aGvHD. Blocking of miR-17 and miR-19b, which are included in the miR-17–92 complex, by systemic administration of antagomiRs (locked nucleic acid-modified oligonucleotides) significantly reduced aGvHD severity (105).

To better understand the regulation of neovascularization during GvHD, Leonhardt and colleagues focused on the role of miR-100 and showed that in intestinal tissue biopsies from patients undergoing allo-HSCT, miR-100 was downregulated when aGvHD evolved suggesting that this miRNA has a role as a negative regulator of aGvHD (108). MiR-100 was also downregulated in the inflamed intestinal tissue of mice developing aGvHD. In the mouse model, functional inactivation of miR-100 with antagomiRs enhanced aGvHD severity, indicating a protective role for miR-100 by blocking inflammatory neovascularization during aGvHD (108).

Other studies focusing on miR-34 showed that the miR-34 family mimics p53 effects by inducing cell cycle arrest and apoptosis in response to DNA damage (109). In the case of Fanconi anemia (FA), an inherited disorder characterized by developmental defects, genomic instability and progressive bone marrow failure (110), miR-34a expression in patient gut biopsies after HSCT was significantly higher in aGvHD grades II–IV compared to grade 0–I or with non-transplanted FA patient gut biopsies (111).

Association between Polymorphisms and Gene Expression Levels in Cytokine and Chemokine-Coding Genes with the Outcome of HSCT

Cytokine Gene Expression Variation and Its Impact on the Outcome of HSCT

Cytokines, such as INF-γ, IL-2, and TNF-α produced by Th_1 cells, contribute to the induction phase of aGvHD (112). A number of polymorphisms in genes encoding IL-10, TNF-α, and IL-6 have been linked to an increased risk of GvHD. IL-2 has a role as a T cell growth factor and treatment, and prophylaxis of aGvHD involves frequently inhibition of IL-2 production by cyclosporine A (113). Moreover, in both animal and human studies, administration of monoclonal antibodies against the IL-2 receptor after HSCT have prevented aGvHD occurrence (114, 115). On the other hand, emerging data show that IL-2 is also necessary for the generation and the maintenance of $CD4^+CD25^+Foxp3^+$ T_{regs}, so that inhibiting IL-2 may have a negative effect on the development of long-term tolerance after allo-HSCT (116, 117). Another cytokine of critical importance during aGvHD is IFN-γ, a pro-inflammatory cytokine, produced by several cell types such as activated T cells, NK, and NKT cells. IFN-γ and IL-2 both play a role in T cell proliferation, stimulation of cytotoxic T lymphocyte (CTL) and NK cell responses and production of IL-1 and TNF-α (118). A number of studies have reported a correlation between the expression of IFN-γ and severity of aGvHD (119–121). IFN-γ production occurs early in the cytokine cascade of GvHD. Acute GvHD is augmented by IFN-γ, which leads to the maturation of DC and stimulation of macrophages to generate cytokines and NO (118).

IFNG (IFN-γ) mRNA expression in the conjunctiva of patients was associated with dry-eye cGvHD (98) and in $CD8^+$ T cells with cGvHD (122). Ichiba and colleagues studied the regulation of 7,329 genes in the hepatic tissue of mice on days 7 and 35, after allogeneic and syngeneic bone marrow transplantation (100). On day 7, 456 genes and on day 35, 554 genes were regulated. Interestingly, *Ifng* mRNA expression was not upregulated during hepatic GvHD on day 7 and no expression of *Ifng* was observed in the liver on day 35. However, the expression of many genes that are inducible by IFN-γ, such as interferon regulatory factor-1 (*Irf1*) and *Irf7* were increased (100). Both *IFNG* and *IL2* mRNA were increased in the peripheral blood mononuclear cells (PBMCs) of GvHD patients that received a donor lymphocyte infusion for the treatment of relapsed leukemia after allogeneic HSCT and *IL2* mRNA expression correlated with the progression of GvHD (121). In another study, genes that contribute to control of inflammation, such as the IL-1 decoy receptor *IL1R2*, as well as pro-fibrotic genes have been found to be overexpressed in PBMCs of patients with cGvHD (123).

Gene expression of *TNF*, encoding TNF-α, a critical pro-inflammatory cytokine, was also elevated during aGvHD in PBMCs of patients (93). TNF-α is one of the most important factors involved in the pathogenesis of aGvHD, and it is important at various stages during the progression of the disease. The importance of this molecule in aGvHD was firstly described in a mouse model (124). Since then, several studies have shown that the neutralization of TNF-α can reduce symptoms of aGvHD (125). Tumor necrosis factor superfamily, member 10 (*TNFSF10*) mRNA expression was also elevated during aGvHD in human PBMCs (95).

Other cytokines regulated in GvHD include IL-15. In a murine aGvHD model, donor IL-15 was crucial for the development for aGvHD (126). Investigations on the role of IL-15 suggested that IL-15 could induce aGVHD by activating T cells and NK cells (127). In conjunctiva of patients with GvHD, *IL15* mRNA was significantly increased (98). *IL27* mRNA

was strongly downregulated during aGvHD in PBMCs from patients (93). Previous reports indicate that IL-27 exhibits a pro-inflammatory response, is involved in activating Th_1 cells, and enhances the immunological response to tumor cells (128). IL-35 is an inhibitory cytokine secreted by T_{regs}. The exact role of IL-35 in aGvHD is not known, although overexpression of IL-35 during murine aGvHD reduced its severity by suppressing activation of effector $CD4^+$ T cells and expansion of $CD4^+Foxp3^+$ T_{regs} in target organs of aGvHD, while preserving a GvL effect (129). IL-35 could be a potential therapeutic target for prevention of aGvHD (129, 130).

SNPs in Cytokine Coding Genes and Association with HSCT Outcome

Given the dysregulation of many cytokines during acute and chronic GvHD, it is not surprising that SNPs in these genes have been associated with the outcome of HSCT. SNP association studies in HSCT have been recently reviewed by Dickinson and Norden (8). Since the original work by Middleton and colleagues (131), large cohort candidate gene association studies have been reported on SNPs in more than 20 genes that code for cytokines and other molecules involved in the biology of HSCT (132–135). Moreover, SNPs originally identified in *NOD2* for their association with Crohn's disease have since been associated with HSCT outcomes (136, 137). Individuals carrying just one variant of rs2066844 (SNP8), rs2066845 (SNP12), or rs41450053 (SNP13) have a twofold to fourfold increased risk of developing Crohn's disease, which increases to approximately 20-fold in individuals who are homozygotes or compound heterozygotes (138, 139). NOD2 is mainly involved in defense against infection; it recognizes pathogen-associated patterns and induces a cytokine response, and is itself regulated by pro-inflammatory cytokines (140).

Goussetis and colleagues retrospectively analyzed specific polymorphisms in genes for IL-10, IL-6, TNF-α, and IFN-γ in a pediatric cohort of 57 HLA-identical sibling myeloablative transplants and found a significant association between the *IL10* promoter haplotype polymorphisms at positions −1082, −819, and −592 with the occurrence of severe aGVHD (grades III–IV). Recipients with the haplotype *GCC* had a decreased risk of severe aGVHD in comparison with patients with other *IL10* haplotypes (141). Chien and colleagues identified two SNPs in *IL10*, such as rs1800871 and rs1800872, which were associated with a 30% decrease of the risk for grade III–IV aGVHD (139). Moreover, the donor allele *C* for rs1800795 in *IL6* was associated with a 20–50% increase in the risk for grade II–IV aGVHD, and the *IL2* polymorphism rs2069762 in the donor genotype was associated with a 1.3-fold increase in risk of grade III–IV aGVHD (139).

Chemokine Gene Expression Variation and Its Impact on the Outcome of HSCT

Many genes encoding chemokines are regulated during GvHD. CXCR3 is an important chemokine receptor involved in lymphocyte recruitment and is expressed on T cells. CXCL9, CXCL10, and CXCL11, the ligands for CXCR3, are induced by the Th_1 cytokines IFN-γ and TNF-α (142). CXCL9 is expressed by effector $CD4^+$ Th_1 cells and $CD8^+$ CTL and has been shown to affect the migration of T_{eff} to inflamed tissue during progression of GvHD (142). *CXCL10* and *CXCL11* mRNA expression were increased in patient skin biopsies with aGvHD (grades II–III) when compared to patients without or grade I GvHD (143). The mRNA expression of *CXCL9* and *CXCL10*, along with their receptor *CXCR3*, was increased in cGvHD in conjunctival biopsies of 10 patients when compared to 10 healthy controls (97). Elevated mRNA expression of the CXCR3 ligands, *CXCL9* and *CXCL10* in target organs of GvHD, shows that *CXCR3* could have a role in GvHD (144). *CXCL8*, encoding IL-8, was upregulated in PBMC from patients who developed aGVHD (92). Moreover, *Cxcl9* (101) and *Cxcl10* were also elevated during murine aGvHD (100). Interestingly, the use of CXCR3-transfected T_{regs}, as a novel therapeutic strategy, resulted in decreased severity of GvHD due to attraction of T_{regs} to the target tissues of GvHD (145).

Other chemokines involved in the stimulation and activation of T cells in lymphoid tissue (*Cxcl1*, *Cxcl2*, *Cxcl9*, and *Cxcl20*, *Ccl2*, *Ccl5*, *Ccl6*, *Ccl7*, *Ccl8*, *Ccl9*, *Ccl11*, and *Ccl29*) and chemokine receptors (*Ccr1* and *Ccr5*) were elevated in the skin of mice during acute GvHD (99). The chemokines CCL2, CCL3, CCL4, and CCL5 are involved in the migration of donor cells to the target organs during GvHD (146). *CCR5* mRNA was also increased during in aGvHD human PBMCs (95) and in murine skin during GvHD (102). In addition, *Ccl2*, *Ccl5*, *Ccl17*, *Cxcl9 (Mig)*, *Cxcl10 (IP-10)*, and *Cxcl11 (1-TAC)* mRNAs were also significantly regulated in mouse skin during GvHD (102). In conjunctival biopsies of patients with GvHD, the gene expression of *CCL24*, *CCL18*, and *CCL2* was highly increased (98). Another chemokine mRNA, *Ccl5* (RANTES), was elevated in the skin of mice during aGvHD (99) and profoundly upregulated during hepatic aGvHD (100).

Differential Expression of Genes Involved in Antigen Processing and Presentation during GvHD

The role of MHC molecules is of critical importance to the development of GvHD. Both class I (HLA-A, B, and C in human) and class II (HLA-DR, DQ, and DP in human) determine not only the histocompatibility but are also responsible for controlling T cell recognition (147). Expression of class II HLA molecules by professional APCs, mainly in the gastrointestinal tract epithelium and skin, allows $CD4^+$ T cells to recognize foreign antigens, possibly contributing to the specific organ sites of aGvHD (148). During the afferent phase of the pathogenesis of aGvHD, the release of cytokines such as IFN-γ leads to an increased expression of MHC molecules. On day 7 of hepatic aGvHD in mice, MHC class II genes, including *I-A*α, *I-A*β, *I-E*α, and *I-E*β, were overexpressed and remained upregulated at day 35 of aGvHD. On the other hand, the expression of the MHC class I genes was not regulated in this study. However, the genes that encode alternative proteasome subunits and that alternate peptide production associated with MHC class I molecules proteasome subunit beta 9 (*Psbm9*) and *Psbm8* (also known as lower molecular mass peptides LMP2 and LMP7) were increased in mouse liver during aGvHD (100). Moreover, *Tap1* and *Tap2* mRNAs were increased in mouse skin (99) as well as in liver during aGvHD (100). TAP1 and TAP2 are transporters associated with antigen processing 1 and 2, responsible for translocating peptides into the

endoplasmic reticulum before loading on MHC class I molecules. In a rat skin explant model, an increase in expression of *Tap1* as well as *Psbm8* and *Ubd* mRNA during graft-versus-host reaction was observed (103). UBD, also known as FAT10, is involved in the proteasomal degradation of cytosolic proteins by providing a ubiquitin-independent signal (149). The differential expression of the MHC I and II genes in addition to genes involved in antigen processing that have been observed during GvHD is in agreement with the important role of MHC genes for HSCT outcomes.

Involvement of the miHAg in Immune Responses during HSCT

Mismatches of polymorphic peptides between donor and recipients cause miHag that can also elicit an alloimmune response (150). The extent of the desired GvL versus the unwanted GvHD responses is dependent on the expression profiles of these genes. In humans, miHag are mostly restricted by HLA class I molecules. Previously, mismatches for HA-1, HA-2, and HA-5 between donor and recipient have been described to be associated with an increased risk of GvHD (151). In a gene expression profiling study to identify the risk of GvHD and relapse posttransplant, the mRNA expression of miHag was assessed in 311 HLA-matched siblings from a single center (152). The *HA-8* gene was expressed in almost all tissues, whereas *ACC-1* gene had a restricted profile. Nonetheless, both HA-8 and ACC-1 miHag mismatches were found to be associated with occurrence of cGvHD (152).

Notably, whole exome sequencing studies have been performed recently to estimate the alloreactive potential between donors and recipients in HSCT. It has been found that non-synonymous and non-conservative SNPs were twice as frequent in HLA-matched unrelated compared to related donor–recipient pairs (153). The information on SNPs between donor and recipient can be used to predict candidate miHags by algorithms taking peptide binding to HLA class I molecules and the tissue distribution of the respective proteins into account (154). Modeling of T cell responses to these miHags potentially can help to identify more favorable donors or to adapt the immunosuppressive treatment after HSCT (155, 156).

Gene Expression Patterns in T Cells Associated with the Outcome of HSCT

Baron and colleagues compared the gene expression profiles of 50 allo-HSCT donors in $CD4^+$ and $CD8^+$ T cells to identify donors that are stronger allo-responders and could elicit a stronger GvHD response than others could. They suggested genes that regulate the transforming growth factor-β signaling and cell proliferation, in donor T cells, as the dangerous donor trait responsible for the occurrence of cGvHD in the corresponding recipients (94). Low levels of *SMAD3* mRNA, which encodes a transcription factor that is activated in response to TFG-β in $CD4^+$ T cells, was associated with the absence of GvHD, while high levels of *SMAD3* were necessary but not sufficient for GvHD occurrence (94).

$CD8^+$ T cells are important effectors in aGvHD (157), and perforin is a cytotoxic effector protease produced by CTL and NK cells. High expression of perforin 1 (*PRF1*) mRNA in $CD8^+$ T cells was found to be associated with the incidence of GVHD in patients (94). In the skin of mice during aGvHD, granzyme B (*Gzmb*) was significantly elevated, in addition to the downstream effector caspase 7 (*Casp7*) (99). Other genes upregulated included the pro-apoptotic members of the BCL2 family, BCL2-antagonist/killer 1 (*Bak1*), BLC2-like 11 (*Bcl2l11*), and BCL2-associated X protein (*Bax*) (99). Thus, expression profiling can indicate ongoing pathophysiological processes contributing to GvHD, such as cellular cytotoxicity.

Notably, Sadeghi and colleagues observed an increase in gene expression of the adhesion molecules intracellular adhesion molecule 1 (*Icam1*) and vascular cell adhesion molecule 1 (*Vcam1*) during murine hepatic aGvHD. Increased expression of *Vcam1* mRNA was also observed in the liver and kidney compared to the muscle during murine aGvHD, whereas *Icam1* mRNA was upregulated only in the liver (101). Both adhesion molecules are expressed on endothelial cells and are critical for the migration of leukocytes to tissues during inflammation (158). Furthermore, *Icam1* and *Vcam1* genes were also upregulated in mouse skin during aGvHD, along with other adhesion molecules *Cd18* or integrin subunit beta 2 (*Itgb2*), *Ly69* or integrin beta 7 (*Itgb7*), and *Psgl1* or selectin platelet ligand (*Selplg*) (99).

The expression of costimulatory molecules that have a role in regulating T cell activation, differentiation, and proliferation has been studied to determine their role in GvHD. CD28 and CD28/cytotoxic T lymphocyte antigen 4 (CTLA4) are the most well characterized costimulatory and inhibitory molecules, respectively (159). Both are present on T cells, while their ligands CD80 (B7-1) and CD86 (B7-2) are expressed primarily on APCs (160). *CD28* mRNA was increased during cGvHD in PBMCs of patients (96). Interestingly, SNPs in *CTLA4* could have an impact on its function. In patients, the presence of the *A* allele in both rs231775 and rs3087243 was associated with a reduced risk of aGvHD after HSCT (161). Another study showed that recipients with the +49*A*/*G* allele had a significantly lower disease-free survival and overall survival in comparison to recipients with the *A*/*A* genotype (162). In addition to the +49*A*/*G* polymorphism, −1722, −1661, −318 polymorphisms in *CTLA4* were also evaluated after allo-HSCT, and a significant association between *GA* genotype (*CTLA4* −1661) and GvHD was shown in males with GvHD compared to males without GvHD (163). In addition, inducible T cell costimulator (ICOS), a member of the CTLA4 family that is expressed on activated T cells, was shown to be associated with the occurrence of aGvHD (19). The exact role of ICOS in GvHD is not clear; however, *ICOS* mRNA was downregulated in aGvHD patients. In contrast, *ICOS* mRNA was elevated during cGvHD in activated T cells in canines (164). Furthermore, blockade of ICOS *in vitro* during mixed lymphocyte reaction (MLR) resulted in immunosuppression, suggesting that ICOS plays a role in graft rejection and blockade of ICOS could be a potential therapeutic strategy (164). Cuzzola and colleagues also observed an increase in mRNA expression of *ICOS* in patients responding to anti-GvHD therapies as well as other genes that are regulated by ICOS, including Th2 cytokines (*IL4, STAT6*, and *IL18*) (19).

In addition to gene and miRNA expression studies in T cells, the characterization of the T cell receptor (TCR) repertoire in patients who underwent HSCT might be informative to assess

risks of GvHD or relapse. It has been reported recently that these complications were associated with a lower TCR repertoire and the expansion of certain T cell clones (165).

Gene Expression Profiles in B Cells Associated with Outcome of HSCT

B cells have been found to be important in contributing to cGvHD; however, the mechanisms involved in maintaining their activation are not known (166). Depletion of B cells reduced the incidence of cGvHD in mice (167). Elevated B cell-activating factor (*BAFF*), also known as tumor necrosis factor superfamily member 13b (*TNFSF13B*), mRNA levels were observed in patients with cGvHD and correlated to B cell activation (168). *BAFF* mRNA expression was also significantly upregulated in clinical GvHD patient biopsies in comparison to those with no GvHD (143). A differential pattern for gene expression for several genes was observed in the purified B cells from cGvHD patients on comparison with the B cells from healthy counterparts. Four of the genes, *IL12A*, interferon regulatory factor 4 (*IRF4*), *CD40*, and interferon gamma receptor 2 (*IFNGR2*), were downregulated whereas B cell linker (*BLNK*) mRNA was upregulated in B cells in patients with cGvHD (168). BLNK has been found to be important in proliferation and survival of B lymphocytes (169).

GENOME-WIDE ASSOCIATION STUDIES (GWAS) FOR HSCT OUTCOME

As a result of the Human Genome and the International HapMap Projects in the early 2000s (4, 5), GWAS became possible, expanding dramatically our capacity to understand genetic variability. A GWAS study of non-HLA SNPs in allogeneic HSCT was reported, which identified a number of SNP genotypes associated with severe aGvHD using a cohort of 1,298 patient donor pairs (139). The *IL6* donor genotype for rs1800795 was confirmed to be associated an increased risk of severe aGvHD. In addition other genes associated with aGvHD, *IL2*, methylene tetrahydrofolate reductase (*MTHFR*), Heparanase (*HPSE*), and cytotoxic T-lymphocyte-associated protein 4 (*CTLA4*) were identified in this GWAS cohort and illustrate (**Figure 6**) the fact that genomic control of immunoregulatory cytokines could alter the function of cells which in turn aid or reduce successful transplant outcome (170).

The Ogawa group has performed GWAS study in large cohort involving 1,589 patients and donors to identify miHAg associated with aGvHD (171). They identified three new loci that were significantly associated with severe (grade II–IV) aGvHD including the SNP rs17473423 within the *KRAS* locus. In a further GWAS study on a smaller identification cohort of 68 patients and a validation cohort of 100 patients, two GvHD susceptibility loci (rs17114803 and rs17114808) within the "suppressor of fused homolog" (*SUFU*) gene have been found (172). The incidence of aGvHD was significantly higher in patients that were homozygous for *CC* at SUFU rs17114808, than in heterozygous patients. Functional studies showed that ectopic expression of SUFU in DCs reduced expression of HLA-DR and suppression of MLR, whereas an increased HLA-DR expression and enhanced MLR was observed on silencing of SUFU (172). In the future, GWAS studies in HSCT will require larger multicenter cohorts but are expected to reveal new genetic associations for HSCT outcomes (8, 147).

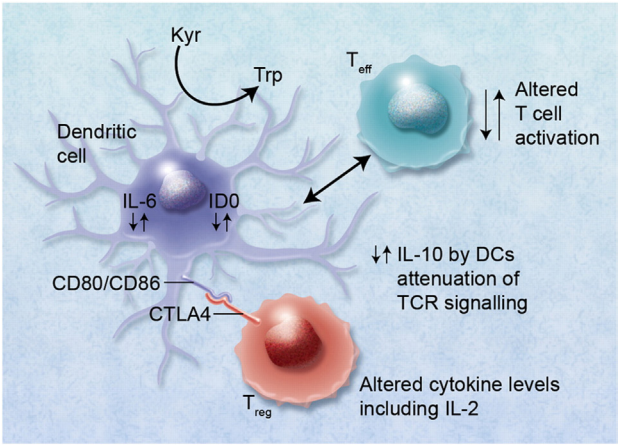

FIGURE 6 | Alterations in cytokine levels, such as interleukin (IL)-10, IL-6, and IL-2, *via* immunoregulatory single-nucleotide polymorphisms (SNPs) can lead to altered immunoregulatory function of regulatory T cells (T_{regs}) and effector T cells (T_{eff}). Binding of cytotoxic T lymphocyte antigen 4 (CTLA4) with its receptor (possibly also *via* functional single-nucleotide polymorphisms) with CD80/CD86 proteins on dendritic cells (DCs) can lead to induction of indoleamine 2,3 dioxygenase (IDO) and the catabolism of tryptophan into proapoptotic metabolites causing immunosuppression of T_{eff}. Altered binding of CTLA4 may also lead to reduced immunosuppression *via* T_{regs} and GvHD. High IL-6 levels induced in DCs by T_{reg} interaction can also cause alteration of T_{regs} to Th_{17} cells and may lead to exacerbation of GvHD. The figure has been taken from Ref. (170). Professional illustration by Alice Y. Chen.

PROBLEMS WITH GENE ASSOCIATION STUDIES IN HSCT

Genetic association studies have inherent difficulties when it comes to validation of results. Only robust genetic markers are able to be validated across HSCT cohorts. This is due to heterogeneity of transplants with regards to diagnosis, conditioning regimens, type of stem cells (e.g., peripheral blood, cord blood, or bone marrow), sibling or matched unrelated transplants and risk factors for outcome (CMV positivity; female to male donors, age of the transplant cohort) all of which alter the biology of the transplant itself. Problems associated with non-HLA genomics in HSCT have also been recently reviewed (8).

One example of this is within our own studies on SNP polymorphisms as risk factors for survival in chronic myeloid leukemia (CML). In the first study, presence of interleukin 1 receptor antagonist (*IL1RN*) allele 2 genotype in the donor (indicating downregulation of IL-1), absence of donor *IL10 ATA*/*ACC* genotype (indicating more downregulation of IL-10) and absence of tumor necrosis factor superfamily member 1B (*TNFSF1B*) 196R in the patient (indicating increased levels of soluble TNF-RII and decreased levels of TNF-α), all were

associated with decreased survival and increased transplant relate mortality (173). In a validation cohort of matched unrelated transplants, none of the SNPs could be validated, and a comparison of the cohorts demonstrated differences in survival and clinical characteristics (174).

In addition, in a larger heterogeneous cohort, including CML and lymphoma (2), we developed a clinical and genetic score, which included the European Bone Marrow Transplantation (EBMT) Group score. This score incorporates clinical risk factors such as age of the patient and donor; time to transplant and type of transplant (175–178). Using a statistical analysis that included a bootstrap estimate of prediction error (179, 180), three further SNPs were associated with survival. A protective effect for the *IL10* genotype *ACC*/*ACC* in the donors was observed, while estrogen receptor 1 (*ESR1*) rs9340799 in the patient, *IL6* rs18000795 in the donors, and *TIRAP* (or *MAL*) rs177374 in the patient were associated with poorer survival (2). The subsequent clinical and genetic score assigned to each patient was shown to have a better predictive value than the EBMT score alone. In a more recent cohort studying acute leukemia transplant patients (181), three polymorphisms, presence of toll-interleukin 1 receptor domain containing adaptor protein (*TIRAP*) (alternatively named *MAL*) allele *T* (rs8177374) in the patient, absence of the glucocorticoid receptor (*GCR*) haplotype (consisting of rs6198, rs33389, and rs33388) *ACT* in the patient and absence of *HSPA1L* (or *HSP70-hom*) +2437 (rs2227956) allele *C* in the patient were associated with decreased survival. The subsequent clinical and genetic score assigned to each patient was shown to have a better predictive value than the EBMT score alone.

Interestingly, in all cohorts, the SNPs associated with reduced survival were all involved in downregulating the immune response, suggesting that this downregulation may be linked to reduced GvL responses and therefore lower overall survival.

These studies indicate that although replication of the exact genomic profiles may be difficult to achieve, the overall influence of genomics on the biology of the transplant is comparable and leads to a similar outcome, demonstrating that genomic studies are important for understanding the overall biology of the transplant.

CONCLUSION

In this review, we have shown the differential expression patterns of a variety of mRNA, different miRNAs and SNPs in specific genes that have a significant impact on transplant outcome and development of GvHD. In addition, several SNP–mRNA–miRNA regulatory networks have been found to contribute to post-HSCT outcomes. Taken together, these findings demonstrate how expression of specific miRNAs can target the genes of key immune response modulators, directly affecting expression of mRNAs that influence the aGvHD response. However, mRNA and miRNA expression studies have not yet revealed a set of genes or miRNAs that can be used as reliable biomarkers for predicting the outcome of HSCT across different transplantation centers. Further, preferably multicentre, studies are required to determine SNPs and to analyze miRNA and mRNA expression in parallel in cohorts of HSCT patients to further elucidate genetic risks of HSCT. Such combined approaches have the potential to improve clinical practise of HSCT and eventually to benefit patients.

AUTHOR CONTRIBUTIONS

RG and PS drafted the manuscript; RC and JN commented and edited the draft; AD and RD supervised and revised the manuscript; and all authors approved the final version.

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REFERENCES

1. Harkensee C, Oka A, Onizuka M, Middleton PG, Inoko H, Hirayasu K, et al. Single nucleotide polymorphisms and outcome risk in unrelated mismatched hematopoietic stem cell transplantation: an exploration study. *Blood* (2012) 119:6365–72. doi:10.1182/blood-2012-01-406785
2. Balavarca Y, Pearce K, Norden J, Collin M, Jackson G, Holler E, et al. Predicting survival using clinical risk scores and non-HLA immunogenetics. *Bone Marrow Transplant* (2015) 50:1445–52. doi:10.1038/bmt.2015.173
3. Sachidanandam R, Weissman D, Schmidt SC, Kakol JM, Stein LD, Marth G, et al. A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms. *Nature* (2001) 409:928–33. doi:10.1038/35057149
4. International Hapmap Consortium. The international HapMap project. *Nature* (2003) 426:789–96. doi:10.1038/nature02168
5. International Hapmap Consortium. A haplotype map of the human genome. *Nature* (2005) 437:1299–320. doi:10.1038/nature04226
6. International Hapmap Consortium, Frazer KA, Ballinger DG, Cox DR, Hinds DA, Stuve LL, et al. A second generation human haplotype map of over 3.1 million SNPs. *Nature* (2007) 449:851–61. doi:10.1038/nature06258
7. Engle LJ, Simpson CL, Landers JE. Using high-throughput SNP technologies to study cancer. *Oncogene* (2006) 25:1594–601. doi:10.1038/sj.onc.1209368
8. Dickinson AM, Norden J. Non-HLA genomics: does it have a role in predicting haematopoietic stem cell transplantation outcome? *Int J Immunogenet* (2015) 42:229–38. doi:10.1111/iji.12202
9. Hudson TJ. Wanted: regulatory SNPs. *Nat Genet* (2003) 33:439–40. doi:10.1038/ng0403-439
10. Macfarlane LA, Murphy PR. MicroRNA: biogenesis, function and role in cancer. *Curr Genomics* (2010) 11:537–61. doi:10.2174/138920210793175895
11. Garzon R, Calin GA, Croce CM. MicroRNAs in cancer. *Annu Rev Med* (2009) 60:167–79. doi:10.1146/annurev.med.59.053006.104707
12. Slaby O, Svoboda M, Michalek J, Vyzula R. MicroRNAs in colorectal cancer: translation of molecular biology into clinical application. *Mol Cancer* (2009) 8:102. doi:10.1186/1476-4598-8-102
13. Ryan BM, Robles AI, Harris CC. Genetic variation in microRNA networks: the implications for cancer research. *Nat Rev Cancer* (2010) 10:389–402. doi:10.1038/nrc2867
14. Atarod S, Dickinson AM. MicroRNAs: the missing link in the biology of graft-versus-host disease? *Front Immunol* (2013) 4:420. doi:10.3389/fimmu.2013.00420

15. Iorio MV, Croce CM. MicroRNAs in cancer: small molecules with a huge impact. *J Clin Oncol* (2009) 27:5848–56. doi:10.1200/JCO.2009.24.0317
16. Hoffmann P, Ermann J, Edinger M, Fathman CG, Strober S. Donor-type CD4(+)CD25(+) regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation. *J Exp Med* (2002) 196:389–99. doi:10.1084/jem.20020399
17. Beres AJ, Drobyski WR. The role of regulatory T cells in the biology of graft versus host disease. *Front Immunol* (2013) 4:163. doi:10.3389/fimmu.2013.00163
18. Mavin E, Ahmed SS, O'boyle G, Turner B, Douglass S, Norden J, et al. Regulatory T cells inhibit CD8(+) T-cell tissue invasion in human skin graft-versus-host reactions. *Transplantation* (2012) 94:456–64. doi:10.1097/TP.0b013e31826205d6
19. Cuzzola M, Fiasche M, Iacopino P, Messina G, Martino M, Console G, et al. A molecular and computational diagnostic approach identifies FOXP3, ICOS, CD52 and CASP1 as the most informative biomarkers in acute graft-versus-host disease. *Haematologica* (2012) 97:1532–8. doi:10.3324/haematol.2011.059980
20. Magenau JM, Qin X, Tawara I, Rogers CE, Kitko C, Schlough M, et al. Frequency of CD4(+)CD25(hi)FOXP3(+) regulatory T cells has diagnostic and prognostic value as a biomarker for acute graft-versus-host-disease. *Biol Blood Marrow Transplant* (2010) 16:907–14. doi:10.1016/j.bbmt.2010.02.026
21. Rezvani K, Mielke S, Ahmadzadeh M, Kilical Y, Savani BN, Zeilah J, et al. High donor FOXP3-positive regulatory T-cell (Treg) content is associated with a low risk of GVHD following HLA-matched allogeneic SCT. *Blood* (2006) 108:1291–7. doi:10.1182/blood-2006-02-003996
22. Wolf D, Wolf AM, Fong D, Rumpold H, Strasak A, Clausen J, et al. Regulatory T-cells in the graft and the risk of acute graft-versus-host disease after allogeneic stem cell transplantation. *Transplantation* (2007) 83:1107–13. doi:10.1097/01.tp.0000260140.04815.77
23. Zorn E, Kim HT, Lee SJ, Floyd BH, Litsa D, Arumugarajah S, et al. Reduced frequency of FOXP3+ CD4+CD25+ regulatory T cells in patients with chronic graft-versus-host disease. *Blood* (2005) 106:2903–11. doi:10.1182/blood-2005-03-1257
24. Miura Y, Thoburn CJ, Bright EC, Phelps ML, Shin T, Matsui EC, et al. Association of Foxp3 regulatory gene expression with graft-versus-host disease. *Blood* (2004) 104:2187–93. doi:10.1182/blood-2004-03-1040
25. Koreth J, Matsuoka K, Kim HT, Mcdonough SM, Bindra B, Alyea EP III, et al. Interleukin-2 and regulatory T cells in graft-versus-host disease. *N Engl J Med* (2011) 365:2055–66. doi:10.1056/NEJMoa1108188
26. Beres AJ, Haribhai D, Chadwick AC, Gonyo PJ, Williams CB, Drobyski WR. CD8+ Foxp3+ regulatory T cells are induced during graft-versus-host disease and mitigate disease severity. *J Immunol* (2012) 189:464–74. doi:10.4049/jimmunol.1200886
27. Sawamukai N, Satake A, Schmidt AM, Lamborn IT, Ojha P, Tanaka Y, et al. Cell-autonomous role of TGFbeta and IL-2 receptors in CD4+ and CD8+ inducible regulatory T-cell generation during GVHD. *Blood* (2012) 119:5575–83. doi:10.1182/blood-2011-07-367987
28. Heinrichs J, Li J, Nguyen H, Wu Y, Bastian D, Daethanasanmak A, et al. CD8(+) Tregs promote GVHD prevention and overcome the impaired GVL effect mediated by CD4(+) Tregs in mice. *Oncoimmunology* (2016) 5:e1146842. doi:10.1080/2162402X.2016.1146842
29. Picarda E, Bezie S, Venturi V, Echasserieau K, Merieau E, Delhumeau A, et al. MHC-derived allopeptide activates TCR-biased CD8+ Tregs and suppresses organ rejection. *J Clin Invest* (2014) 124:2497–512. doi:10.1172/JCI71533
30. Eastell T, Group BS, Hinks A, Thomson W. SNPs in the FOXP3 gene region show no association with juvenile idiopathic arthritis in a UK Caucasian population. *Rheumatology (Oxford)* (2007) 46:1263–5. doi:10.1093/rheumatology/kem129
31. Shen Z, Chen L, Hao F, Wang G, Liu Y. Intron-1 rs3761548 is related to the defective transcription of Foxp3 in psoriasis through abrogating E47/c-Myb binding. *J Cell Mol Med* (2010) 14:226–41. doi:10.1111/j.1582-4934.2008.00370.x
32. Piao Z, Kim HJ, Choi JY, Hong CR, Lee JW, Kang HJ, et al. Effect of FOXP3 polymorphism on the clinical outcomes after allogeneic hematopoietic stem cell transplantation in pediatric acute leukemia patients. *Int Immunopharmacol* (2016) 31:132–9. doi:10.1016/j.intimp.2015.12.022
33. Tiercy JM. Immunogenetics of hematopoietic stem cell transplantation: the contribution of microsatellite polymorphism studies. *Int J Immunogenet* (2011) 38:365–72. doi:10.1111/j.1744-313X.2011.01026.x
34. Bassuny WM, Ihara K, Sasaki Y, Kuromaru R, Kohno H, Matsuura N, et al. A functional polymorphism in the promoter/enhancer region of the FOXP3/Scurfin gene associated with type 1 diabetes. *Immunogenetics* (2003) 55:149–56. doi:10.1007/s00251-003-0559-8
35. Noriega V, Martinez-Laperche C, Buces E, Pion M, Sanchez-Hernandez N, Martin-Antonio B, et al. The genotype of the donor for the (GT)n polymorphism in the promoter/enhancer of FOXP3 is associated with the development of severe acute GVHD but does not affect the GVL effect after myeloablative HLA-identical allogeneic stem cell transplantation. *PLoS One* (2015) 10:e0140454. doi:10.1371/journal.pone.0140454
36. Rouas R, Fayyad-Kazan H, El Zein N, Lewalle P, Rothe F, Simion A, et al. Human natural Treg microRNA signature: role of microRNA-31 and microRNA-21 in FOXP3 expression. *Eur J Immunol* (2009) 39:1608–18. doi:10.1002/eji.200838509
37. Baumjohann D, Ansel KM. MicroRNA-mediated regulation of T helper cell differentiation and plasticity. *Nat Rev Immunol* (2013) 13:666–78. doi:10.1038/nri3494
38. Kohlhaas S, Garden OA, Scudamore C, Turner M, Okkenhaug K, Vigorito E. Cutting edge: the Foxp3 target miR-155 contributes to the development of regulatory T cells. *J Immunol* (2009) 182:2578–82. doi:10.4049/jimmunol.0803162
39. Lu LF, Thai TH, Calado DP, Chaudhry A, Kubo M, Tanaka K, et al. Foxp3-dependent microRNA155 confers competitive fitness to regulatory T cells by targeting SOCS1 protein. *Immunity* (2009) 30:80–91. doi:10.1016/j.immuni.2008.11.010
40. Cobb BS, Hertweck A, Smith J, O'connor E, Graf D, Cook T, et al. A role for Dicer in immune regulation. *J Exp Med* (2006) 203:2519–27. doi:10.1084/jem.20061692
41. Jeker LT, Zhou X, Gershberg K, De Kouchkovsky D, Morar MM, Stadthagen G, et al. MicroRNA 10a marks regulatory T cells. *PLoS One* (2012) 7:e36684. doi:10.1371/journal.pone.0036684
42. Jalapothu D, Boieri M, Crossland RE, Shah P, Butt IA, Norden J, et al. Tissue-specific expression patterns of microRNA during acute graft-versus-host disease in the rat. *Front Immunol* (2016) 7:361. doi:10.3389/fimmu.2016.00361
43. Atarod S, Ahmed MM, Lendrem C, Pearce KF, Cope W, Norden J, et al. miR-146a and miR-155 expression levels in acute graft-versus-host disease incidence. *Front Immunol* (2016) 7:56. doi:10.3389/fimmu.2016.00056
44. Pontoppidan PL, Jordan K, Carlsen AL, Uhlving HH, Kielsen K, Christensen M, et al. Associations between gastrointestinal toxicity, micro RNA and cytokine production in patients undergoing myeloablative allogeneic stem cell transplantation. *Int Immunopharmacol* (2015) 25:180–8. doi:10.1016/j.intimp.2014.12.038
45. Ranganathan P, Heaphy CE, Costinean S, Stauffer N, Na C, Hamadani M, et al. Regulation of acute graft-versus-host disease by microRNA-155. *Blood* (2012) 119:4786–97. doi:10.1182/blood-2011-10-387522
46. Chen S, Smith BA, Iype J, Prestipino A, Pfeifer D, Grundmann S, et al. MicroRNA-155-deficient dendritic cells cause less severe GVHD through reduced migration and defective inflammasome activation. *Blood* (2015) 126:103–12. doi:10.1182/blood-2014-12-617258
47. Stanczyk J, Pedrioli DM, Brentano F, Sanchez-Pernaute O, Kolling C, Gay RE, et al. Altered expression of MicroRNA in synovial fibroblasts and synovial tissue in rheumatoid arthritis. *Arthritis Rheum* (2008) 58:1001–9. doi:10.1002/art.23386
48. Thai TH, Calado DP, Casola S, Ansel KM, Xiao C, Xue Y, et al. Regulation of the germinal center response by microRNA-155. *Science* (2007) 316:604–8. doi:10.1126/science.1141229
49. Taganov KD, Boldin MP, Chang KJ, Baltimore D. NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc Natl Acad Sci U S A* (2006) 103:12481–6. doi:10.1073/pnas.0605298103
50. Boldin MP, Taganov KD, Rao DS, Yang L, Zhao JL, Kalwani M, et al. miR-146a is a significant brake on autoimmunity, myeloproliferation, and cancer in mice. *J Exp Med* (2011) 208:1189–201. doi:10.1084/jem.20101823
51. Yang L, Boldin MP, Yu Y, Liu CS, Ea CK, Ramakrishnan P, et al. miR-146a controls the resolution of T cell responses in mice. *J Exp Med* (2012) 209:1655–70. doi:10.1084/jem.20112218
52. Stickel N, Prinz G, Pfeifer D, Hasselblatt P, Schmitt-Graeff A, Follo M, et al. MiR-146a regulates the TRAF6/TNF-axis in donor T cells during GVHD. *Blood* (2014) 124:2586–95. doi:10.1182/blood-2014-04-569046

53. Zhao JL, Starczynowski DT. Role of microRNA-146a in normal and malignant hematopoietic stem cell function. *Front Genet* (2014) 5:219. doi:10.3389/fgene.2014.00219
54. Lu Y, Hippen KL, Lemire AL, Gu J, Wang W, Ni X, et al. miR-146b antagomir-treated human Tregs acquire increased GVHD inhibitory potency. *Blood* (2016) 128:1424–35. doi:10.1182/blood-2016-05-714535
55. Xu W, Xu J, Liu S, Chen B, Wang X, Li Y, et al. Effects of common polymorphisms rs11614913 in miR-196a2 and rs2910164 in miR-146a on cancer susceptibility: a meta-analysis. *PLoS One* (2011) 6:e20471. doi:10.1371/journal.pone.0020471
56. Isernhagen A, Malzahn D, Bickeboller H, Dressel R. Impact of the MICA-129Met/Val dimorphism on NKG2D-mediated biological functions and disease risks. *Front Immunol* (2016) 7:588. doi:10.3389/fimmu.2016.00588
57. Isernhagen A, Malzahn D, Viktorova E, Elsner L, Monecke S, Von Bonin F, et al. The MICA-129 dimorphism affects NKG2D signaling and outcome of hematopoietic stem cell transplantation. *EMBO Mol Med* (2015) 7:1480–502. doi:10.15252/emmm.201505246
58. Bauer S, Groh V, Wu J, Steinle A, Phillips JH, Lanier LL, et al. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* (1999) 285:727–9. doi:10.1126/science.285.5428.727
59. Groh V, Rhinehart R, Randolph-Habecker J, Topp MS, Riddell SR, Spies T. Costimulation of CD8alphabeta T cells by NKG2D via engagement by MIC induced on virus-infected cells. *Nat Immunol* (2001) 2:255–60. doi:10.1038/85321
60. Gannage M, Buzyn A, Bogiatzi SI, Lambert M, Soumelis V, Dal Cortivo L, et al. Induction of NKG2D ligands by gamma radiation and tumor necrosis factor-alpha may participate in the tissue damage during acute graft-versus-host disease. *Transplantation* (2008) 85:911–5. doi:10.1097/TP.0b013e31816691ef
61. Boukouaci W, Busson M, Peffault De Latour R, Rocha V, Suberbielle C, Bengoufa D, et al. MICA-129 genotype, soluble MICA, and anti-MICA antibodies as biomarkers of chronic graft-versus-host disease. *Blood* (2009) 114:5216–24. doi:10.1182/blood-2009-04-217430
62. Isernhagen A, Malzahn D, Monecke S, Schilling D, Shah P, Multhoff G, et al. Functional consequences of genetic polymorphisms in the NKG2D receptor signaling pathway and putative gene interactions. *Receptors Clin Investig* (2016) 3:e1269. doi:10.14800/rci.1269
63. Espinoza JL, Takami A, Onizuka M, Sao H, Akiyama H, Miyamura K, et al. NKG2D gene polymorphism has a significant impact on transplant outcomes after HLA-fully-matched unrelated bone marrow transplantation for standard risk hematologic malignancies. *Haematologica* (2009) 94:1427–34. doi:10.3324/haematol.2009.008318
64. Shafi S, Vantourout P, Wallace G, Antoun A, Vaughan R, Stanford M, et al. An NKG2D-mediated human lymphoid stress surveillance response with high interindividual variation. *Sci Transl Med* (2011) 3:113ra124. doi:10.1126/scitranslmed.3002922
65. Lo PH, Urabe Y, Kumar V, Tanikawa C, Koike K, Kato N, et al. Identification of a functional variant in the MICA promoter which regulates MICA expression and increases HCV-related hepatocellular carcinoma risk. *PLoS One* (2013) 8:e61279. doi:10.1371/journal.pone.0061279
66. Ashiru O, Lopez-Cobo S, Fernandez-Messina L, Pontes-Quero S, Pandolfi R, Reyburn HT, et al. A GPI anchor explains the unique biological features of the common NKG2D-ligand allele MICA*008. *Biochem J* (2013) 454:295–302. doi:10.1042/BJ20130194
67. Isernhagen A, Schilling D, Monecke S, Shah P, Elsner L, Walter L, et al. The MICA-129Met/Val dimorphism affects plasma membrane expression and shedding of the NKG2D ligand MICA. *Immunogenetics* (2016) 68:109–23. doi:10.1007/s00251-015-0884-8
68. Kitcharoen K, Witt CS, Romphruk AV, Christiansen FT, Leelayuwat C. MICA, MICB, and MHC beta block matching in bone marrow transplantation: relevance to transplantation outcome. *Hum Immunol* (2006) 67:238–46. doi:10.1016/j.humimm.2006.02.012
69. Parmar S, Del Lima M, Zou Y, Patah PA, Liu P, Cano P, et al. Donor-recipient mismatches in MHC class I chain-related gene A in unrelated donor transplantation lead to increased incidence of acute graft-versus-host disease. *Blood* (2009) 114:2884–7. doi:10.1182/blood-2009-05-223172
70. Askar M, Sun Y, Rybicki L, Zhang A, Thomas D, Kalaycio M, et al. Synergistic effect of major histocompatibility complex class I-related chain a and human leukocyte antigen-DPB1 mismatches in association with acute graft-versus-host disease after unrelated donor hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant* (2014) 20:1835–40. doi:10.1016/j.bbmt.2014.07.019
71. Carapito R, Jung N, Kwemou M, Untrau M, Michel S, Pichot A, et al. Matching for the nonconventional MHC-I MICA gene significantly reduces the incidence of acute and chronic GVHD. *Blood* (2016) 128:1979–86. doi:10.1182/blood-2016-05-719070
72. Fuerst D, Neuchel C, Niederwieser D, Bunjes D, Gramatzki M, Wagner E, et al. Matching for the MICA-129 polymorphism is beneficial in unrelated hematopoietic stem cell transplantation. *Blood* (2016) 128:3169–76. doi:10.1182/blood-2016-05-716357
73. Anderson E, Grzywacz B, Wang H, Wang T, Haagenson M, Spellman S, et al. Limited role of MHC class I chain-related gene A (MICA) typing in assessing graft-versus-host disease risk after fully human leukocyte antigen-matched unrelated donor transplantation. *Blood* (2009) 114:authorrely4754–5. doi:10.1182/blood-2009-08-239301
74. Askar M, Sobecks R, Wang T, Haagenson M, Majhail N, Madbouly A, et al. MHC class I chain-related gene A (MICA) donor-recipient mismatches and MICA-129 polymorphism in unrelated donor hematopoietic cell transplantations has no impact on outcomes in acute lymphoblastic leukemia, acute myeloid leukemia, or myelodysplastic syndrome: a center for International Blood and Marrow Transplant Research Study. *Biol Blood Marrow Transplant* (2017) 23:436–44. doi:10.1016/j.bbmt.2016.11.021
75. Raulet DH, Gasser S, Gowen BG, Deng W, Jung H. Regulation of ligands for the NKG2D activating receptor. *Annu Rev Immunol* (2013) 31:413–41. doi:10.1146/annurev-immunol-032712-095951
76. Stern-Ginossar N, Gur C, Biton M, Horwitz E, Elboim M, Stanietsky N, et al. Human microRNAs regulate stress-induced immune responses mediated by the receptor NKG2D. *Nat Immunol* (2008) 9:1065–73. doi:10.1038/ni.1642
77. Eissmann P, Evans JH, Mehrabi M, Rose EL, Nedvetzki S, Davis DM. Multiple mechanisms downstream of TLR-4 stimulation allow expression of NKG2D ligands to facilitate macrophage/NK cell crosstalk. *J Immunol* (2010) 184:6901–9. doi:10.4049/jimmunol.0903985
78. Wang B, Wang Q, Wang Z, Jiang J, Yu SC, Ping YF, et al. Metastatic consequences of immune escape from NK cell cytotoxicity by human breast cancer stem cells. *Cancer Res* (2014) 74:5746–57. doi:10.1158/0008-5472.CAN-13-2563
79. Xie J, Liu M, Li Y, Nie Y, Mi Q, Zhao S. Ovarian tumor-associated microRNA-20a decreases natural killer cell cytotoxicity by downregulating MICA/B expression. *Cell Mol Immunol* (2014) 11:495–502. doi:10.1038/cmi.2014.30
80. Yang H, Lan P, Hou Z, Guan Y, Zhang J, Xu W, et al. Histone deacetylase inhibitor SAHA epigenetically regulates miR-17-92 cluster and MCM7 to upregulate MICA expression in hepatoma. *Br J Cancer* (2015) 112:112–21. doi:10.1038/bjc.2014.547
81. Kishikawa T, Otsuka M, Yoshikawa T, Ohno M, Takata A, Shibata C, et al. Regulation of the expression of the liver cancer susceptibility gene MICA by microRNAs. *Sci Rep* (2013) 3:2739. doi:10.1038/srep02739
82. Ohno M, Otsuka M, Kishikawa T, Shibata C, Yoshikawa T, Takata A, et al. Specific delivery of microRNA93 into HBV-replicating hepatocytes downregulates protein expression of liver cancer susceptible gene MICA. *Oncotarget* (2014) 5:5581–90. doi:10.18632/oncotarget.2143
83. Yadav D, Ngolab J, Lim RS, Krishnamurthy S, Bui JD. Cutting edge: down-regulation of MHC class I-related chain A on tumor cells by IFN-gamma-induced microRNA. *J Immunol* (2009) 182:39–43. doi:10.4049/jimmunol.182.1.39
84. Min D, Lv XB, Wang X, Zhang B, Meng W, Yu F, et al. Downregulation of miR-302c and miR-520c by 1,25(OH)2D3 treatment enhances the susceptibility of tumour cells to natural killer cell-mediated cytotoxicity. *Br J Cancer* (2013) 109:723–30. doi:10.1038/bjc.2013.337
85. Fielding CA, Aicheler R, Stanton RJ, Wang EC, Han S, Seirafian S, et al. Two novel human cytomegalovirus NK cell evasion functions target MICA for lysosomal degradation. *PLoS Pathog* (2014) 10:e1004058. doi:10.1371/journal.ppat.1004058
86. Elias S, Mandelboim O. Battle of the midgets: innate microRNA networking. *RNA Biol* (2012) 9:792–8. doi:10.4161/rna.19717

87. Jasinski-Bergner S, Mandelboim O, Seliger B. The role of microRNAs in the control of innate immune response in cancer. *J Natl Cancer Inst* (2014) 106:dju257. doi:10.1093/jnci/dju257
88. Espinoza JL, Takami A, Yoshioka K, Nakata K, Sato T, Kasahara Y, et al. Human microRNA-1245 down-regulates the NKG2D receptor in natural killer cells and impairs NKG2D-mediated functions. *Haematologica* (2012) 97:1295–303. doi:10.3324/haematol.2011.058529
89. Boieri M, Shah P, Dressel R, Inngjerdingen M. The role of animal models in the study of hematopoietic stem cell transplantation and GvHD: a historical overview. *Front Immunol* (2016) 7:333. doi:10.3389/fimmu.2016.00333
90. Weber JA, Baxter DH, Zhang S, Huang DY, Huang KH, Lee MJ, et al. The microRNA spectrum in 12 body fluids. *Clin Chem* (2010) 56:1733–41. doi:10.1373/clinchem.2010.147405
91. Xiao B, Wang Y, Li W, Baker M, Guo J, Corbet K, et al. Plasma microRNA signature as a noninvasive biomarker for acute graft-versus-host disease. *Blood* (2013) 122:3365–75. doi:10.1182/blood-2013-06-510586
92. Verner J, Kabathova J, Tomancova A, Pavlova S, Tichy B, Mraz M, et al. Gene expression profiling of acute graft-vs-host disease after hematopoietic stem cell transplantation. *Exp Hematol* (2012) 40(899–905):e895. doi:10.1016/j.exphem.2012.06.011
93. Buzzeo MP, Yang J, Casella G, Reddy V. A preliminary gene expression profile of acute graft-versus-host disease. *Cell Transplant* (2008) 17:489–94. doi:10.3727/096368908785096042
94. Baron C, Somogyi R, Greller LD, Rineau V, Wilkinson P, Cho CR, et al. Prediction of graft-versus-host disease in humans by donor gene-expression profiling. *PLoS Med* (2007) 4:e23. doi:10.1371/journal.pmed.0040023
95. Takahashi N, Sato N, Takahashi S, Tojo A. Gene-expression profiles of peripheral blood mononuclear cell subpopulations in acute graft-vs-host disease following cord blood transplantation. *Exp Hematol* (2008) 36:1760–70. doi:10.1016/j.exphem.2008.07.007
96. Lai P, Weng J, Lu Z, Guo R, Luo C, Wu S, et al. Gene expression profiling-based identification of CD28 and PI3K as new biomarkers for chronic graft-versus-host disease. *DNA Cell Biol* (2011) 30:1019–25. doi:10.1089/dna.2011.1284
97. Westekemper H, Meller S, Citak S, Schulte C, Steuhl KP, Homey B, et al. Differential chemokine expression in chronic GVHD of the conjunctiva. *Bone Marrow Transplant* (2010) 45:1340–6. doi:10.1038/bmt.2009.346
98. Cocho L, Fernandez I, Calonge M, Martinez V, Gonzalez-Garcia MJ, Caballero D, et al. Gene expression-based predictive models of graft versus host disease-associated dry eye. *Invest Ophthalmol Vis Sci* (2015) 56:4570–81. doi:10.1167/iovs.15-16736
99. Sugerman PB, Faber SB, Willis LM, Petrovic A, Murphy GF, Pappo J, et al. Kinetics of gene expression in murine cutaneous graft-versus-host disease. *Am J Pathol* (2004) 164:2189–202. doi:10.1016/S0002-9440(10)63776-5
100. Ichiba T, Teshima T, Kuick R, Misek DE, Liu C, Takada Y, et al. Early changes in gene expression profiles of hepatic GVHD uncovered by oligonucleotide microarrays. *Blood* (2003) 102:763–71. doi:10.1182/blood-2002-09-2748
101. Sadeghi B, Al-Chaqmaqchi H, Al-Hashmi S, Brodin D, Hassan Z, Abedi-Valugerdi M, et al. Early-phase GVHD gene expression profile in target versus non-target tissues: kidney, a possible target? *Bone Marrow Transplant* (2013) 48:284–93. doi:10.1038/bmt.2012.120
102. Zhou L, Askew D, Wu C, Gilliam AC. Cutaneous gene expression by DNA microarray in murine sclerodermatous graft-versus-host disease, a model for human scleroderma. *J Invest Dermatol* (2007) 127:281–92. doi:10.1038/sj.jid.5700517
103. Novota P, Zinöcker S, Norden J, Wang XN, Sviland L, Opitz L, et al. Expression profiling of major histocompatibility and natural killer complex genes reveals candidates for controlling risk of graft versus host disease. *PLoS One* (2011) 6:e16582. doi:10.1371/journal.pone.0016582
104. Wang Y, Zhao X, Ye X, Luo H, Zhao T, Diao Y, et al. Plasma microRNA-586 is a new biomarker for acute graft-versus-host disease. *Ann Hematol* (2015) 94:1505–14. doi:10.1007/s00277-015-2414-z
105. Wu Y, Heinrichs J, Bastian D, Fu J, Nguyen H, Schutt S, et al. MicroRNA-17-92 controls T-cell responses in graft-versus-host disease and leukemia relapse in mice. *Blood* (2015) 126:1314–23. doi:10.1182/blood-2015-02-627356
106. Serody J. GVHD and miR: good things in small packages. *Blood* (2015) 126:1265–7. doi:10.1182/blood-2015-07-657114
107. Jiang S, Li C, Olive V, Lykken E, Feng F, Sevilla J, et al. Molecular dissection of the miR-17-92 cluster's critical dual roles in promoting Th1 responses and preventing inducible Treg differentiation. *Blood* (2011) 118:5487–97. doi:10.1182/blood-2011-05-355644
108. Leonhardt F, Grundmann S, Behe M, Bluhm F, Dumont RA, Braun F, et al. Inflammatory neovascularization during graft-versus-host disease is regulated by alphav integrin and miR-100. *Blood* (2013) 121:3307–18. doi:10.1182/blood-2012-07-442665
109. He L, He X, Lowe SW, Hannon GJ. microRNAs join the p53 network – another piece in the tumour-suppression puzzle. *Nat Rev Cancer* (2007) 7:819–22. doi:10.1038/nrc2232
110. Kee Y, D'andrea AD. Molecular pathogenesis and clinical management of Fanconi anemia. *J Clin Invest* (2012) 122:3799–806. doi:10.1172/JCI58321
111. Wang L, Romero M, Ratajczak P, Leboeuf C, Belhadj S, Peffault De Latour R, et al. Increased apoptosis is linked to severe acute GVHD in patients with Fanconi anemia. *Bone Marrow Transplant* (2013) 48:849–53. doi:10.1038/bmt.2012.237
112. Reddy P. Pathophysiology of acute graft-versus-host disease. *Hematol Oncol* (2003) 21:149–61. doi:10.1002/hon.716
113. Qian L, Wu Z, Shen J. Advances in the treatment of acute graft-versus-host disease. *J Cell Mol Med* (2013) 17:966–75. doi:10.1111/jcmm.12093
114. Ferrara JL, Marion A, Mcintyre JF, Murphy GF, Burakoff SJ. Amelioration of acute graft vs host disease due to minor histocompatibility antigens by in vivo administration of anti-interleukin 2 receptor antibody. *J Immunol* (1986) 137:1874–7.
115. Blaise D, Olive D, Hirn M, Viens P, Lafage M, Attal M, et al. Prevention of acute GVHD by in vivo use of anti-interleukin-2 receptor monoclonal antibody (33B3.1): a feasibility trial in 15 patients. *Bone Marrow Transplant* (1991) 8:105–11.
116. Zeiser R, Beilhack A, Negrin RS. Acute graft-versus-host disease-challenge for a broader application of allogeneic hematopoietic cell transplantation. *Curr Stem Cell Res Ther* (2006) 1:203–12. doi:10.2174/157488806776956896
117. Gavin MA, Rasmussen JP, Fontenot JD, Vasta V, Manganiello VC, Beavo JA, et al. Foxp3-dependent programme of regulatory T-cell differentiation. *Nature* (2007) 445:771–5. doi:10.1038/nature05543
118. Jaksch M, Mattsson J. The pathophysiology of acute graft-versus-host disease. *Scand J Immunol* (2005) 61:398–409. doi:10.1111/j.1365-3083.2005.01595.x
119. Dickinson AM, Sviland L, Hamilton PJ, Usher P, Taylor P, Jackson G, et al. Cytokine involvement in predicting clinical graft-versus-host disease in allogeneic bone marrow transplant recipients. *Bone Marrow Transplant* (1994) 13:65–70.
120. Tanaka J, Imamura M, Kasai M, Sakurada K, Miyazaki T. Cytokine gene expression after allogeneic bone marrow transplantation. *Leuk Lymphoma* (1995) 16:413–8. doi:10.3109/10428199509054427
121. Das H, Imoto S, Murayama T, Mizuno I, Sugimoto T, Taniguchi R, et al. Kinetic analysis of cytokine gene expression in patients with GVHD after donor lymphocyte infusion. *Bone Marrow Transplant* (2001) 27:373–80. doi:10.1038/sj.bmt.1702799
122. Poloni A, Sartini D, Emanuelli M, Trappolini S, Mancini S, Pozzi V, et al. Gene expression profile of cytokines in patients with chronic graft-versus-host disease after allogeneic hematopoietic stem cell transplantation with reduced conditioning. *Cytokine* (2011) 53:376–83. doi:10.1016/j.cyto.2010.12.008
123. Kohrt HE, Tian L, Li L, Alizadeh AA, Hsieh S, Tibshirani RJ, et al. Identification of gene microarray expression profiles in patients with chronic graft-versus-host disease following allogeneic hematopoietic cell transplantation. *Clin Immunol* (2013) 148:124–35. doi:10.1016/j.clim.2013.04.013
124. Piguet PF, Grau GE, Allet B, Vassalli P. Tumor necrosis factor/cachectin is an effector of skin and gut lesions of the acute phase of graft-vs.-host disease. *J Exp Med* (1987) 166:1280–9. doi:10.1084/jem.166.5.1280
125. Cooke KR, Hill GR, Crawford JM, Bungard D, Brinson YS, Delmonte J Jr, et al. Tumor necrosis factor-alpha production to lipopolysaccharide stimulation by donor cells predicts the severity of experimental acute graft-versus-host disease. *J Clin Invest* (1998) 102:1882–91. doi:10.1172/JCI4285
126. Blaser BW, Roychowdhury S, Kim DJ, Schwind NR, Bhatt D, Yuan W, et al. Donor-derived IL-15 is critical for acute allogeneic graft-versus-host disease. *Blood* (2005) 105:894–901. doi:10.1182/blood-2004-05-1687
127. Kumaki S, Minegishi M, Fujie H, Sasahara Y, Ohashi Y, Tsuchiya S, et al. Prolonged secretion of IL-15 in patients with severe forms of acute graft-versus-host disease after allogeneic bone marrow transplantation in children. *Int J Hematol* (1998) 67:307–12. doi:10.1016/S0925-5710(97)00117-5

128. Shimizu M, Shimamura M, Owaki T, Asakawa M, Fujita K, Kudo M, et al. Antiangiogenic and antitumor activities of IL-27. *J Immunol* (2006) 176:7317–24. doi:10.4049/jimmunol.176.12.7317
129. Liu Y, Wu Y, Wang Y, Cai Y, Hu B, Bao G, et al. IL-35 mitigates murine acute graft-versus-host disease with retention of graft-versus-leukemia effects. *Leukemia* (2015) 29:939–46. doi:10.1038/leu.2014.310
130. Zhang XH, Zhou Y, Zhang JM, Zhou SY, Wang M, Feng R, et al. IL-35 inhibits acute graft-versus-host disease in a mouse model. *Int Immunopharmacol* (2015) 29:383–92. doi:10.1016/j.intimp.2015.10.025
131. Middleton PG, Taylor PR, Jackson G, Proctor SJ, Dickinson AM. Cytokine gene polymorphisms associating with severe acute graft-versus-host disease in HLA-identical sibling transplants. *Blood* (1998) 92:3943–8.
132. Cavet J, Middleton PG, Segall M, Noreen H, Davies SM, Dickinson AM. Recipient tumor necrosis factor-alpha and interleukin-10 gene polymorphisms associate with early mortality and acute graft-versus-host disease severity in HLA-matched sibling bone marrow transplants. *Blood* (1999) 94:3941–6.
133. Socie G, Loiseau P, Tamouza R, Janin A, Busson M, Gluckman E, et al. Both genetic and clinical factors predict the development of graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. *Transplantation* (2001) 72:699–706. doi:10.1097/00007890-200108270-00024
134. Mullighan CG, Bardy PG. New directions in the genomics of allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant* (2007) 13:127–44. doi:10.1016/j.bbmt.2006.10.018
135. Espinoza JL, Takami A, Nakata K, Onizuka M, Kawase T, Akiyama H, et al. A genetic variant in the IL-17 promoter is functionally associated with acute graft-versus-host disease after unrelated bone marrow transplantation. *PLoS One* (2011) 6:e26229. doi:10.1371/journal.pone.0026229
136. Holler E, Rogler G, Brenmoehl J, Hahn J, Herfarth H, Greinix H, et al. Prognostic significance of NOD2/CARD15 variants in HLA-identical sibling hematopoietic stem cell transplantation: effect on long-term outcome is confirmed in 2 independent cohorts and may be modulated by the type of gastrointestinal decontamination. *Blood* (2006) 107:4189–93. doi:10.1182/blood-2005-09-3741
137. Grube M, Brenmoehl J, Rogler G, Hahn J, Herr W, Holler E. Donor nucleotide-binding oligomerization-containing protein 2 (NOD2) single nucleotide polymorphism 13 is associated with septic shock after allogeneic stem cell transplantation. *Biol Blood Marrow Transplant* (2015) 21:1399–404. doi:10.1016/j.bbmt.2015.05.011
138. Economou M, Pappas G. New global map of Crohn's disease: genetic, environmental, and socioeconomic correlations. *Inflamm Bowel Dis* (2008) 14:709–20. doi:10.1002/ibd.20352
139. Chien JW, Zhang XC, Fan W, Wang H, Zhao LP, Martin PJ, et al. Evaluation of published single nucleotide polymorphisms associated with acute GVHD. *Blood* (2012) 119:5311–9. doi:10.1182/blood-2011-09-371153
140. Rosenstiel P, Fantini M, Brautigam K, Kuhbacher T, Waetzig GH, Seegert D, et al. TNF-alpha and IFN-gamma regulate the expression of the NOD2 (CARD15) gene in human intestinal epithelial cells. *Gastroenterology* (2003) 124:1001–9. doi:10.1053/gast.2003.50157
141. Goussetis E, Varela I, Peristeri I, Kitra V, Spanou K, Moraloglou O, et al. Cytokine gene polymorphisms and graft-versus-host disease in children after matched sibling hematopoietic stem cell transplantation: a single-center experience. *Cell Mol Immunol* (2011) 8:276–80. doi:10.1038/cmi.2011.4
142. Groom JR, Luster AD. CXCR3 ligands: redundant, collaborative and antagonistic functions. *Immunol Cell Biol* (2011) 89:207–15. doi:10.1038/icb.2010.158
143. Ahmed SS, Wang XN, Norden J, Pearce K, El-Gezawy E, Atarod S, et al. Identification and validation of biomarkers associated with acute and chronic graft versus host disease. *Bone Marrow Transplant* (2015) 50:1563–71. doi:10.1038/bmt.2015.191
144. Bouazzaoui A, Spacenko E, Mueller G, Miklos S, Huber E, Holler E, et al. Chemokine and chemokine receptor expression analysis in target organs of acute graft-versus-host disease. *Genes Immun* (2009) 10:687–701. doi:10.1038/gene.2009.49
145. Hasegawa H, Inoue A, Kohno M, Lei J, Miyazaki T, Yoshie O, et al. Therapeutic effect of CXCR3-expressing regulatory T cells on liver, lung and intestinal damages in a murine acute GVHD model. *Gene Ther* (2008) 15:171–82. doi:10.1038/sj.gt.3303051
146. Castor MG, Pinho V, Teixeira MM. The role of chemokines in mediating graft versus host disease: opportunities for novel therapeutics. *Front Pharmacol* (2012) 3:23. doi:10.3389/fphar.2012.00023
147. Hansen JA, Chien JW, Warren EH, Zhao LP, Martin PJ. Defining genetic risk for graft-versus-host disease and mortality following allogeneic hematopoietic stem cell transplantation. *Curr Opin Hematol* (2010) 17:483–92. doi:10.1097/MOH.0b013e32833eb770
148. Goker H, Haznedaroglu IC, Chao NJ. Acute graft-vs-host disease: pathobiology and management. *Exp Hematol* (2001) 29:259–77. doi:10.1016/S0301-472X(00)00677-9
149. Hipp MS, Kalveram B, Raasi S, Groettrup M, Schmidtke G. FAT10, a ubiquitin-independent signal for proteasomal degradation. *Mol Cell Biol* (2005) 25:3483–91. doi:10.1128/MCB.25.9.3483-3491.2005
150. Warren EH, Zhang XC, Li S, Fan W, Storer BE, Chien JW, et al. Effect of MHC and non-MHC donor/recipient genetic disparity on the outcome of allogeneic HCT. *Blood* (2012) 120:2796–806. doi:10.1182/blood-2012-04-347286
151. Dickinson AM, Charron D. Non-HLA immunogenetics in hematopoietic stem cell transplantation. *Curr Opin Immunol* (2005) 17:517–25. doi:10.1016/j.coi.2005.07.017
152. Turpeinen H, Ojala PJ, Ojala K, Miettinen M, Volin L, Partanen J. Minor histocompatibility antigens as determinants for graft-versus-host disease after allogeneic haematopoietic stem cell transplantation. *Int J Immunogenet* (2013) 40:495–501. doi:10.1111/iji.12051
153. Sampson JK, Sheth NU, Koparde VN, Scalora AF, Serrano MG, Lee V, et al. Whole exome sequencing to estimate alloreactivity potential between donors and recipients in stem cell transplantation. *Br J Haematol* (2014) 166:566–70. doi:10.1111/bjh.12898
154. Jameson-Lee M, Koparde V, Griffith P, Scalora AF, Sampson JK, Khalid H, et al. In silico derivation of HLA-specific alloreactivity potential from whole exome sequencing of stem-cell transplant donors and recipients: understanding the quantitative immunobiology of allogeneic transplantation. *Front Immunol* (2014) 5:529. doi:10.3389/fimmu.2014.00529
155. Toor AA, Kobulnicky JD, Salman S, Roberts CH, Jameson-Lee M, Meier J, et al. Stem cell transplantation as a dynamical system: are clinical outcomes deterministic? *Front Immunol* (2014) 5:613. doi:10.3389/fimmu.2014.00613
156. Abdul Razzaq B, Scalora A, Koparde VN, Meier J, Mahmood M, Salman S, et al. Dynamical system modeling to simulate donor T cell response to whole exome sequencing-derived recipient peptides demonstrates different alloreactivity potential in HLA-matched and -mismatched donor-recipient pairs. *Biol Blood Marrow Transplant* (2016) 22:850–61. doi:10.1016/j.bbmt.2015.11.1103
157. Theiss-Suennemann J, Jörss K, Messmann JJ, Reichardt SD, Montes-Cobos E, Lühder F, et al. Glucocorticoids attenuate acute graft-versus-host disease by suppressing the cytotoxic capacity of CD8(+) T cells. *J Pathol* (2015) 235:646–55. doi:10.1002/path.4475
158. Ren G, Zhao X, Zhang L, Zhang J, L'huillier A, Ling W, et al. Inflammatory cytokine-induced intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in mesenchymal stem cells are critical for immunosuppression. *J Immunol* (2010) 184:2321–8. doi:10.4049/jimmunol.0902023
159. Alegre ML, Frauwirth KA, Thompson CB. T-cell regulation by CD28 and CTLA-4. *Nat Rev Immunol* (2001) 1:220–8. doi:10.1038/35105024
160. Manickasingham SP, Anderton SM, Burkhart C, Wraith DC. Qualitative and quantitative effects of CD28/B7-mediated costimulation on naive T cells in vitro. *J Immunol* (1998) 161:3827–35.
161. Karabon L, Markiewicz M, Partyka A, Pawlak-Adamska E, Tomkiewicz A, Dzierzak-Mietla M, et al. A CT60G>A polymorphism in the CTLA-4 gene of the recipient may confer susceptibility to acute graft versus host disease after allogeneic hematopoietic stem cell transplantation. *Immunogenetics* (2015) 67:295–304. doi:10.1007/s00251-015-0840-7
162. Mossallam GI, Samra MA. CTLA-4 polymorphism and clinical outcome post allogeneic hematopoietic stem cell transplantation. *Hum Immunol* (2013) 74:1643–8. doi:10.1016/j.humimm.2013.08.002
163. Iravani-Saadi M, Karimi MH, Yaghobi R, Geramizadeh B, Ramzi M, Niknam A, et al. Polymorphism of costimulatory molecules (CTLA4, ICOS, PD.1 and CD28) and allogeneic hematopoietic stem cell transplantation in Iranian patients. *Immunol Invest* (2014) 43:391–404. doi:10.3109/08820139.2013.879594
164. Sato M, Storb R, Loretz C, Stone D, Mielcarek M, Sale GE, et al. Inducible costimulator (ICOS) up-regulation on activated T cells in chronic

graft-versus-host disease after dog leukocyte antigen-nonidentical hematopoietic cell transplantation: a potential therapeutic target. *Transplantation* (2013) 96:34–41. doi:10.1097/TP.0b013e318295c025
165. Yew PY, Alachkar H, Yamaguchi R, Kiyotani K, Fang H, Yap KL, et al. Quantitative characterization of T-cell repertoire in allogeneic hematopoietic stem cell transplant recipients. *Bone Marrow Transplant* (2015) 50:1227–34. doi:10.1038/bmt.2015.133
166. Shimabukuro-Vornhagen A, Hallek MJ, Storb RF, Von Bergwelt-Baildon MS. The role of B cells in the pathogenesis of graft-versus-host disease. *Blood* (2009) 114:4919–27. doi:10.1182/blood-2008-10-161638
167. Schultz KR, Paquet J, Bader S, Hayglass KT. Requirement for B cells in T cell priming to minor histocompatibility antigens and development of graft-versus-host disease. *Bone Marrow Transplant* (1995) 16:289–95.
168. Allen JL, Tata PV, Fore MS, Wooten J, Rudra S, Deal AM, et al. Increased BCR responsiveness in B cells from patients with chronic GVHD. *Blood* (2014) 123:2108–15. doi:10.1182/blood-2013-10-533562
169. Xu S, Tan JE, Wong EP, Manickam A, Ponniah S, Lam KP. B cell development and activation defects resulting in xid-like immunodeficiency in BLNK/SLP-65-deficient mice. *Int Immunol* (2000) 12:397–404. doi:10.1093/intimm/12.3.397
170. Dickinson AM. SNPs and GVHD prediction: where to next? *Blood* (2012) 119:5066–8. doi:10.1182/blood-2012-03-409078
171. Sato-Otsubo A, Nannya Y, Kashiwase K, Onizuka M, Azuma F, Akatsuka Y, et al. Genome-wide surveillance of mismatched alleles for graft-versus-host disease in stem cell transplantation. *Blood* (2015) 126:2752–63. doi:10.1182/blood-2015-03-630707
172. Bari R, Hartford C, Chan WK, Vong Q, Li Y, Gan K, et al. Genome-wide single-nucleotide polymorphism analysis revealed SUFU suppression of acute graft-versus-host disease through downregulation of HLA-DR expression in recipient dendritic cells. *Sci Rep* (2015) 5:11098. doi:10.1038/srep11098
173. Dickinson AM, Pearce KF, Norden J, O'brien SG, Holler E, Bickeboller H, et al. Impact of genomic risk factors on outcome after hematopoietic stem cell transplantation for patients with chronic myeloid leukemia. *Haematologica* (2010) 95:922–7. doi:10.3324/haematol.2009.016220
174. Pearce KF, Lee SJ, Haagenson M, Petersdorf EW, Norden J, Collin MP, et al. Analysis of non-HLA genomic risk factors in HLA-matched unrelated donor hematopoietic cell transplantation for chronic myeloid leukemia. *Haematologica* (2012) 97:1014–9. doi:10.3324/haematol.2011.053611
175. Gratwohl A, Hermans J, Goldman JM, Arcese W, Carreras E, Devergie A, et al. Risk assessment for patients with chronic myeloid leukaemia before allogeneic blood or marrow transplantation. Chronic Leukemia Working Party of the European Group for blood and marrow transplantation. *Lancet* (1998) 352:1087–92. doi:10.1016/S0140-6736(98)03030-X
176. Gratwohl A, Brand R, Apperley J, Crawley C, Ruutu T, Corradini P, et al. Allogeneic hematopoietic stem cell transplantation for chronic myeloid leukemia in Europe 2006: transplant activity, long-term data and current results. An analysis by the Chronic Leukemia Working Party of the European Group for Blood and Marrow Transplantation (EBMT). *Haematologica* (2006) 91:513–21.
177. Gratwohl A, Stern M, Brand R, Apperley J, Baldomero H, De Witte T, et al. Risk score for outcome after allogeneic hematopoietic stem cell transplantation: a retrospective analysis. *Cancer* (2009) 115:4715–26. doi:10.1002/cncr.24531
178. Gratwohl A. The EBMT risk score. *Bone Marrow Transplant* (2012) 47:749–56. doi:10.1038/bmt.2011.110
179. Schoenfeld D. Partial residuals for the proportional hazards regression model. *Biometrika* (1982) 69:239–41. doi:10.1093/biomet/69.1.239
180. Efron B. Estimating the error rate of a prediction rule: improvement on cross-validation. *J Am Stat Assoc* (1983) 78:316–31. doi:10.1080/01621459.1983.10477973
181. Pearce KF, Balavarca Y, Norden J, Jackson G, Holler E, Dressel R, et al. Impact of genomic risk factors on survival after haematopoietic stem cell transplantation for patients with acute leukaemia. *Int J Immunogenet* (2016) 43:404–12. doi:10.1111/iji.12295

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REVIEW
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Recent Developments in Cellular Immunotherapy for HSCT-Associated Complications

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Allogeneic hematopoietic stem cell transplantation is associated with serious complications, and improvement of the overall clinical outcome of patients with hematological malignancies is necessary. During the last decades, posttransplant donor-derived adoptive cellular immunotherapeutic strategies have been progressively developed for the treatment of graft-versus-host disease (GvHD), infectious complications, and tumor relapses. To date, the common challenge of all these cell-based approaches is their implementation for clinical application. Establishing an appropriate manufacturing process, to guarantee safe and effective therapeutics with simultaneous consideration of economic requirements is one of the most critical hurdles. In this review, we will discuss the recent scientific findings, clinical experiences, and technological advances for cell processing toward the application of mesenchymal stromal cells as a therapy for treatment of severe GvHD, virus-specific T cells for targeting life-threating infections, and of chimeric antigen receptors-engineered T cells to treat relapsed leukemia.

Keywords: mesenchymal stromal cells, immunomodulation, extracellular vesicles, infection, adoptive transfer, chimeric antigen receptor, T cells, cell manufacture

INTRODUCTION

The medical need for improved therapeutic options to successfully treat patients with hematologic malignancies is high. Allogeneic hematopoietic stem cell transplantation (HSCT) is the only curative treatment for patients with hematologic malignancies, but the success of the therapy is limited by several severe side effects. One major obstacle with the highest transplant-related mortality rate is the recurrence of the underlying disease, due to failure in effective eradication of malignant cells by the reconstituted allogeneic immune system, mediated largely by T cells. The leading cause of non-relapse mortality is graft-versus-host disease (GvHD), an inflammatory immune reaction against healthy tissue of the patient, induced by donor-derived T cells and triggered by major and minor histocompatibility antigen differences between HSCT recipient and donor. Due to immunosuppressive treatment of the patient for prophylaxis and posttransplant therapy of GvHD, the appearance of life-threatening opportunistic infections is responsible for a substantial rate of non-relapse mortality. Thus, one of the biggest challenges for an effective treatment with allogeneic HSCT is maintaining the balance between tolerance of the host, elimination of the malignancy, and protection against infections.

Engineering of the allograft itself is one possible strategy to reduce the risk for development of GvHD and concomitantly remain the favorable immune reaction toward the tumor and infectious pathogens. The incidence and severity of GvHD can be reduced by *ex vivo* T cell removal either achieved *via* $CD34^+$ hematopoietic stem cell enrichment or active depletion of T cells, but these approaches have been associated with the risk for occurrence of graft rejection, relapse, and infections due to the missing T cells. However, for matched sibling donor transplantation in acute myeloid leukemia, it has been shown recently that *ex vivo* T cell depletion can reduce the incidence of chronic GvHD significantly without affecting the relapse rate (1, 2). The most novel procedures in graft manipulation aim for the elimination of potential alloreactive T cells only, allowing antiviral and antitumor T cells to remain in the transplant supporting tumor elimination and providing protection against infections (3–8).

Another strategy to control allogeneic HSCT-related complications is the adaptive transfer of *ex vivo* selected donor-derived immune cell populations after transplantation. At first, donor lymphocyte infusions (DLI) were established to prevent and treat relapses, but, subsequently, controlling infections became an important matter for concern (9, 10). DLI contain allogeneic T cells and are therefore associated with an increased risk for the onset of GvHD. These observations initiated the development of several adoptive therapies with selected immune cell populations depleted of alloreactive cells. Strategies that are followed include the adoptive therapy of regulatory T cells (Tregs) and mesenchymal stromal cells (MSCs) for treatment of GvHD, dendritic cell (DC) vaccination and natural killer (NK) cell transfer to support antitumor responses, as well as application of T cells to control infections or to induce antitumor responses (11–13).

Despite the differences in cell type and the underlying medical problem, which require specific considerations during the translational phase, various hurdles are common for all cellular immunotherapies. At present, a variety of clinical protocols, including cell manufacturing processes, have been generated for each of the three therapeutic approaches and reached a stage of evaluation within clinical trials. However, the obstacles, prior to clinical application which remain, include the establishment of standardized clinical protocols and understanding the therapeutic mechanisms. Nevertheless, the promising and beneficial clinical outcomes of early-phase clinical studies, the enormous achievements in scientific understanding of immune interventions, and the innovative technical advances in cell manipulation and processing has led to a huge growth in interest in cellular immunotherapy, especially in the area of hematological diseases. To offer these new therapeutic options as standard-of-care treatments for all patients, various aspects have to be considered for the implementation into clinical practice, in particular with regard to the cell manufacturing. Cell-processing protocols, often developed in research laboratories using tools and technologies available or suitable for research application only, need to be process engineered to good manufacturing practice (GMP) prior to clinical application.

This review will discuss the challenges and recent progresses made toward clinical application of MSCs for the management of GvHD, antiviral T cells for the treatment of opportunistic viral infections, and chimeric antigen receptors (CAR)-engineered T cells as an adoptive therapy for leukemia relapses. These three examples allow us to not only to highlight technological and clinical advances of the individual therapy but also discuss general aspects of translation, especially with regard to cell processing.

CLINICAL APPLICATION WITH MESENCHYMAL STROMAL CELLS FOR THE MANAGEMENT OF GvHD

Mesenchymal stromal cells are multipotent progenitor cells, which can be acquired from various adult tissues, primarily bone marrow (BM) (14). Their immunomodulatory property has empowered them to play an important role as a cellular therapy for GvHD (15). GvHD is a frequent and potentially life-threatening complication after allogeneic HSCT, affecting 40–60% of patients, and a leading cause of non-relapse mortality (16, 17). Despite significant advances in the understanding of GvHD pathogenesis and the development of transplantation medicine, corticosteroids remain the first-line treatment of GvHD, but with only an approximately 50% response rate. Patients who fail the standard steroid treatment have an overall survival rate of only 5–30% (18–20). Apart from the low response rate, steroid treatment also bears the risk of increased leukemia relapse and opportunistic infections. To improve the efficacy of GvHD management, several cellular immunotherapies have been developed using MSCs as well as DCs and Tregs (17, 21, 22).

Lessons Learned from Recent Clinical Trials

Since the first case report in which infusion of haploidentical MSCs showed a beneficial outcome in the treatment of severe treatment-refractory acute GvHD (aGvHD) (23), an increasing number of clinical trials have been conducted to evaluate the effect of MSC infusion on GvHD for over a decade (17, 24, 25). The outcome of early clinical trials has been well reviewed. This article mainly collates recent clinical studies, reported between 2010 and 2015, on the prophylactic and therapeutic use of MSCs for aGvHD. The relevant information is summarized in **Table 1** (26–29) and **Table 2** (15, 30–38), respectively.

These reports have shown encouraging results indicative of positive steps taken toward the development of a more refined MSC therapy, although significant improvements are still needed. First, recent clinical studies have shown a clear trend toward replacing fetal calf serum (FCS) with human platelet lysate (hPL) to generate MSCs. Until the first clinical trial utilizing hPL-expanded MSCs to treat aGvHD being reported in 2009 (25), all clinical trials in the HSCT setting were performed using MSCs expanded in FCS-containing medium, a condition no longer accepted under current regulatory GMP requirements. As illustrated in **Table 2**, 40% (4/10) of clinical studies published between 2010 and 2015 have used MSCs expanded

TABLE 1 | Prophylactic use of MSCs to prevent GvHD.

MSCs	HSCs	MSC group	Ctrl group	Observation on GvHD incidence/severity	Reference
UCB 0.5 × 10^6/kg Single dose	BM, PBSC Haploidentical Without TCD	21	None	9 of 21 patients developed aGvHD (II–IV)	(27)
UCB 0.5 × 10^6/kg Single dose	BM, PBSC Haploidentical	50	None	12 of 50 patients developed aGvHD (II–IV)	(26)
BM-PL of HSC donor 0.9–1.3 × 10^6/kg Single dose	BM Donor type NR	19	18 Randomized	1 of 19 patients had aGvHD in MSC group 6 of 18 patients had aGvHD (II–IV) in Ctrl group	(28)
BM, third party 0.9–1.3 × 10^6/kg Single dose	PBSC MMR or MMU	20	16 Historic	9 of 20 patients had aGvHD (II–IV) in MSC group 9 of 16 patients had aGvHD (II–IV) in Ctrl group	(29)

BM, bone marrow; PBSC, peripheral blood stem cells; UCB, umbilical cord blood; HSCs, hematopoietic stem cells; NR, not reported; BM-PL, platelet lysate expanded MSC; MMR, HLA-mismatch related donors; MMU, HLA-mismatched unrelated donors; TCD, T cell depletion.

TABLE 2 | Therapeutic use of MSC infusion for steroid-resistant/refractory aGvHD.

MSCs	HSCs	No. Pts	Clinical outcome	Reference
BM, third party 1 × 10^6/kg 2–8 infusions	BM, PBSC, CUB HLA identical Haploidentical HLA-mismatched	28	CR: 61% OR: 75%	(15)
BM-PL, third party 1.5 × 10^6/kg 1–5 infusions	BM, PBSC, UCB HLA identical Haploidentical HLA-mismatched	40	CR: 27.5% OR: 67.5%	(30)
BM-PL, third party 1.1 × 10^6/kg 2–4 infusions	NR	25	CR: 46% OR: 71%	(31)
BM, third party 2 × 10^6/kg 8–12 infusions	BM, PBSC, UCB, DLI HLA-matched HLA-mismatched	75	CR: NR OR: 61.3%	(35)
BM, third party n = 34 1–2 × 10^6/kg 1–13 infusions	BM, PBSC, UCB, DLI HLA identical, MUD Haploidentical	37	CR: 65% OR: 86%	(36)
BM, third party 1.1 × 10^6/kg 1–4 infusions	BM, PBSC, UCB HLA identical, MUD Haploidentical, UCB	50	CR: 34% IR: 66%	(37)
BM, third party 1.7–2.3 × 10^6/kg 2–8 infusion	PBSC MUD	12	CR: 58.3% OR: 91.7%	(38)
BM-AS/AS + PL haplo- & RD 1–2 × 10^6/kg 1–4 infusions	BM, PBSC HLA-matched HLA-mismatched	10	CR: 10% OR: 70%	(32)
BM, third party 8 × 10^6/kg n = 2 2 × 10^6/kg n = 10 8–12 infusions	BM, PBSC, UCB HLA-matched HLA-mismatched	12	CR: 58% OR: 75%	(34)
BM-PL, third party 1.2 × 10^6/kg 1–5 infusions	BM, PBSC, UCB HLA-matched HLA-mismatched	11	CR: 23.8% OR: 71.4%	(33)

No. Pts, number of patients; BM, bone marrow; PBSC, peripheral blood stem cells; UCB, umbilical cord blood; DLI, donor lymphocyte infusions; MUD, HLA-matched unrelated donor; RD, related donors; HSC, hematopoietic stem cells; NR, not reported; BM-PL, platelet lysate expanded MSC; BM-AS, human serum expanded MSC; CR, complete response; OR, overall response; IR, initial response.

in hPL or human serum (30–33), which provides evidence and confidence for a xeno-free era of MSC production. Second, in 90% (9/10) of recent clinical trials, MSCs have been generated from third-party donors (**Table 2**), and some patients received different batches of MSCs derived from two or more donors (15, 34, 35). This has further strengthened the concept response rates

are independent on HLA-matching and reinforced the feasibility of using pre-manufactured "off-the-shelf" MSCs as a therapeutic agent (34, 35). On the other hand, recent clinical studies have also exposed significant limitations in the field. Although the reported response rates indicate some effect of MSCs on GvHD, their therapeutic efficacy remains ambiguous with complete and overall response rates varying from 10 to 65% and 61 to 91%, respectively, across the studies (**Table 2**). This can be attributed to multifactorial factors such as small patient cohort, lack of uniform efficacy measure and appropriate control groups in the analysis, heterogeneity in patient/MSC populations, and varying HSCT regimens. The lack of standardized protocols for MSC production and differences in dose/timing of MSC delivery could also contribute to the inconsistent results. These limitations highlight the need to interpret reported therapeutic efficacy with caution and preclude a definitive conclusion for the efficacy of MSCs in the treatment of GvHD.

Collectively, although the therapeutic efficacy of MSCs remains controversial, clinical studies consistently suggest that MSCs are safe to infuse in humans with no acute toxicity and no ectopic tissue formation, irrespective of their origin, culture conditions, and doses (17, 34, 39, 40). No association has been observed between MSC therapy and organ complications, death, or malignancy (41). This safety record allows future trials to be conducted using improved trial design and optimized practical procedures. Due to their immunosuppressive nature, whether MSC infusion could increase the risk of leukemia relapse and/or infectious diseases has been an area of concern. Results from clinical studies are highly controversial (40, 42, 43). This subject has been extensively discussed in a recent review (44). To date, MSC therapy in HSCT settings remains exploratory and experimental.

Manufacturing of GMP-Compliant MSC Products

Among a spectrum of challenges, GMP-compliant cell production is one of the most critical steps. Translation of pre-clinical MSC amplification into clinical-grade large-scale expansion presents a big challenge for the development of a successful therapy. As with any cell therapy, the manufacturing process of MSCs for human use must follow GMP conditions and appropriate regulations to ensure product efficacy and safety. To achieve this, specialized GMP facilities, equipments, and trained staff are required. In addition, the unique characteristics of MSCs regarding cell source and cell culturing, including cell seeding, expansion, and culture medium, have to be considered. MSCs are mainly generated from BM, but umbilical cord and adipose tissue are also considered as well as a reliable source. Due to the low frequency of MSCs in BM (0.001–0.01%), large-scale *ex vivo* expansion is a pre-requisite to achieve the required cell dose of about 1–2 × 10^6/kg, in total around 100–200 million cells/patient prior to clinical application (45). A very important factor to allow for a good expansion of MSCs is the density of plating the cells. As MSC are adherent cells, their growth is inhibited by reaching confluence. As a consequence, successive passaging of the cells has to be performed, and, typically after 3 weeks of culture, the proliferation rate and the differentiation potential declines. Furthermore, the increasing age of the donor is reported to be linked with a reduced expansion and multipotency (46). Details on standardization of the production of clinically applied products and further requirements have been summarized in several reviews (47, 48).

Development of Xeno-Free Expansion Medium

For the purpose of human applications, The International Society of Cellular Therapy (ISCT) recommends that reagents used for cell processing be free of xenogeneic products, due to the potential for infections, and that expansion be limited to early passages, due to the theoretical risk of cell senescence and malignant transformation. Conventionally, FCS is used for MSCs expansion for research applications and most clinical trials so far. FCS is a complex mixture of mitogenic factors which contribute to the maintenance and proliferation of MSCs *in vitro* (49). It is by nature ill-defined and exhibits batch-to-batch variability (50). It could be associated with the transmission of prions and undefined zoonosis as well as an increased risk of triggering adverse immune reactions resulting in the elimination of infused MSC, especially when multiple infusions are required (51). Therefore, the use of FCS is being criticized and strongly discouraged by the regulatory agencies, which urge for the development of GMP-compliant media, either serum- or animal-free, that can be standardized and used in both, research and clinical trials.

Over the last decade, numerous laboratories have been focused on the development of medium formulations that are either serum-free or use human blood-derived products, such as human autologous or pooled allogeneic serum, cord blood, and platelet derivatives (49, 51–55). Despite promising results with these culture supplements, the use of platelet derivatives, more specifically hPL has illustrated the best results. hPLs are manufactured by platelet disruption, using freeze/thaw protocols. Relatively standardized batches of hPL are produced by pooling platelet concentrates of several healthy donors (56). Repeated freezing/thawing of platelet concentrates allows the release of growth factors at a higher level that those in most FCS batches, such as basic fibroblast growth factor (bFGF), insulin growth factor 1 (IGF1), platelet-derived growth factor (PDGF), and transforming growth factor beta (TGF-β) (57). Several studies have demonstrated the use of hPL for MSC expansion provides increased proliferative capacity, while maintaining differentiation and immunomodulatory properties (57–59). These promising results have prompted the use of hPL-generated MSCs in clinical applications. Currently, 11 registered clinical studies are ongoing utilizing hPL-expanded MSCs for the treatment of GvHD, Crohn's disease, and diabetes (www.clinicaltrials.gov; as for 10/2016). Meanwhile, GMP-grade complete media specially developed for MSC expansion are commercially available, which also achieve higher expansion rate and thereby shorten the production time and the associated risk of product contamination (48).

Culture Systems and Product Release

Classically, MSC expansion is performed in open culture systems using numerous plastic culture flasks or cell stacks. Manual

handling steps for sequential cell passaging are labor intensive and time consuming, as well as bearing the risk for contamination. In this respect, automated and closed devices would simplify the manufacturing and increase product safety. Suitable bioreactors for MSC expansion on the market are the Quantum® (Terumo) and Scinus Cell Expansion™ (Xpand Biotechnology). In addition, the CliniMACS Prodigy® (Miltenyi Biotec) allows automated cell processing starting from sample preparation to cell culture and magnetic cell separation until the final formulation of the cellular product in a closed system by using single-use tubing sets (60). The instrument has the capability for preparation of mononuclear cells from BM samples using high-density gradient centrifugation prior to cell expansion. Additionally, magnetic enrichment steps for MSCs could be integrated into the manufacturing process, either before or after the expansion phase to further increase the purity of the cellular product.

Regarding the quality control for product release, the ISCT recommendation is to test for three characteristics of MSCs: (1) adherence to plastic; (2) expression of defined MSC cell surface markers, including positivity for CD73, CD90, and CD105 but negative for hematopoietic cell markers CD14, CD19, CD34, CD45; and (3) differentiation ability toward osteoblastic, chondrogenic, and adipocytic linages (61). Further tests, such as immunopotency assays and cytogenetic analysis remain at the discretion of the regulatory authorities (62). Ultimately, the most pressing issue relating to therapeutic efficacy is the fact that currently no standardized immune potency assay exists for quality control. This is partly complicated by their complex mechanism of action and the lack of understanding regarding MSC distribution and overall fate after infusion. However, a recent publication has described three tests defined in an ISCT workshop as potential release criteria: quantitative RNA analysis of selected gene products related to the cell's immunomodulatory function, flow cytometry analysis of functionally relevant surface markers, and a protein-based assay of the MSC secretome (63). Together, these could provide appropriate guidance for releasing products, however not enough evidence currently exists to support their definitive use. Furthermore, a comprehensive understanding in the mechanisms of action of MSCs holds the key to successful development of future MSC therapies.

MSC-Derived Extracellular Vesicles

Extracellular vesicles (EVs) are nanovesicles secreted by various cell types and are composed of a phospholipid bilayer, including transmembrane proteins and cell-specific receptors, enclosing cytoplasmic components. EVs are responsible for the horizontal transfer of bioactive proteins and genetic material, by internalization into endocytic compartments, fusion with plasma membranes, and/or by recognition of specific receptors (64). EVs can be easily isolated from cell culture medium and have been detected in a wide variety of bodily fluids (65–75). There are three major types of EVs: exosomes, microvesicles, and apoptotic bodies (76). A general description of these types and their corresponding characteristics can be found in **Table 3**. The two main types of EVs are microvesicles and exosomes, of which the latter are the most abundant.

General Features of MSC-EVs

Mesenchymal stromal cells-extracellular vesicles are constitutively secreted by MSCs and can be identified by transmission electron microscopy as cup-shaped nanovesicles with sizes ranging from 20–150 nm in diameter (**Figure 1**). They are rich in adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1), lysosomal-associated membrane 2 (LAMP-2), tetraspanins (e.g., CD9, CD63, CD81), integrins (e.g., CD49C, CD49D), heat-shock proteins, cytoskeletal proteins, and membrane trafficking proteins, such as "Ras-related in brain" (67) and annexins (77, 78). Moreover, they express cell-specific molecules, including CD29, CD73, CD44, and CD105, and enclose proteins involved in MSC self-renewal and differentiation (GF, Wnt, TGF-β, MAPK, BMP, etc) (77). MSC-EVs also carry a variety of genetic

TABLE 3 | Nomenclature and classification of the different types of vesicles.

Characteristics	Exosomes	Microvesicles	Apoptotic bodies
Size	20–100 nm	50–1000 nm	500–5000 nm
Shape	Cup shaped	Irregular	Heterogeneous
Sedimentation	100,000 × *g*	Size dependent at 100,000 × *g*, 10,000 × *g*, and 2000 × *g*	Size dependent at 100,000 × *g*, 10,000 × *g*, and 2000 × *g*
Sucrose gradient	1.13–1.19 g/ml	1.04–1.07 g/ml	1.16 and 1.28 g/ml
Markers	Tetraspanins (CD63/CD9), Alix, TSG1, ESCRT components, flotilin	Integrins, tetraspanins, selectins, and CD40 ligand	Histones
Lipids	Cholesterol, sphingomyelin, ceramide, lipid rafts, phosphatidylserine	Phosphatidylserine	High amounts of phosphatidylserine
Origin	Endolysosomal pathway; intraluminal budding into multivesicular bodies and released by fusion of the multivesicular bodies with the cell membrane	Cell surface; outward budding of cell membrane	Cell surface; outward blebbing of apoptotic cell membrane
Contents	mRNA, microRNA, and other non-coding RNAs; cytoplasmic and membrane proteins (including HSP and cell-specific receptors)	mRNA, microRNA (miRNA), and other non-coding RNAs; cytoplasmic proteins and membrane proteins, including cell-specific receptors	Nuclear fractions and cell organelles

ESCRT, endosomal sorting complexes required for transport; MVB, multivesicular bodies; HSP, heat-shock protein; mRNA, messenger RNA.
Table has been adapted from published literatures (90, 259, 260).

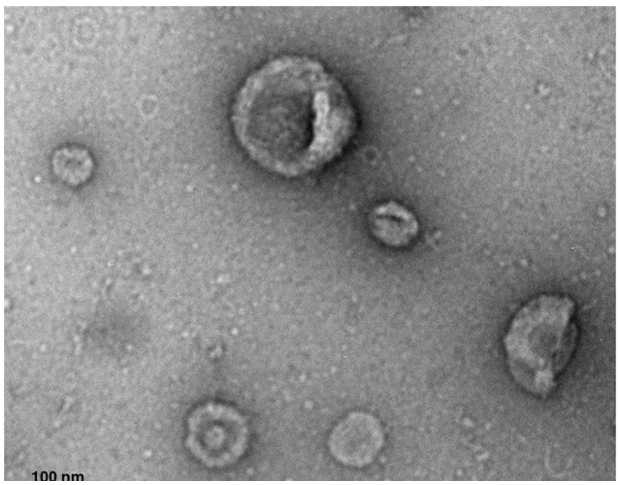

FIGURE 1 | Transmission electron microscopy micrograph of whole-mounted extracellular vesicles-purified human MSCs. MSC-EVs exhibit a spheroid, cup-shaped morphology. Scale bar shows 100 nm. Photography courtesy of Monica Reis.

material, including mRNA and non-coding RNA [pre-microRNA (miRNA), miRNA, tRNA, piRNA] (79–81). Significant importance has been given to MSC-EV shuttled miRNA, which has been shown to be functionally active and involved in the regulation of genes related to organ development, cell survival, and differentiation (82–85). The lipid composition of MSC-EV is still unknown; however, very recently, Lai and colleagues have reported an enrichment of phosphatidylserine (86). This lipid has been identified on the surface of various types of EVs, derived from various types of cells, and has been described as an evolutionary conserved immunosuppressive signal which promotes tolerance and prevents the activation of the immune system (87). Recently, Wei et al. have demonstrated that phosphatidylserine on the surface of MSC-derived microvesicles is essential for their uptake by human umbilical vein endothelial cells (HUVECs), however, the role of this lipid in MSC-EV-derived immunosuppression is still unexplored (88).

Common Procedures for EV Purification

Currently, differential ultracentrifugation represents the gold standard and most commonly used protocol for EV purification. This protocol involves several centrifugation steps at different speeds to eliminate cell debris and protein contaminants (89). EV sedimentation is usually accomplished by ultracentrifugation of the pre-cleared biofluid at speeds of 100,000 × *g*. This protocol varies across users which may lead to inconsistences in EV yields. In some protocols, EV sedimentation is accomplished at higher-speed ultracentrifugation (e.g., 140,000 × *g*) and longer centrifugations (e.g., 4–7 h). Alternatively, the last ultracentrifugation step can be replaced by microfiltration or followed by an extra purification step, e.g., sucrose-gradient centrifugation, which yields a cleaner EV population without co-precipitation or protein contaminants (89). Other EV purification methodology includes the use of commercially available kits based on polymer-precipitation and immune-capture using antibody-coated magnetic beads (90). The commercially available kits precipitate a wide range of vesicles, however, it may display concomitant precipitation of protein contaminants, while the immunolabeled beads only precipitates a restricted fraction of EVs and neglects others (90). Laboratories worldwide have been focused on the refinement of protocols to allow for a more robust purification and yield a purer EV population.

Therapeutic Potential of MSC-Derived EVs

Since the initial identification of EVs in the conditioning medium of MSCs, increasing studies have demonstrated that MSC-EVs harness therapeutic effects. MSC-derived EVs have been shown to recapitulate the therapeutic effect of the parent cells in animal models of cardiac, kidney, and brain injuries and the observation MSCs have restricted migration and survival potential argues for the clinical use of EVs (91–94). The importance of MSC-EVs has also been identified as one of the mechanisms of MSC immunomodulation. MSC-EVs have been reported to modulate proliferation and differentiation of T cells, B cells, and monocytes (**Table 4**). Budoni et al. demonstrated that the effect of MSCs on B cell proliferation and differentiation could be fully reproduced by MSC-EVs and that this was inhibited in the presence of MSC-EVs in a CpG-stimulated peripheral blood mononuclear cell coculture system, in a dose-dependent manner (95). The effect of MSC-EVs on T cells was initially investigated by Mokarizadeh et al. in 2012. MSC-EVs were shown to express regulatory receptors, such as programed death ligand 1 (PD-L1), galectin-1, and membrane-bound TGF-β1, and were able to inhibit auto-reactive lymphocyte proliferation, promote the production of IL-10 and TGF-β, and increase apoptosis of recipient T cells (96). MSC-EVs seemed to induce tolerogenic signaling by prompting the generation of $CD4^+CD25^+FoxP3^+$ Tregs (96). These findings were further corroborated by different independent studies which showed that MSC-EVs were capable of reducing proliferation and IFN-γ release of *in vitro* stimulated T cells in a dose-dependent manner and that one of the main mechanisms of MSC-EV to regulate T-cell proliferation and activation was the generation of *de novo* Tregs (97–99). Zhang et al. demonstrated that this effect was indirect and that MSC-EVs were preferentially taken up by splenocytes, which in turn polarized activated $CD4^+$ T cells to that of a $CD4^+CD25^+FoxP3^+$ Treg phenotype. In this study, the authors proposed that MSC-EVs are responsible for the activation of TLR-dependent signaling in macrophages, which leads to the induction of an $IL\text{-}12^{lo}IL\text{-}10^{hi}$ M2 phenotype. These M2 macrophages are then responsible for the generation of Tregs (100). Additionally, infusion of MSC-EVs led to enhanced survival of allogeneic skin grafts in mice (100). Recently, Favaro et al. demonstrated that MSC-EVs internalized by DCs impaired their *in vitro* maturation, with reduced expression of maturation markers CD86, CD80, and CD83, and an increase in IL-10 production by the EV-conditioned DCs (101).

Mesenchymal stromal cells-extracellular vesicles have also been tested in the context of HSCT and GvHD. A recent study has provided initial evidence that MSC-EV treatment combined with HSCs contributes to faster reconstitution of the hematopoietic

TABLE 4 | Summary of the immunomodulatory potential of MSC-EVs.

Target cells	Experimental approach	Source of EVs and isolation method	Results	Reference
PBMC	*In vitro* coculture	Human umbilical cord MSC UC (Sed.: 10,000 × *g*) and Exoquick	↓ Proliferation of $CD8^+$ and $CD4^+$ ↑ Percentage of $CD4^+CD25^+FoxP3^+$ Tregs ↑ TGF-β1 and IL-10; ↓ IFN-γ, IL-6, TNF-α	(99)
Colon cells	TNBS-induced colitis model	Human BM-MSCs UC (Sed.: 100,000 × *g*)	↓ Pro-inflammatory cytokine levels in injured colons Suppression of apoptosis Inhibition of NF-kBp65 signal transduction pathways	(261)
T lymphocytes	*In vitro* coculture	Human ASCs UC (Sed.: 100,000 × *g*)	Decreased T-cell activation and proliferation	(97)
Auto-reactive lymphocytes	EAE mice	Murine BM-MSCs UC (Sed.: 100,000 × *g*)	EVs express PD-L1, galectin-1, and TGF-β1 Inhibition auto-reactive T-cell responses ↑ Apoptosis ↑ $CD4^+CD25^+FoxP3^+$ Tregs	(96)
PBMC from type I diabetes patients	*In vitro* coculture	Human BM-MSC UC (Sed.: 100,000 × *g*)	↓ IFN-γ production and ↑ TGF-β, IL-10, IL-6, and PGE2 ↓ Level of Th17 cells and ↑ $FoxP3^+$ Tregs	(98)
B lymphocytes	*In vitro* coculture	Human BM-MSC UC (Sed.: 100,000 × *g*) and UF	Inhibition of B-cell proliferation and differentiation	(95)
THP-1 MΦ	*In vitro* coculture and *in vivo* injection of EVs in a mouse model of allogeneic skin grafting	Human ESC-MSC HPLC	↑ Anti-inflammatory cytokines ↓ Pro-inflammatory cytokines TLR-dependent induction of M2-like phenotype Treg cell expansion	(100)
	In vitro coculture	LPS treated UC-MSC UC (Sed.: 100,000 × *g*)	MΦ polarization *via* delivery of Let-7b by EVs and inhibition of TLR4 signaling pathway	(84)
moDCs from type I diabetic patients	*In vitro* coculture	Human BM-MSC UC (Sed.: 100,000 × *g*)	EV-conditioned DCs exhibited immature phenotype ↑ IL-10, IL-6, and TGFβ ↓ IL-17 and Th17 cells Treg expansion	(101)

Sed., sedimentation rate; UC, ultracentrifugation; UF, ultrafiltration; Tregs, regulatory T cells; EAE, experimental autoimmune encephalomyelitis; TNBS, 2,4,6 trinitrobenzene sulfonic acid; HPLC, high performance liquid chromatography; BM-MSC, bone marrow-derived MSC; ASC, adipocyte-derived stem cells; NF-kBp65, nuclear Factor kappa B p65; TGF-β1, transforming growth factor beta 1; IL-10, interleukin 10; IFN-γ, interferon gamma; IL-6, interleukin 6; TNF-α, tumor necrosis factor alpha; PD-L1, programed death ligand 1; PGE2, prostaglandin E2; TLR, toll-like receptor; IL-17, interleukin 17; Th, T-helper cell; MΦ, macrophage; moDCs, monocyte-derived dendritic cells; LPS, lipopolysaccharide; FoxP3, forkhead box P3.

microenvironment. In this study, MSC-EVs were shown to be enriched in miRNAs that promote UCB-$CD34^+$ migration and engraftment in the BM niche (83). Amarnath et al. detected CD73-expressing EVs in MSC recipients in a mouse model of GvHD. These EVs were found to metabolize extracellular ATP into adenosine and, as a consequence, to inhibit Th1 cell effector function (102). In 2014, Kordelas et al. were the first to administer MSC-EVs in a steroid-refractory GvHD patient. MSC-EV preparations were shown to contain high concentrations of anti-inflammatory molecules IL-10, TGF-β, and HLA-G and were administered to the patient at intervals of 2 or 3 days for a period of 2 weeks. MSC-EV administration was well tolerated and no side effects were reported. The patient exhibited a 50% decrease in the production of the pro-inflammatory cytokines IL-1β, TNF-α, and IFN-γ and concomitant a reduction of diarrhea and cutaneous and mucosal GvHD, which remained stable for more than 4 months post MSC-EV treatment (103).

Future Perspectives of MSC Therapy

Donor Source and the Use of Freeze–Thawed MSC Products

A long standing debate is the donor source of MSCs, particularly autologous versus allogeneic, and single-donor versus pooled donor batches (also called "master cell stocks"). Largely, the pros and cons of each relate to development costs and product safety. Autologous MSCs are innately safe from an immunological/infective perspective and obviate the search for a third-party donor. However, allogeneic MSCs would allow for product preparation in advance for infusion as an "off the shelf" treatment, without delays for the recipient. The advantages of master cell stocks are seemingly obvious, as they would allow mass production of MSCs for clinical use in multiple patients; as opposed to the need to isolate, expand, and quality check a batch of MSCs for every single recipient. However, not only would MSC production at an industrial scale prove costly, the potential for the contamination of multiple individuals with a single batch would require even more rigorous product analysis to ensure safety, which would only increase development costs further.

Another area of controversy is the clinical response and efficacy of using fresh (from culture) versus thawed MSCs. In earlier clinical trials, MSCs were infused into patients as thawed products, due to the benefit of cryopreservation allowing for long-term storage and use at a later date. However, recently the clinical effectiveness and safety of these products have been questioned (104). *In vitro*, it has been shown that post-thaw MSCs display a weaker immunomodulatory profile compared to their pre-freeze counterparts due to a heat-shock response, particularly in relation to weak IDO secretion (105). This seems to correlate

with clinical outcomes, with reports of double the response rates in fresh compared to thawed MSCs for the treatment of HSCT complications (106). Despite aforementioned evidence, a recent study has examined the effect of cryopreservation on human MSC viability, immunomodulatory potency, and performance in an ischemia/reperfusion injury model. This study has observed that with modifications to standard cryopreservation methods over 95% MSC viability could be achieved upon thawing. These thawed high viability MSCs maintained their function in suppressing human mononuclear cell activation. Furthermore, the study has demonstrated that when viability is maintained, MSCs retained their therapeutic potency in an *in vivo* ischemia/reperfusion injury mode (107). This controversial evidence highlights potential risks as well as achievable hopes for an off-the-shelf therapy. Further studies are warranted to provide the field with a more definitive view.

From a safety perspective, concerns have also been raised regarding the possibility that post-thaw MSCs are associated with an increased rate of the so-called instant blood-mediated inflammatory reaction (IBMIR) (106). As seen with islets of Langerhans cells, this physiological process involves activation of a number of components, mainly the coagulation and complement cascades, leading to leukocyte and platelet activation, and subsequent tissue damage (108, 109). The extent of this, however, remains unclear, and more importantly, this has not been shown to have a negative impact on the safety profile of MSCs.

Mechanisms of Action of MSCs

Despite their potential therapeutic benefits in GVHD treatment, the exact mechanisms of action of MSCs are yet to be fully elucidated. Increasing evidence has led to a common consensus that the efficacy of MSC therapy could be predominantly attributable to the release of soluble factors rather than long-term engraftment (110, 111). The MSC secretome includes an array of bioactive proteins, such as cytokines, growth factors, and chemokines. Their functions and interactions, together with relevant literatures, have been summarized in **Figure 2**. Ultimately, establishing a comprehensive understanding of how MSCs work holds the key to the development of successful MSC therapies.

Considerations in Using MSC-EVs for Therapies

Current research suggests MSC-EV-based therapy could potentially have significant clinical relevance. In comparison with MSCs, MSC-EVs are non-self-replicating hence no risk of aneuploidy, less likely to be modified by inflammatory environment and have a lower possibility of immune rejection owing to their small size and lower expression of membrane-bound molecules, including membrane histocompatibility molecules. MSC-EVs are also more stable than the parent cells, by virtue of their encapsulated cargo, EVs provide added protection to their contents from *in vivo* degradation, thus preventing problems associated with rapid breakdown of soluble molecules, such as cytokines, growth factors, and RNAs. In contrast to cell-based therapy, MSC-EV therapy can be easier to manufacture and safer, as they are devoid of cells and hence impose no danger of ectopic tissue formation. Additionally, they can be stored in non-toxic cryopreservatives at −20°C for 6 months with maintenance of biological activity (112). Despite these advantages, for clinical translation to be considered, it is essential to elucidate on the biological properties and the constituents of these vesicles, in terms of proteins and RNAs. MSC-EVs, as cellular products, are influenced by the secreting cells; therefore, it is inevitable that MSC heterogeneity will impact on EV cargo and biological effects. Distinct MSCs have been shown to display different abilities to produce cytokines and to respond to inflammatory licensing (113). Moreover, donor age and gender also affect the functional characteristics of MSCs (114). Current studies have not clarified the effect of inter-individual variability of MSC-EVs, and only a few studies have shown the effect of MSC licensing with inflammatory cytokines on the immunomodulatory potential to the EVs (84, 96). Furthermore, considerations regarding the immunomodulatory potency of the vesicles in relation to their cellular counterparts need to be taken into account. A recent report on the immunosuppressive effect of BM-MSCs and their derived EVs has shown the latter were considerably less potent in suppressing T cell proliferation and preventing B cell differentiation (115). EVs were also seen to be not as effective in modulating DC maturation as their parent cells (101). In the future, it will be important to investigate the effect of MSC variability and licensing on the molecular signature of their derived vesicles. This notwithstanding, data indicate MSC-EVs are capable, at least in part, of mediating immunomodulatory responses; however, further research is needed to unravel their mode of action, the development of standardized EV purification, characterization, and potency assays.

IMMUNOTHERAPY WITH ANTIVIRAL T CELLS TO TREAT INFECTIOUS COMPLICATIONS

Opportunistic infections are serious complications affecting the morbidity and mortality of transplant patients (116). The most common infections in immunocompromised transplant recipients are caused by viral, bacterial, parasitic, and fungal pathogens (117). In immunocompetent individuals, the majority of these pathogens are controlled by the immune system, but in immunocompromised patients they can lead to prolonged recovery or hospitalization due to recurrent reactivations and can even influence the overall survival (116). The most important risk factors for post-HSCT infections are immunodeficiency and mucosal injury caused by conditioning regimen pre-transplantation (118), allogeneic transplantation with T cell depletion (119), delayed immune reconstitution due to immunosuppressive therapy for GvHD, and the pathogen serostatus of donor/recipients pairs (120). Taking the risk factors into account, preventive and preemptive treatments against these pathogens are important to promote engraftment, avoid relapse, and improve the overall survival. Bacterial, fungal, and parasitic infections can be treated with antibiotics, antifungals, or antiparasitic medications, but reconstitution of specific immunity is important. Latent virus reactivations or *de novo* infections can be treated with antiviral medications, but reactivation is only treated successfully or prevented by the recovery of anti-virus-specific T cells. The

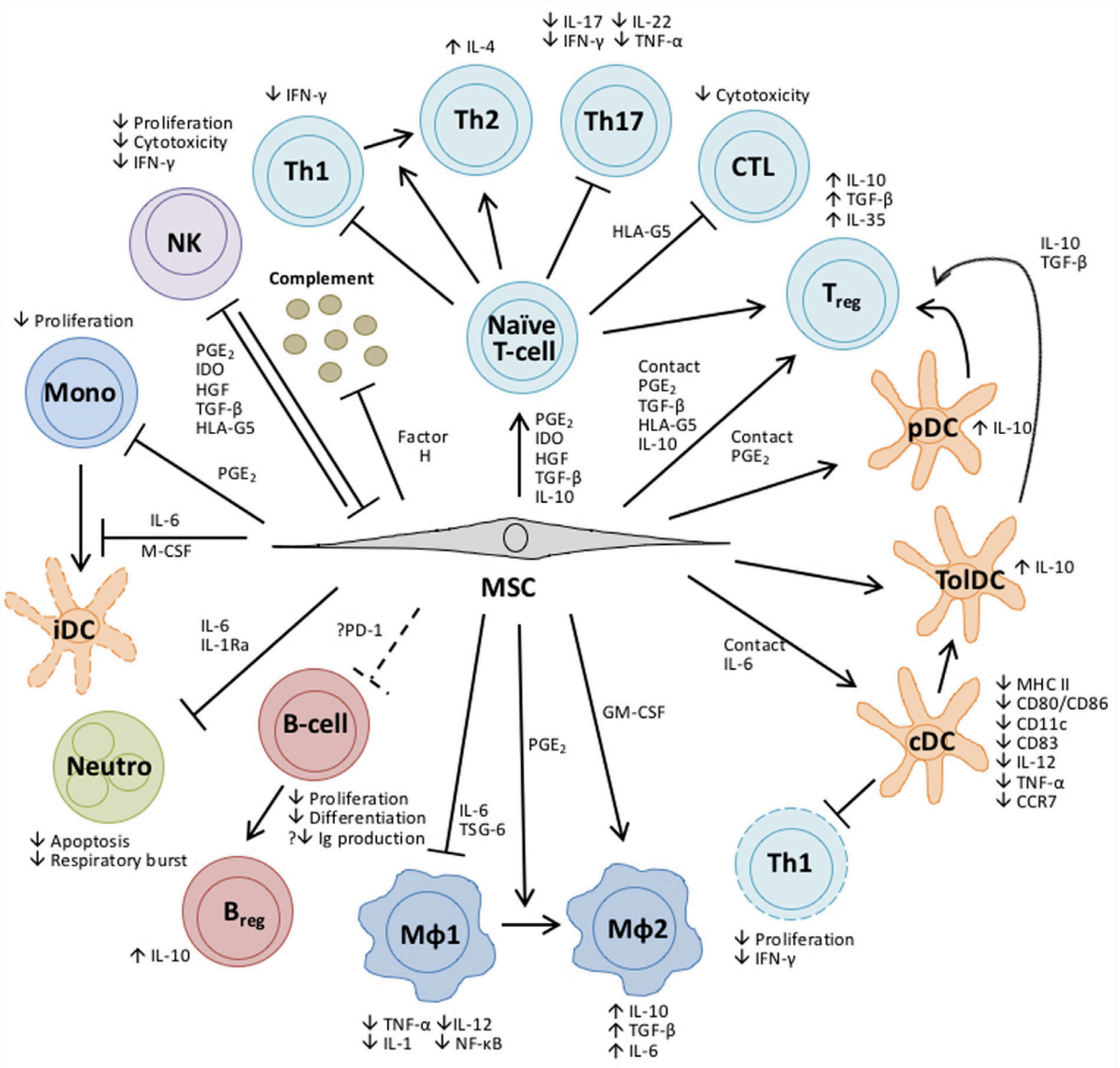

FIGURE 2 | Overview of the bioactive molecules secreted by MSCs and their impact on cells of the innate and adaptive immune response. Some bioactive molecules are constitutively expressed by MSCs, while others are "licensed" by exposure to an inflammatory environment or upon TLR stimulation (241). Depending upon the bioactive secretion profile, MSCs can skew the differentiation of $CD4^+$ T-helper cells into various T-cell subsets, each with distinct cytokine and gene expression profiles, can promote the generation of regulatory T cells (Tregs) and inhibit the proliferation of cytotoxic T cells (242–244). MSCs can modulate the development of conventional and plasmacytoid DC (245–247) while DCs generated in the presence of MSCs have functional properties typical of tolerogenic DCs (248–250). Similarly, MSCs can polarize macrophages of the classical M1 pro-inflammatory phenotype to that of an alternative anti-inflammatory M2 phenotype (215), or directly induce this alternative phenotype by coculture (251). In contrast to other cell types, MSC modulation of B-cell function is poorly understood and the findings are contentious. Results from *in vitro* experiments show that while MSCs impair the proliferation and terminal differentiation of B cells (252) they have also been shown to stimulate antibody secretion (253). More recently, data have emerged which suggests that MSCs can promote the induction of regulatory B cells (Breg) (254). Neutrophils are an important mediator of the innate response and MSCs have been shown to enhance their survival through an IL-6-mediated mechanism, concomitant with the downregulation of reactive oxygen species, thereby conserving the pool of neutrophils primed to respond rapidly to infection (255). MSCs inhibit the proliferation and differentiation of monocytes to immature dendritic cells (DCs) (245). Natural Killer (NK) cells and MSCs have a reciprocal relationship; MSCs can inhibit the proliferation and cytotoxicity of resting NK cells and their cytokine production *in vitro,* while activated NK cells can be cytotoxic to MSCs (256). MSCs constitutively secrete Factor H which inhibits complement activation (257), conversely the complement activation products C3a and C5a released upon tissue damage are chemotactic factors for MSCs (258), recruiting them to sites of injury. Abbreviations: CCR, C-C chemokine; CD, cluster of differentiation; cDC, conventional dendritic cell; CTL, cytotoxic T-lymphocyte; GM-CSF, granulocyte-macrophage colony-stimulating factor; HGF, hepatocyte growth factor; HLA, human leukocyte antigen; IDO, indoleamine 2,3-dioxygenase; IFNγ, interferon-γ; Ig, immunoglobulin; IL, interleukin; MΦ, macrophage; MHC, major histocompatibility complex; Mono, monocyte; Neutro, neutrophil; NF-κB, nuclear factor kappa B; PD-1, programed cell death protein-1; pDC, plasmacytoid dendritic cell; PGE_2, prostaglandin E_2; TGFβ, transforming growth factor β; Th, T-helper cell; TNFα, tumor necrosis factor α; tolDC, tolerogenic dendritic cell; TSG, TNF-α-stimulated protein.

prophylaxis and the treatment of transplanted patients with traditional drugs might be effective by killing the pathogens or control replication; however, virus infections or invasive fungal infections (121) are often refractory to these treatments due to limited activity, drug resistance, or short-term drug protection (122, 123). Furthermore, antiviral and antifungal drugs have demonstrated significant toxicity, which raises a real concern for HSCT patients undergoing intensive drug treatments (124, 125).

Cytomegalovirus (CMV) is a latent herpes virus, which may lead to mild diseases at first contact or remains silent during most of the life of immune competent individuals. CMV is latently expressed in 30–60% of the population (126). CMV persists life-long in infected individuals in endothelial and epithelial cells, but is usually controlled by T cells specific for CMV (127). Thus, in immunocompetent individuals, the infection with CMV is not problematic, in immunocompromised individuals, like HSCT patients, it can trigger severe diseases. The most common manifestations of CMV disease are gastrointestinal complications, pneumonia and interstitial pneumonitis, hepatitis, retinitis, and encephalitis (128). Furthermore, several studies have reported a correlation between CMV infection and reactivation with the onset or aggravation of GvHD, which makes the treatment of these patients even more difficult considering that the immunosuppression required for GvHD will increase CMV reactivation (129).

Epstein–Barr virus (EBV) is a herpes virus spread in more than 90% of the adult population with a life-long latency in B lymphocytes (130). EBV *de novo* infection or reactivation affects about 11 and 46%, respectively, of patients undergoing HSCT (131). The most life-threatening condition related to EBV infection in immunocompromised patients is the posttransplant lymphoproliferative disease (PTLD) (132).

Adenovirus (AdV) is a common latent virus, which presents at least 51 serotypes having various clinical manifestations, which make the decision for a therapeutic strategy more complicated than for other viruses. The infection occurs frequently during the childhood, but the most susceptible individuals are pediatric patients after HSCT (120). In these patients, AdV infection can cause hepatitis, pneumonia, encephalitis, myocarditis, gastroenteritis, or nephritis and when disseminated is associated with more than 50% of mortality risk (120, 133).

Cytomegalovirus, EBV, and AdV are the major viral pathogens involved in infection complications after HSCT. Other critical non-viral infections occurring in HSCT patients are invasive fungal infections mainly caused by fungal pathogens, such as *Aspergillus* and *Candida*. The mortality among posttransplant patients with IFI is between 1 and 13% and occurs in the majority of the cases within the first year after HSCT (134).

Toward Adaptive T Cell Transfer for Treatment of Viral Infections

Viral reactivation is the result of impaired function of the immune system, thus adoptive transfer of virus-specific T cells can help to restore virus-specific immunity after HSCT. Over the last 20 years, adoptive T cell therapy has become a potential alternative to pharmacologic treatments for patients with refractory posttransplant infections (135–138). Donor lymphocyte transfusion has been largely used in HSCT patients to prevent relapse by providing graft-versus-leukemia effect (GvL) although the development of GvHD has unfortunately been a concomitant risk (139, 140). In the early 1990s, it became evident that the practice of DLI was at the same time advantageous for the treatment of virus infections due to the presence of anti-virus reactive memory T cells among the lymphocytes from seropositive donors (141). Despite considerable benefits, the treatment of virus infections with DLI has demonstrated limitations concerning both safety and efficacy issues, due to the high presence of alloreactive T cells and to the low frequency of antigen-specific T cells (142, 143). These findings contributed to strategies increasing the number of antigen-specific T cells by selecting the donor target cytotoxic T cells and depleting the alloreactive T cells as an alternative immunotherapy for the reconstitution of the anti-pathogen immunity with a reduced risk of triggering GvHD (**Figure 3**). One of the pioneering studies published by Riddell et al. demonstrated the successful reconstitution of antiviral-specific T cell immunity in HSCT patients at high risk of developing CMV disease by the prophylactic transfusion of *in vitro* expanded CMV-specific $CD8^+$ T cell clones (143). Although they could show the reconstitution of CMV-specific immunity, the expansion of virus-specific T cell clones had several drawbacks for integration into clinical practice. Since that time, innovative technological developments as well as novel basic immunological findings to improve and to disseminate the treatment of infectious diseases by adaptive anti-pathogen T cell transfer were developed.

In Vitro GMP Manufacturing of Antiviral T Cell Products

Basically two different strategies for depletion of potential alloreactive T cells and concomitant enrichment of relevant virus-specific T cells are established for the generation of GMP-grade antiviral T cell products (**Figure 4**). One strategy relies on

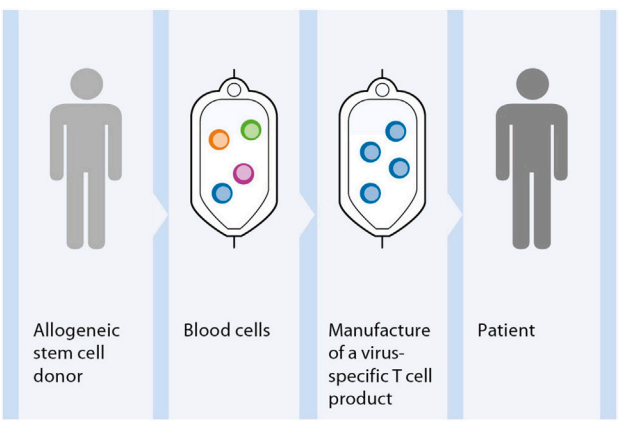

FIGURE 3 | Principle approach of adoptive T cell therapy for treatment of viral infections. Out of peripheral blood of the HSCT donor the virus-specific T cells are selected. The generated T cell product is infused into the patient suffering of viral complications after allogenic HSCT.

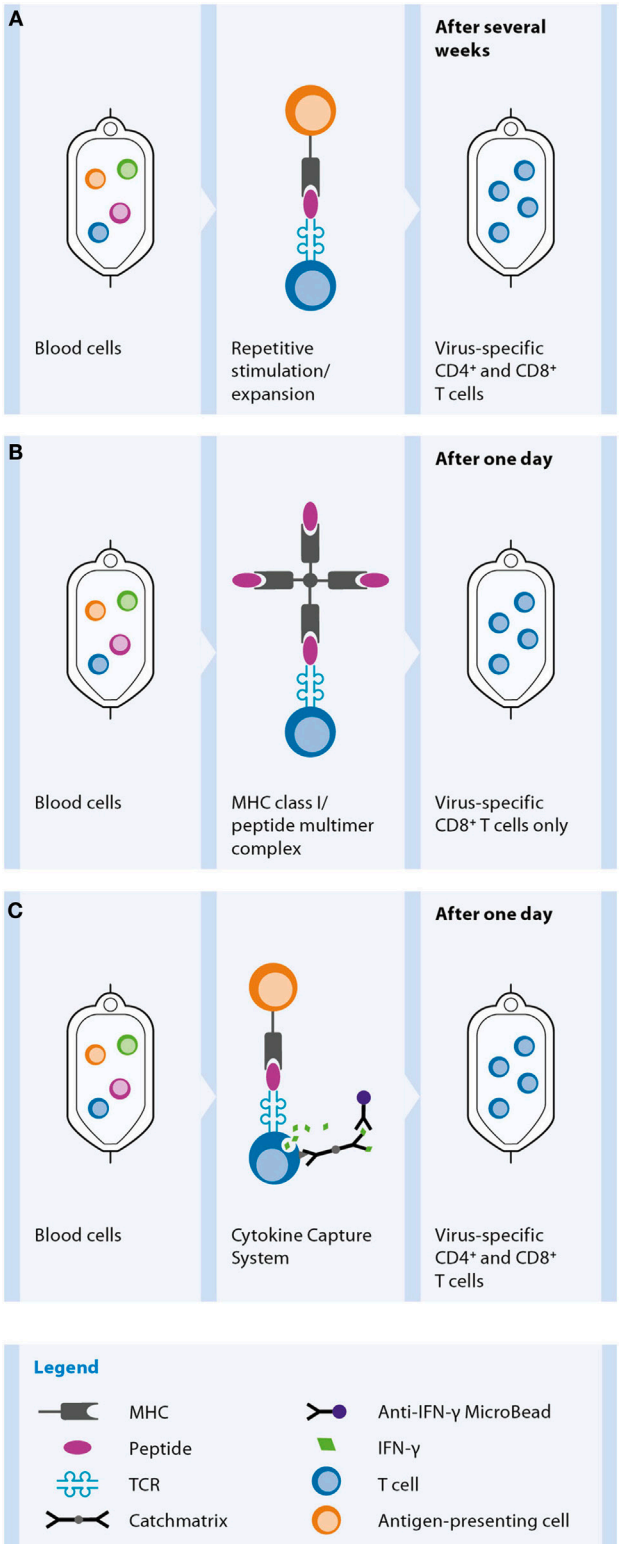

FIGURE 4 | Methods for *in vitro* generation of a virus-specific T cell product. For the *in vitro* manufacture process blood is used as the cellular source, mostly derived from the stem cell donor. Selection of virus-specific T cell and thereby depletion of potentially alloreactive T cells from the blood

(Continued)

FIGURE 4 | Continued

can be achieved by different methods. **(A)** Activation and expansion: peripheral blood cells are incubated with viral antigen. Antigen-presenting cells (APC) phagocytose, process, and present the antigen as peptides on MHC molecules. Virus-specific T cells recognize their cognate viral antigenic peptide *via* the TCR, get activated, and later on start proliferating for several days. In many applications, additional repetitive antigen restimulations are performed to further increase the expansion and thereby the number and the purity of the virus-specific T cell population. Dependent on the viral antigen and APC used for the process, either $CD4^+$ and/or $CD8^+$ T cells are contained in the product. **(B)** MHC class I/peptide multimer technology: virus-specific T cells within peripheral blood become labeled by a MHC class I/peptide multimer reagent, which binds to the TCR of the viral peptide-specific T cells. After an additional labeling step with magnetic beads the $CD8^+$ virus-specific T cells are magnetically enriched. **(C)** Cytokine-capture assay: peripheral blood cells are incubated with viral antigen, e.g., a peptide pool, for 4 h. APC present the peptides on MHC molecules to virus-specific T cells, which start producing IFN-γ. Cells are labeled with a catch matrix consisting of a CD45 antibody conjugated to an Anti-IFN-γ antibody. In this way, secreted IFN-γ is specifically captured on the cell surface of the activated virus-specific T cells. Subsequently, the cell-bound IFN-γ is detected with Anti-IFN-γ magnetic particles and the virus-specific T cells are magnetically enriched. Both $CD4^+$ and $CD8^+$ T cells are obtained by this method.

conventional *in vitro* stimulation of blood cells with viral antigen and *in vitro* propagated antigen-presenting cells (APC), like EBV-transformed B cells, and repetitive restimulation and long-term culture to gain T cell clones or lines (143, 144) (**Figure 4A**). Despite successful optimization and simplification of multiple steps within this manufacturing over the last year to yield clinically practical protocols resulting in effective and safe T cell lines, a main disadvantage of these cell products is the long and laborious preparation time of at least 10 days (145). The development of new magnetic selection methods to obtain the rare virus-specific T cells out of peripheral blood, based on either IFN-γ secretion [CliniMACS® Cytokine Capture System (CCS) (IFN-gamma)] enables or peptide/MHC multimer labeling, allowed significant reduction of the preparation time of the cellular product under GMP conditions from one to two working days.

The peptide/MHC multimer technology allows selection of peptide-specific $CD8^+$ T cells out of a blood sample according to the magnetic labeling of the antigen-specific T cell receptor (TCR), without the need of prior *in vitro* stimulation step (**Figure 4B**). The CliniMACS® CCS (IFN-gamma) requires stimulation of peripheral blood samples with antigen like peptides or proteins for about 4–16 h to induce IFN-γ production by the virus-specific T cells (**Figure 4C**). The secreted IFN-γ is specifically caught onto the cell surface of antigen-activated T cells using a capture matrix. The subsequent recognition of IFN-γ-secreting cells with magnetic beads conjugated to anti-IFN-γ antibodies enables its enrichment.

Both methods yield rapid and effective production of antigen-specific T cells. The advantage of the CCS over peptide/MHC multimer technology is a parallel stimulation and selection of antigen-reactive $CD4^+$ as well as $CD8^+$ T cells. Although $CD8^+$ cytotoxic T lymphocytes (CTLs) are responsible for the fast antiviral response, it has been shown that the presence and help of antigen-specific $CD4^+$ T cells is essential to activate the

CTL and maintain long-term immunity (146). Moreover, the CCS enables generation of a cell product consisting of multiple viral epitopes of either one or more antigenic viral proteins. Whereas the number of available peptide/MHC multimer reagents is limited to the most common HLA/epitope specificities, the cytokine-capture assay is suitable for isolation of specific T cells independent of HLA allotypes. A disadvantage of the IFN-γ secretion assay technology compared to peptide/MHC multimer technology is the need for a short-term (4 h) incubation phase for antigenic stimulation. However, exactly this technological feature makes it possible to generate tailored T cell products for patients by choosing on the required viral antigen, either peptides, pools of peptides, proteins, and even use of multiple antigens. Meanwhile a whole panel of viral protein antigens is available as pre-pooled GMP-grade peptide cocktails, covering $CD4^+$ as well as $CD8^+$ T cell epitopes without HLA restriction.

Despite the possibilities of adaptive virus-specific T cell therapy the number of clinical sites, which have GMP manufacturing unit and processes and thus offering such a treatment option to patients, is limited. One of the general obstacles of cell therapy is the complexity of the clinical manufacturing. Beside the demands on the infrastructure with clean rooms and various instruments, the generation of antigen-specific T cell products requires several different handling and intervention steps during the production process and skilled and well-trained operators are needed. To guarantee robust and reliable processes as well as safe and effective clinical products, a standardization of the cell manufacturing is essential, which can be accomplished by automation. A newly developed cell processing device, the CliniMACS Prodigy®, enables to run the complete CCS in an automated and closed system (60, 147). The cells are processed from the first until the last step within a closed and single-use tubing set. All process steps, i.e., cell preparation, cell stimulation, labeling and washing steps, magnetic enrichment, and final formulation are performed automatically. Only a minimum of operator action is necessary to set-up sterile connections of all starting materials (blood sample, antigen, buffers, cell culture media, labeling, and separation reagents) to the tubing set, for programing the desired time of the process end, and for cell sampling to allow their quality control.

Quality of the Cellular Products Determines Clinical Outcome

Clinical Benefits Are Detected upon Transfer of Low Numbers of T Cells

The cell numbers obtained with either system for rapid magnetic *ex vivo* selection of virus-specific T cells is limited due to the low frequency of virus-specific T cells within peripheral blood. *In vitro* expansion of the specific T cells was considered to be essential for a successful adoptive therapy as in early clinical studies the number of transferred T cells were as high as several million cell/m^2 body surface area (148, 149). However, various investigators treated patients with CMV-, EBV-, or AdV-specific T cells directly obtained after *ex vivo* isolation using the CCS and reported clinical efficacy (138, 150–153). Thus, this low number of transferred cells most probably are compensated by their high *in vivo* proliferating capacity in the lymphodepleted host, thus leading to sufficient antigen-specific T cell immunity and successful treatment of viral infections. It has been shown for tumor-infiltrating T cell products that longer periods of *in vitro* expansion reduce the clinical efficacy *in vivo*, hypothesized to be the result of enhanced terminal differentiation of cells (154). The number of virus-specific T cells that can be isolated *ex vivo* using either method depends mainly on the frequency of specific T cells in donors' peripheral blood. Usually the number of enriched T cells allows the transfer of less than 1×10^5/kg body weight of the patient, i.e., the number of transfused T cells is below the number of unselected T cells regarded as critical for GvHD induction (MUD/MRD: 1×10^6/kg body weight). Thus, methods for rapid generation of cellular antiviral T cell products are of advantage compared to long-term cell culturing processes.

Cotransfer of $CD4^+$ T Cells Support *In Vivo* Effector Function of $CD8^+$ T Cells

Controversial data on the protective role of $CD4^+$ and $CD8^+$ T cells, the benefit of transferring both, antigen-specific $CD4^+$ and $CD8^+$ T cell subsets, or $CD8^+$ T-cells alone still exist and need to be discussed. The prophylactic infusion of CMV-specific $CD4^+$ T cells in patients without CMV-specific T helper response has been shown to increase the frequency of CMV-specific T cells in both $CD4^+$ and $CD8^+$ T cell subpopulations and to eradicate the virus successfully (149). On the other hand, the transfusion of CMV-specific $CD8^+$ T cells has been likewise efficient in clearing the viremia and increasing the frequency of donor CMV-specific $CD8^+$ T cells as well as recruiting $CD4^+$ T cells in the recipients (155). Riddell et al. and Walter et al. have transfused CMV-specific T cell clones and reported a progressive decrease of transferred CMV $CD8^+$ T cell clones in patients who lacked $CD4^+$ T cells (143, 148). Since then, several other studies have demonstrated the critical role of $CD4^+$ T cells in both maintaining the functionality of cytotoxic $CD8^+$ T cells (156, 157) and directly fighting the viral infection (158). Furthermore, in a multicentre study, Leen et al. observed that transfusion of either $CD4^+$ or $CD8^+$ T cells were equally protective against viral infections (159). The $CD4^+$ T cell population remains, however, a controversial issue for adoptive immunotherapy, since several studies have reported a higher alloreactive potential of this T cell subset (160, 161).

Reduced Alloreactivity in *In Vitro*-Generated T Cell Products

The allogeneic reactivity of pathogen-specific T cells has been largely investigated and their potential to elicit GvHD needs still to be clarified, particularly with HLA-mismatched donors. Several *in vitro* studies have reported the cross-reactive potential of expanded virus-specific T cells toward allogeneic-HLA antigens (162–166). Single-viral antigen $CD4^+$ and $CD8^+$ T cell lines or clones, specific for CMV, EBV, VZV, and influenza virus, have shown *in vitro* to recognize and lyse allo-HLA class I and class II molecules also expressed on normal cell subsets (164). Long-time culture and the generation of clones under repeated immune stimulation may contribute to the *in vitro* alloreactivity

of T cell clones, reported. In the clinical setting, this alloreactivity has not been reported, not even in HLA-mismatched clinical conditions (163).

In vitro data clearly showed a high degree reduction of alloreactivity by selection and expansion of CMV- and AdV-specific T cells using the CliniMACS® CCS (IFN-gamma) is achieved (167–169). One limitation of the data above is that the tests were not performed versus the recipients' material. In practice, alloreactivity testing of the donor material versus the recipient material is not feasible due to the time it takes and the necessary collection of the relevant tissue since GvHD can affect the skin, the gut, and the liver. Moreover, there was no alloactivation reported in AdV-specific T cells stimulated with third-party HLA-matched unrelated donor cells in a mixed lymphocyte reaction (MLR) setting when compared with autologous stimulation, but a residual 28% of alloreactivity was shown in the HLA-mismatched MLR setting (150). Very recently, our team has demonstrated that CMV-CTL isolated by IFN-γ secretion assay and further *in vitro* expansion did not induce relevant cutaneous GvH tissue damage in the *in vitro* skin explant model while maintaining high level of antiviral activity (170). At low cell doses (5×10^5) none of CMV-CTLs led to GvH reactions in the HLA-mismatched recipient's skin, whereas at the high cell dose (1×10^6) two of nine CMV-CTLs induced a mild GvH skin damage (**Figure 5**). Our observations contribute to further elucidate the knowledge on the immunogenicity of antiviral T cells supporting simultaneously their safety use in the clinical practice.

Furthermore, it is important to correlate the phenotype and functionality of the infused cells with the clinical outcome.

Clinical Trials Using *Ex Vivo* Magnetically Enriched Virus-Specific T Cells

Clinical trials to date have confirmed safety and efficacy of the adoptive transfer of virus-specific T cells. The **Tables 5–7** summarize the data of clinical studies performed with donor-derived CMV-, EBV-, and AdV-specific T cell products, either using T cell lines or directly *ex vivo* isolated T cells, administered for therapeutic or pre-emptive treatment after HSCT. We are going to discuss in more detail below the virus clearance and kinetics of virus-specific immune recovery after application of cellular therapies based on the two methods for *ex vivo* isolation of virus-specific T cells, namely the IFN-γ secretion assay and the peptide/MHC multimer selection technologies.

Clinical Trials Using CliniMACS® Cytokine Capture System (IFN-Gamma)

Several studies used the IFN-γ secretion assay to select antigen-specific T cells (**Tables 5–7**). Feuchtinger and colleagues published the clinical experience on 13 patients treated with the infusion of pp65-specific IFN-γ-secreting $CD4^+$ and $CD8^+$ cells for refractory CMV infections or CMV disease after HSCT (171). It was observed that *in vivo* expansion of transferred cells was correlated with clearance or significant reduction of viremia. Furthermore, expansion was seen in $CD4^+$ and $CD8^+$ T cells and cells could be detected *in vivo* within an average of 3–6 weeks.

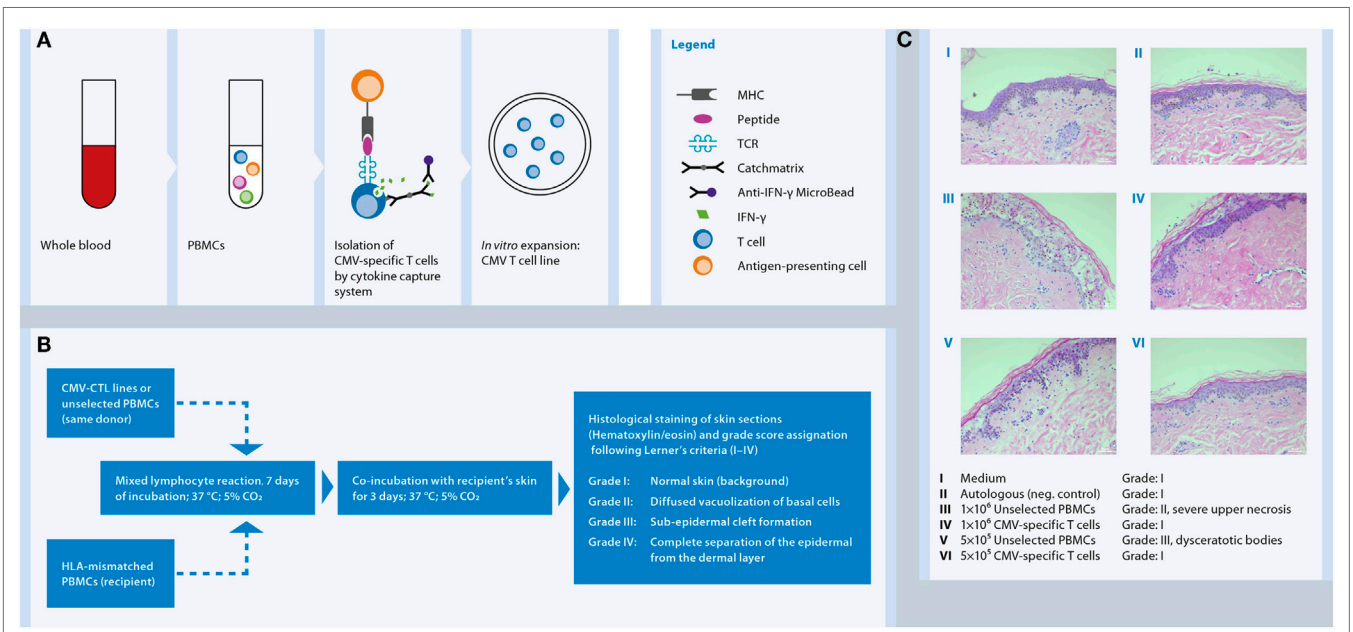

FIGURE 5 | *In vitro* human skin explant assay as a model to investigate the potential of third-party CMV-specific T cells to elicit GvHR in an HLA-mismatched system. **(A)** CMV-specific T cells were isolated from blood of seropositive donors by IFN-γ secretion assay and expanded *in vitro* between 2 and 4 weeks with IL-2 and irradiated feeder cells. **(B)** CMV-specific T cell lines and unselected PBMCs from the same donor where exposed to HLA-mismatched PBMCs (recipient's cells) in a mixed lymphocyte reaction for 7 days followed by incubation with recipient's skin for further 3 days. Then skin biopsies were collected, fixed in formalin, and stained with hematoxylin and eosin. **(C)** The histopathological damage in the skin biopsies displays a readout of the allogeneic-HLA reactions caused by T cell activation. The images show that CMV-specific T cells do not cause GvHR (Grade I) as opposed to Unselected PBMCs (Grade II and III) from the same donor.

TABLE 5 | Clinical trials with therapeutic treatment of CMV-specific T cells.

Reference	Method	No. pts	Results	Dose
Einsele et al. (149)	*In vitro* stimulation and expansion of CMV-specific polyclonal CD4+ and CD8+ T cells	8	5/7 evaluable pts eliminated infection	10^7 cells/m^2
Peggs et al. (262)	*In vitro* stimulation and expansion of CMV-specific polyclonal CD4+ and CD8+ T cells	16	Pre-emptive therapy: 8/16 did not require antiviral treatment	0.2–1 × 10^5 T cells/kg
Bao et al. (263)	*In vitro* stimulation and expansion of CMV-specific polyclonal CD4+ and CD8+ T cells	7	3/7 pts cleared infection 1/7 pts reduced viral load	2.5–5 × 10^5 CMV-specific CD3+ cells/kg
Blyth et al. (264)	*In vitro* stimulation and expansion of CMV-specific polyclonal CD4+ and CD8+ T cells	21	Pre-emptive therapy: 13/21 did not require antiviral treatment	2 × 10^7 CMV CTLs/m^2
Koehne et al. (265)	*In vitro* stimulation and expansion of CMV-specific polyclonal CD4+ and/or CD8+ T cells	16	14/16 pts eliminated infection	5 × 10^5–3 doses with 1 × 10^6 T cells/kg
Total	***In vitro* stimulation and expansion**	**68**	**23/30 responders (w/o pre-emptive therapy)**	
Feuchtinger et al. (171)	Direct isolation of CMV-specific CD8+ and CD4+ T cells using the CCS	18	15/18 responders	1.2–166 × 10^3 cells/kg
Peggs et al. (137)	Direct isolation of CMV-specific CD8+ and CD4+ T cells using the CCS	11	Pre-emptive therapy: 2/11 did not require antiviral treatment	10^4 CD3+ T cells/kg
Meij et al. (266)	Direct isolation of CMV-specific CD8+ and CD4+ T cells using the CCS	6	6/6 patients eliminated infection	0.9 × 10^4–3.1 × 10^5 cells/kg
Total	**Direct isolation using the CCS**	**35**	**21/24 responders (w/o pre-emptive therapy)**	
Cobbold et al. (267)	Direct isolation of CMV-specific CD8+ T cells using MHC-I-tetramers	9	8/9 patients eliminated infection	1.2–33 × 10^3 cells/kg
Schmitt et al. (155)	Direct isolation of CMV-specific CD8+ T cells using MHC-I-streptamers	2	Control of CMV-viremia in both patients	0.37 and 2.2 × 10^5 cells/kg
Uhlin et al. (268)	Direct isolation of CMV-specific CD8+ T-cells using MHC-I-pentamers	5	4/5 responders	0.8–24.6 × 10^4 cells/kg
Total	**Direct isolation using MHC-I-multimers**	**16**	**14/16 responders**	

TABLE 6 | Clinical trials with therapeutic treatment of EBV-specific T cells.

Reference	Method	No. pts	Results	Dose
Rooney et al. (144)	*In vitro* stimulation and expansion of EBV-specific CD8+ T cells	10	Therapy: 3/3 responders Prophylaxis: 7/7 virus free	0.2–1.2 × 10^8 cells/m^2
Haque et al. (269)	*In vitro* stimulation and expansion of EBV-specific CD8+ and CD4+ T cells	8	4/8 Remission	10^6 cells/kg
Haque et al. (270)	*In vitro* stimulation and expansion of EBV-specific CD8+ and CD4+ T cells	33	14/33 complete remission 3/33 partial response	2 × 10^6 cells/kg
Heslop et al. (271)	*In vitro* stimulation and expansion of EBV-specific CD8+ T cells	114	Therapy: 11/13 complete response Prophylaxis: All PTLD free	1–5 × 10^7 cells/m^2
Doubrovina et al. (272)	DLI or *in vitro* stimulation and expansion of EBV-specific CD8+ T cells	19	13/19 complete response	10^6 cells/kg
Gallot et al. (273)	*In vitro* stimulation and expansion of EBV-specific CD8+ and CD4+ T cells	11	4/10 responders	5 × 10^6 cells/kg
Total	***In vitro* stimulation and expansion**		**52/86 responders (w/o prophylaxis)**	
Moosman et al. (152)	Direct isolation of EBV-specific CD8+ and CD4+ T cells using the CCS	6	3/6 responders	0.4–9.7 × 10^4 cells/kg
Icheva et al. (151)	Direct isolation of EBV-specific CD8+ and CD4+ T cells using the CCS	10	7/10 responders	0.15–53.8 × 10^3 cells/kg
Total	**Direct isolation using the CCS**	**16**	**10/16 responders**	
Uhlin et al. (268)	Direct isolation of EBV-specific CD8+ T cells using MHC-I-pentamers	1	1/1 complete response	1.8 × 10^4 cells/kg
Total	**Direct isolation using MHC-I-multimers**	**1**	**1/1 responder**	

The transferred pp65-specific T cell immunity could be detected for more than 6 months after infusion in single patients (171). Moosmann and colleagues used the IFN-γ capture assay and stimulation with peptides derived from EBV antigens to generate EBV-specific T cells to treat PTLD induced by EBV (152). Three out of six patients had complete and stable remission after failing treatment with rituximab, an anti-CD20 antibody together with low numbers of CD4+ and CD8+ EBV-specific T cells.

TABLE 7 | Clinical trials with therapeutic treatment of AdV-specific T cells.

Reference	Method	No. pts	Results	Dose
Geyeregger et al. (274)	*In vitro* stimulation and expansion of AdV-specific CD8$^+$ and CD4$^+$ T cells	2	1/2 complete response 1/2 partial response	10^4 CD3$^+$ cells/kg
Total	***In vitro* stimulation and expansion**	**2**	**2/2 responders**	
Feuchtinger et al. (150)	Direct isolation of AdV-specific CD8$^+$ and CD4$^+$ T cells using the CCS	9	4/9 responders	1.2–50 × 10^3 cells/kg
Qasim et al. (153)	Direct isolation of AdV-specific CD8$^+$ and CD4$^+$ T cells using the CCS	5	3/5 responders (cleared adenoviremia)	10^4 cells/kg
Feucht et al. (138)	Direct isolation of AdV-specific CD8$^+$ and CD4$^+$ T cells using the CCS	30	21/30 responders	0.3–24 × 10^3 CD3$^+$ cells/kg
Total	**Direct isolation using the CCS**	**44**	**28/44 responders**	
Uhlin et al. (268)	Direct isolation of AdV-specific CD8$^+$ T cells using MHC-I-pentamers	1	No response	3.1 × 10^4 and 1.7 × 10^4 cells/kg
Total	**Direct isolation using MHC-I-multimers**	**1**	**0/1 responder**	

Non-responders suffered from the late-stage disease with multiorgan dysfunction at the time of T cell transfer. In two responders, long-term follow up was possible, showing that EBV-specific T cells rapidly expanded upon transfer, high levels were maintained for approximately 6 months then the numbers declined, according to the characteristic expansion and contraction of antigen-specific T cells, and stabilized at levels characteristic for healthy individuals, providing protection for at least 2 years after transfer. Detailed analyses of cell differentiation markers early after transfer showed that EBV-specific CD8$^+$ T cells had an effector memory phenotype (CCR7$^-$ CD45RA$^-$), which after contraction evolved into central memory (CCR7$^+$ CD45RA$^-$) and terminally differentiated effector cells (CCR7$^-$ CD45RA$^+$). It has also been shown that infusion of AdV-specific IFN-γ$^+$ T cells was successful and their expansion *in vivo* correlated with decreased viral load (138, 150). The analysis of four AdV-specific T cell products before treatment revealed that the majority of cells were of effector memory phenotype, identified based on the expression profiles CCR7$^-$ CD45RA$^-$ and CD62L$^-$ CD45RO$^+$, and a minority of central memory phenotype, characterized as CCR7$^+$ CD45RA$^-$ and CD62L$^+$ CD45RO$^+$ (138). Further investigation on tracking of the infused cells and correlating the phenotype and functionality of the infused cells with the clinical outcome will in addition help to define the optimal conditions for a successful and long-lasting effect of the adoptive transfer.

Clinical Trials Using the Peptide/MHC Multimer-Based Selection

Nowadays, this technique is used for adoptive transfer, since it has been shown that antigen-specific CD8$^+$ T-cells selected with peptide/MHC multimers induced long-lasting immune responses without increasing the risk for GvHD (**Tables 5–7**). The development of "reversible" TCR staining with streptamers allowed selection of the phenotypically and functionally unchanged cells (172, 173). Schmitt and colleagues reported results from the study on two patients treated with CMVpp65-specific T cells for recurrent CMV antigenaemia after HSCT (155). For one of the donors the phenotype and function of cells after transfusion was analyzed. Donor-derived CMV-specific T cells from the cellular product rapidly expanded *in vivo*, showed early after transfusion an effector memory phenotype (CCR7$^-$ CD45RA$^-$), acquired effector phenotype (CCR7$^-$ CD45RA$^+$) at later timepoints, and were capable of secreting IFN-γ upon *in vitro* stimulation. In both patients, clearance of the CMV reactivation without any signs of GvHD was observed. Additionally, Odendahl and colleagues showed in a pre-clinical study the potential of clinical-scale CMV streptamer-selected T cells. In this study, 22 cell products displayed excellent viability, cytotoxicty, and purity with effectively removed selection reagents (174). Recently, a GMP-compliant protocol using the streptamer technology was implemented to enrich EBV- and AdV-specific T cells. Because of the very low frequencies of EBV- and AdV-specific T cells in the starting material, the purity (among CD3$^+$ cells) of the large-scale cell product was poor, up to 44 and 6.7%, respectively. However, an increase in purity was achieved by small-scale selection or simultaneous application of EBV- and AdV-streptamers. An IFN-γ response was seen in most of the products and cells were predominantly of the effector memory (CD62L$^-$ CD45RA$^-$) or central memory phenotype (CD62L$^+$ CD45RA$^-$), thus those cells are suitable for clinical use (175).

Future Perspectives

Generation of Multipathogen-Specific T Cells

Adoptive transfer of multi-antigen-specific T cells is a promising approach in restoring antigen-specific immunity and preventing or treating infectious complications after HSCT. Several strategies have been developed to simultaneously select T cells specific for viral and/or fungal pathogens. Initial studies focused on CMV, EBV, and AdV, using a clinical-grade AdV vector Ad5f35 with expression of the CMV antigen pp65 transgene, which permitted transduction of APC like DCs or EBV-transformed B cells to successfully stimulate and expand virus-specific T cells (164, 176–178). A new enrichment strategy based on the activation-dependent CD154 (CD40L)-expression (transient expression on activated CD4$^+$ and to lesser extent on activated CD8$^+$ T cells) and subsequent expansion of T cell has been introduced to production of multi-pathogen-specific T cells without the need to genetically modify APC. This technique allowed generation of alloantigen-depleted CD4$^+$ and CD8$^+$ T cell lines within 14 days with high specificity for the most common posttransplantation

pathogens. These T cell lines showed extensive proliferative capacity and confirmed functionality *in vitro* (179). Recently, the use of either DNA plasmids or peptide pools to pulse APC has been validated to avoid safety and regulatory issues associated with transduction of APC using viral vectors. The combination of the peptide mixture approach or transfection of DC with plasmids with expansion in gas permeable rapid expansion (G-Rex) bioreactors provided further advances, increasing both feasibility and applicability of T cell therapy (180). These rapidly (10–12 days) expanded multi-virus-specific T cells provided effective antiviral protection in clinical trials (121, 159). Certainly, the short-term activation concomitantly with peptide pools from multiple viral antigens in combination with the CliniMACS® CCS (IFN-gamma) provides the most simplest and fastest way for simultaneous GMP-grade selection of CMV-, EBV-, and AdV-specific $CD4^+$ and $CD8^+$ T cells.

Broadening the Clinical Use of Adoptive T Cell Therapy

Several barriers prevent the broader use of virus-specific T cell therapies after HSCT. One of the main hurdles is associated with the complexity of GMP-grade cell manufacturing. More details and suitable solutions are described for generation of virus-specific T cells in Section "*In Vitro* GMP Manufacturing of antiviral T Cell Products" and of gene-modified T cells in Section "Complexity of the Cell Manufacturing Process" of this article. A second main problem is connected with pathogen-naïve donors and umbilical cord blood transplants. For immunotherapy with cells derived from pathogen-naïve donors or cord blood, *in vitro* priming of the donor T cells with APC pulsed with antigen or genetically modified APC can be introduced (177). Another option is the transfer of virus-specific TCR genes into donor primary T cells by viral vectors (181). The antigen-specific responses in recipient can be boosted also by the vaccination with peptide-loaded donor-derived DC (182). Apart from above mentioned strategies, the selection of the virus-specific T cells from healthy seropositive third-party donors is an attractive alternative. Haque and colleagues showed for the first time that partially matched third-party EBV-CTL led to the control of PTLD after solid organ transplantation (183). Also post-HSCT successful treatments of refractory viral infections (CMV, EBV, AdV) with third-party virus-specific T cells were reported (177, 184). A detailed summary on clinical results of third-party-derived virus-specific T cell administration is found in a recent review written by O'Reilly and colleagues (185). The first promising results using virus-specific T cells from third-party donors initiated the idea of donor registries and biobanks with the cryopreserved antigen-specific T cells, which could provide "off the shelf" immunotherapy product (185).

The introduction of rapid manufacturing technologies such as magnetic enrichment processes for selection of pathogen-specific T cells out of heterogeneous hematological populations offered new possibilities leading to successful application of adoptive T cell transfer in HSCT patients with refractory virus (CMV, EBV, ADV) infections (152, 171, 186) (**Tables 5–7**). More recently adoptive cell transfer has been developed for other virus infections, like Varicella Zoster virus, BK virus, or human herpesvirus 6 (121, 187) as well as for invasive fungal infections with aspergillus or candida (135).

IMMUNOTHERAPY WITH CAR GENE-MODIFIED T CELLS FOR TREATMENT OF LEUKEMIAS

Despite the success of allogeneic HSCT in the quest for a cure of leukemic patients, the demand for alternative and new treatment options is high, as relapse and refractory leukemia remain a major challenge for patients having with very poor prognosis (188–190). How to improve the antitumor immunity, especially in patients who are not eligible for HSCT, need of a bridge therapy prior to transplant, or even after failure of HSCT. In the future, will there be a way even to replace SCT and thereby avoiding transplantation-associated complications?

Elimination of the malignant cells and sustained remissions can be achieved by induction of GvL effects after HSCT, which are based on a donor T cell-mediated immune response. Enhancement of the GvL effects is observed with DLI (191, 192). However, a treatment with the complete repertoire of allogeneic T cells is always accompanied by the substantial risk for the life-threatening GvHD. One way to increase anti-leukemic effects while avoiding GvHD in allogenic transplantation settings is the transfusion of *in vitro* selected T cells, specifically targeting tumor-associated antigens. But the majority of described tumor-associated antigens are not exclusively found in tumor cells, but represents self-antigens, either expressed in other adult healthy tissue or during embryonic development. In general, it is assumed the endogenous T cell repertoire against self-antigens show limited potency to eradicate tumor cells due to low affinity TCR. The most powerful T cells would target either neo-antigens derived from mutated genes within tumor cells or allogenic antigens like minor histocompatibility antigens with restricted expression in hematological cells, e.g., HA-1. These antigens are recognized as foreign proteins by the immune system (i.e., the T cell repertoire for these antigens is not shaped due to negative thymic selection of T cells expressing high-affinity TCRs). Another approach to break self-tolerance is the introduction of a new, high-affinity antigen specificity into the T cells, i.e., by genetic modification with an artificial TCR or with a CAR (12, 193).

Clinical Outcome of CD19 CAR-Transduced T Cell Therapy

Recent success stories of therapy with CAR-modified T cells targeting CD19 in patients with high-risk B cell malignancies, such as chronic lymphocytic leukemia (CLL) or childhood acute lymphoblastic leukemia (ALL), have raised enormous scientific and public expectations. For example, in a clinical trial including 30 children and adults with relapsed or refractory ALL treated with CD19 CAR–transduced T cells 90% of the patients achieved complete remission (194). The development of CAR T cell therapy and a summary of clinical studies and data generated within the past years have been described in several reviews and therefore will not further discussed in this article (195, 196).

Workflow of Adoptive Therapy with CAR-Engineered T Cells

To prepare CAR-modified T cells for the treatment of a leukemic patient, first peripheral blood is drawn from the patient. The T cells are then isolated from the blood and engineered *in vitro* with a CAR targeting a pre-defined antigen on tumor cells. Subsequently, the cells are amplified to obtain a sufficient number of CAR T cells for transfusion into the patient (**Figure 6**). Before administration of CAR T cells, the patient undergoes a non-myeloablative lymphodepletion, which supports the therapy, e.g., by promoting the *in vivo* proliferation and thus the persistence of CAR T cells.

Engineering Potent and Safe CAR-Modified T Cells

Chimeric antigen receptors are artificially constructed receptors introduced into somatic cells, mainly in T cells, by genetic engineering and redirect immune responses toward the tumor. A CAR consists of an extracellular antigen recognition motif, resembling a single-chain fragment of the variable region of an antibody (scFv), directed against a cell surface antigen expressed on a tumor cell (**Figure 7**). The scFv part is linked *via* a transmembrane domain to intracellular signaling structures derived from the TCR and costimulatory receptor(s). If CAR-engineered T cells encounter tumor-associated antigens, the intracellular signaling cascades of the TCR/costimulatory moieties are triggered. Ultimately, this activation results in T cell effector function, i.e., cell proliferation, cytokine secretion, and cytolytic activity (197). Over the last years, the functional properties of CARs have been improved. First-generation CARs lacked the intracellular signaling motifs for costimulation. Effective T cell activation requires at least two types of signals: (i) engagement of the TCR with antigen presented by MHC and (ii) engagement of costimulatory molecules, such as CD28, OX40, and 4-1BB. However, tumors often do not express appropriate ligands for costimulatory molecules. To overcome these restrictions second-generation CARs were developed incorporating the intracellular domains of one costimulatory receptor, either CD28 or 4-1BB. T cells expressing such CARs had a higher capacity to expand, mediate increased tumor killing, and persist *in vivo* for a longer period of time compared to first-generation CARs (198–201). With the aim to further improve the functionality of CAR-modified T cells, so-called "third-generation" CARs, which deliver more than one type of costimulatory signal, are now prepared for clinical trials.

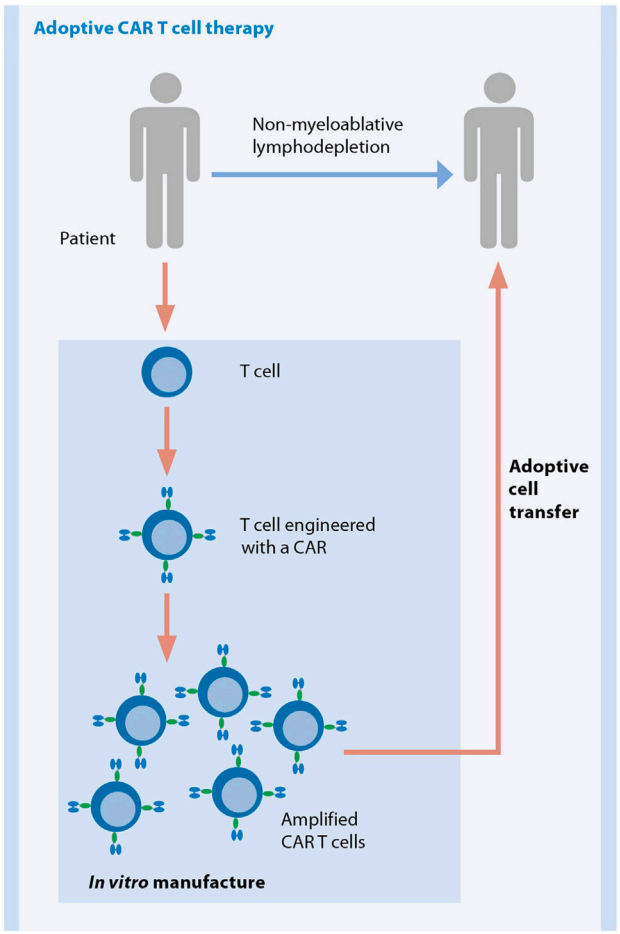

FIGURE 6 | General workflow for adoptive therapy with CAR-modified T cells. Figure courtesy of Prof. Hinrich Abken.

Further efforts concentrate on strategies for design of T cells with the goal to overcome inhibitory T cell signaling, the suppression by the tumor microenvironment, or tumor antigen loss, which is now regularly detected in a subset of patients suffering from relapses after CD19 CAR T cell therapy (202–208).

Other strategies for CAR T cell design aim toward increasing the safety of CAR T cells. One major concern of the therapy is the attack of normal tissues ("on-target, off-tumor" toxicity), which could dependent on the chosen target antigen result in very severe and life-threating toxicity (208). However, the elimination of normal B cells with, e.g., CD19 CAR T cells and the resulting B cell aplasia is regarded as an expected and acceptable "on-target, off-tumor" effect, which is successfully treated with infusion of gamma immunoglobulins. Another toxicity first observed with CD19 CAR T cells is the cytokine release syndrome (202, 209, 210). It is a side-effect of the desired antitumor response induced by CAR T cells leading to mild, but in some cases to severe clinical syndromes, which requires intensive care and therapeutic management of the patients. Severe events are now effectively treated with blocking Anti-IL-6 receptor antibody without influencing the tumor rejection by the CAR T cells (211–213). Nevertheless, the need to prevent or substantially limit the toxicity of the therapy is high and potential solutions are under investigations (208, 214–216).

Advantages of CAR-Engineered T Cells

Chimeric antigen receptors-modified T cells have some crucial advantages over natural T cells and in part also over TCR-engineered T cells, as they can function independently of MHC molecules. First, the affinity of an antibody–antigen binding is in general much higher compared to a TCR–peptide/MHC binding. This provides at least the option to target antigens that are usually not detected by T cells, e.g., carbohydrates and glycolipids, which

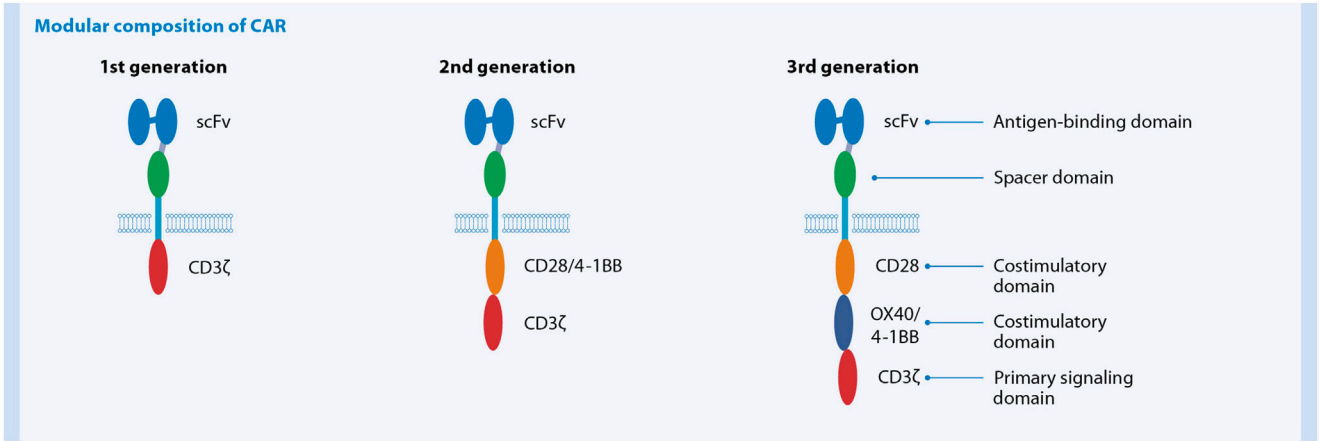

FIGURE 7 | **Structure of different generations of CARs**. Figure courtesy of Prof. Hinrich Abken.

are frequently altered in tumor cells (217–219). Second, loading of antigenic peptide onto MHC requires antigen processing and presentation, and both processes are targets of tumor escape mechanisms resulting in the loss of antigen/MHC expression on malignant cells. Third, a CAR recognizes its antigen independent of individual MHC allotypes, resulting in the universal application in all patients that express this antigen on the cell surface. In contrast a TCR is specific only for the combination of an antigenic peptide in the context with an MHC allele. Due to the MHC polymorphism in the human population, patient-specific or at least a panel of MHC allele/peptide-specific TCRs are needed to cover the human population comprehensively. Last, not only $CD8^+$ T cells, but also $CD4^+$ T cells can be engineered, which allows for T cell help independent of MHC class II expression. A clear disadvantage of CARs is that only cell surface antigens can be targeted, while intracellular tumor antigens remain invisible. However, the recognition of MHC/peptide complexes by CARs is not excluded (220), which might also facilitates access to intracellular tumor antigens.

As learned from the outcome of the clinical application of *ex vivo* expanded melanoma-infiltrating T cells over the last years, the key factors for a successful adoptive T cell therapy to target cancers are the selection of the best possible tumor antigen, the *in vivo* persistence of transferred T cells and their accessibility to the tumor. Beyond that, a reliable and reproducible manufacturing procedure leading to high-quality cellular products is a crucial element of the therapy (193). We will focus our discussion in the next sections on the demands and challenges connected to the manufacturing process and will disclose recent progress toward the implementation of therapy with CAR-engineered T cells into clinical practice.

Complexity of the Cell Manufacturing Process

Currently, therapies with CAR-modified T cells are mainly applied in the context of clinical trails by investigators, according to their own manufacturing process utilizing existing infrastructure with clean rooms, instruments etc. The *in vitro* preparation of CAR T cells is a quite complex process and lasts for several days to weeks. So far, most concepts for CAR T cell therapies are based on autologous cells, which means that each cellular product is manufactured in a single batch in small scale for a single patient. It starts with isolation of peripheral blood cells, e.g., by an initial leukapheresis step. Blood is drawn either from the patient directly (autologous therapy) or – in the case of a patient who received stem cell transplantation – from the stem cell donor (allogeneic therapy). Then T cells are enriched from the blood, activated, and subsequently gene-modified with viral or non-viral vectors encoding the CAR. The CAR-modified T cells are amplified to obtain larger numbers of cells and finally formulated and/or cryopreserved prior to infusion into the patient. Several in-process and quality control analyses of the cell product are required to guarantee the safety and quality of the final cellular end product (**Figure 8**). This multi-step workflow poses high demands on the infrastructure, is labor intensive, and requires various different techniques, devices, reagents, handling steps, and skilled and extensively trained operators. Within a small-scale clinical trial the entire process can be executed in a semi-automated manner with the use of several devices for single process steps according to GMP guidelines. To date only a restricted number of GMP facilities worldwide are able to carry out this manufacturing process. But in the light of the encouraging clinical outcomes, the need for a broadly applicable therapy is high. However, the transformation of such a manufacturing process into a routine and large-scale setting has some pitfalls. An optimization and an upscaling of the manufacturing process is one of the key factors for the dissemination of this therapy.

Manufacture of High-Quality Cell Products Requires Robust and Reproducible Cell Processing

A favorable outcome of cell therapy depends on a robust and reproducible manufacturing processes resulting in safe and clinically effective cell products. Currently, many investigators

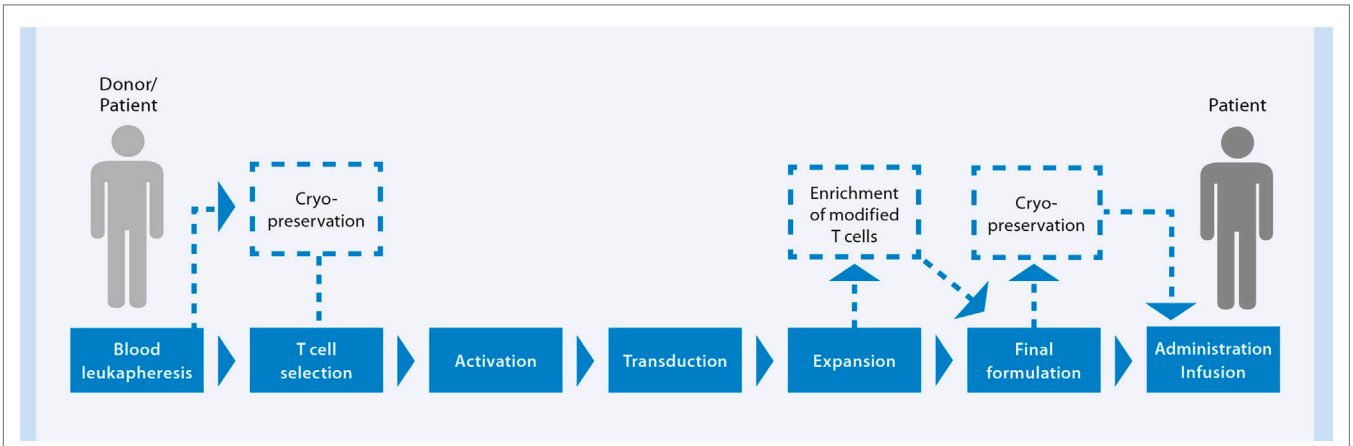

FIGURE 8 | ***In vitro*** **manufacture process of a CAR-engineered T cell product**.

and companies are developing solutions, including instruments, reagents, and consumables, for GMP-grade cell manufacture (221). Robustness of the cell manufacturing process, which will eliminate failure risks and allow standardization, can be improved by several means.

(1) Operation in a "closed" system in contrast to "open" processing minimizes the risk of contamination and therefore failure of production. Maintaining the sterility of the cell product is essential. All interventions during cell processing, like addition or exchange of reagents and buffer/media during washing, feeding, activation, transduction, and sampling steps bear the risk for product contamination. Closed systems are set-up with equipment that allows processing of cell without its exposure to the room environment, but keeping sterile conditions. Suitable closed systems are, e.g., bags with closed tubing pathways and connections. Introduction of material into closed systems is possible, e.g., *via* sterile filters. A suitable simplified and semi-closed cell culture system for CD19 CAR T cell production has been described (222). Closed systems might enable operators to work under less advanced GMP clean room conditions, which is more cost effective and easier to establish.

(2) The use of enriched T cells as starting material for the activation process helps to achieve higher reproducibility in the manufacturing process. Patient's blood samples are highly variable in their cellular composition and one of the most critical parameter for reproducible cell processing. Instead of using the entire blood cell fraction for gene modification, isolation of T cells or even T cell subsets prior to modification is favorable for various reasons. Most patients are heavily pre-treated, which often give rise to abnormal or immunosuppressive blood cell populations or even low-responding T cells (223–225). Moreover, it has been shown that activation and expansion of the T cells is substantially enhanced when T cells were isolated from the blood product to eliminate suppressive influences (222, 226, 227). Currently, particular T cell subpopulations are under investigation with the aim to improve *in vivo* persistence and effector function of adoptively transferred CAR-modified T cells. One strategy is based on the modification of patient's endogenous CMV- or EBV-specific T cell pools, which contain long-living memory cells (228). In addition, persistence of these CAR-modified T cells might be promoted by triggering the natural TCR *in vivo* upon reactivation of those latent viruses. A further advantage of CAR-modified virus-specific T cells is that they provide protection in the case of viral reactivation after lymphodepletion (228–230). A disadvantage of this concept is the need to implement the generation of virus-specific T cells into the manufacturing process, which adds more complexity to the whole process, is time consuming, and might affect functionality of the cells, especially if long-term culturing is required to obtain virus-specific T cell populations. Alternatively, the naive, central memory, or stem memory T cell subset, which have been described to have essential functional advantages, are regarded as an appropriate starting population (231–235).

A straightforward and closed system for GMP-grade and large-scale T cell processing is the combination of Dynabeads® CD3/CD28 CTS™, a large magnet (both offered by Thermo Fisher Scientific), and bags to enrich and concomitantly activate T cells from whole blood products (236). A versatile, reliable platform for closed, clinical-scale magnetic enrichment of either all T cell types or naive and central memory T cell subsets is the CliniMACS® System, encompassing separation reagents and the CliniMACS Plus Instrument developed by Miltenyi Biotec, Bergisch Gladbach, Germany (237, 238). For T cell activation a reagent (MACS® GMP TransAct™ CD3/CD28 Reagent) consisting of a biodegradable polymeric nanomatrix coated with agonists for CD3 and CD28 is available, which allows for efficient viral transduction (237). This reagent is in compliance with relevant GMP guidelines. It can be sterile filtered, which makes it a highly valuable tool for aseptic cell manufacturing.

(3) Simplification of the cell processing by automation improves reproducibility and reduces resources for operators and thus increases productivity. The CAR T cell manufacturing involves various process steps, like cell enrichment, cell culturing, final formulation of the product and in between cell washing, concentration, feeding, and rebuffering. In addition, in-process and quality controls samplings are performed. Several commercially available devices allow the run of single or few steps of the process (221). Nevertheless, multiple instruments and systems need to be implemented for execution of the whole process, which challenge the manufacturer in many ways. The different devices and the procedures need to be adjusted to each other to achieve a feasible and safe process. Substantial manual handling steps and user interactions are required. Additionally, each device demands installations, services, qualifications, and training of operators. A new device, the CliniMACS Prodigy® instrument (Miltenyi Biotec), is designed as an all-in-one solution for automated cell processing in a closed GMP-compliant system (60). A process specifically developed and optimized for the manufacture of CAR T cells on this instrument is now available. With this process, the entire workflow for the manufacture of CAR T cells, starting with T cell enrichment through to final formulation, can be performed in a single-use tubing set with minimal operator interaction. The complex CAR T cell production process includes many different reagents, i.e., T cell separation reagents, activation and expansion reagents, viral vectors, cell culture media, cytokines, and buffers. Importantly, for the use of the CliniMACS Prodigy® all these reagents are developed to efficiently and stably work together as an integrated reagent system. This CliniMACS Prodigy® approach significantly simplifies the manufacturing process. In addition, due to the integrated solution it allows easy implementation in GMP facilities and can boost CAR T cell therapy to a standard-of-care.

Future Perspectives: Commercialized Manufacture of Personalized Engineered Cellular Products

Today, most CAR T cell products are manufactured for phase I/II trials in a limited number either within clinical centers or facilities of commercial providers. At least with entering into phase II/III clinical trials new considerations have to be taken into account as the number of patients to be treated increases to hundreds or thousands per year. Production, infrastructure, and logistics for shipment of cellular materials have to be set up to guarantee the manufacturing of these high quantities in a high-quality and cost-effective manner and with compliance of all the regulatory requirements. To achieve these goals the process needs standardization and scale-up. In the end, a therapy must fulfill economical requirements to be available as a standard-of-care for patients.

Chimeric antigen receptors T cell therapy applied in the moment is cost-intensive as individualized products have to be generated starting with patient-derived cells. Several investigators are currently evaluating options to reduce the costs of cell production by depersonalizing T cell therapy, e.g., using off-the-shelf third-party T cells modified for knock-out of the endogenous HLA class I, TCR and/or CD52 expression for subsequent gene engineering with artificial antigen receptors (239, 240).

In principal, two different models for clinical cell manufacturing are discussed (221). A production line, as established for automated industries, where the manufacturing process for one patient product is structured in sequential operations, which are performed with specialized and dedicated personnel in physically separated spaces of the facility. In line with this concept is, e.g., the Xvivo modular laminar flow system from BioSpherix (Lacona, NY, USA), which enables the transport of cells through a whole series of areas. Due to the high investment for establishing a production line including the efforts required for organization of the infrastructure for cell shipments, a centralized manufacturing in highly specialized large facilities rather than a decentralized, local production at patients' point-of-care is of favorite. The second model relies on devices such as the CliniMACS Prodigy®, to handle one cell sample in one instrument at a time in an automated way and with only a minimum of user interactions. Within one facility numerous devices can be run in parallel and completely independently from each other. The device-based system is in accordance with a centralized as well as a decentralized organized cell manufacturing and therefore an attractive solution for commercial providers having large or smaller facilities, including hospital located sites.

CONCLUSION

Within the last years, the cellular immunotherapy field, especially in the context of hematological malignancies, gained tremendous attention by scientific researchers, clinicians, as well as commercial entities, thanks to the substantial progress made in multiple. The better scientific understanding of immunological mechanism and the novel advanced ideas and technologies for cell engineering and manufacturing have enabled the design of improved clinical approaches, which are currently being evaluated within clinical trials. The next step has to be the translation and broad implementation of these treatments into clinical routine. This requires on the one hand the selection of the best therapeutic options with maximal clinical benefit for the patients and on the other hand that the economical needs are met for all: the pharmaceutical companies and clinical entities involved in bringing the therapy to the patient, and the payers, who reimburse the therapy, i.e., health insurances.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of "The Newcastle Hospitals NHS Foundation Trust Heath Research Authority-NRES Committee North East – Newcastle & North Tyneside 2" with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

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

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REFERENCES

1. Devine SM, Carter S, Soiffer RJ, Pasquini MC, Hari PN, Stein A, et al. Low risk of chronic graft versus host disease and relapse associated with T-cell depleted peripheral blood stem cell transplantation for acute myeloid leukemia in first remission: results of the Blood and Marrow Transplant Clinical Trials Network (BMT CTN) protocol 0303. *Biol Blood Marrow Transplant* (2011) 17(9):1343–51. doi:10.1016/j.bbmt.2011.02.002
2. Pasquini MC, Devine S, Mendizabal A, Baden LR, Wingard JR, Lazarus HM, et al. Comparative outcomes of donor graft CD34+ selection and immune suppressive therapy as graft-versus-host disease prophylaxis for patients with acute myeloid leukemia in complete remission undergoing HLA-matched sibling allogeneic hematopoietic cell transplantation. *J Clin Oncol* (2012) 30(26):3194–201. doi:10.1200/JCO.2012.41.7071
3. Airoldi I, Bertaina A, Prigione I, Zorzoli A, Pagliara D, Cocco C, et al. γδ T-cell reconstitution after HLA-haploidentical hematopoietic transplantation depleted of TCR-αβ(+)/CD19(+) lymphocytes. *Blood* (2015) 125(15):2349–58. doi:10.1182/blood-2014-09-599423
4. Aversa F. T-cell depletion: from positive selection to negative depletion in adult patients. *Bone Marrow Transplant* (2015) 50(S2):S11–3. doi:10.1038/bmt.2015.88
5. Bleakley M, Heimfeld S, Loeb KR, Jones LA, Chaney C, Seropian S, et al. Outcomes of acute leukemia patients transplanted with naive T cell-depleted stem cell grafts. *J Clin Invest* (2015) 125(7):2677–89. doi:10.1172/JCI81229
6. Im HJ, Koh KN, Suh JK, Lee SW, Choi ES, Jang S, et al. Haploidentical HCT using an [alpha][beta] T-cell-depleted graft with targeted [alpha][beta]+ cells by add-back after a reduced intensity preparative regimen containing low-dose TBI. *Bone Marrow Transplant* (2016) 51(9):1217–22. doi:10.1038/bmt.2016.114
7. Lang P, Feuchtinger T, Teltschik HM, Schwinger W, Schlegel P, Pfeiffer M, et al. Improved immune recovery after transplantation of TCRalphabeta/CD19-depleted allografts from haploidentical donors in pediatric patients. *Bone Marrow Transplant* (2015) 50(Suppl 2):S6–10. doi:10.1038/bmt.2015.87
8. Maschan M, Shelikhova L, Ilushina M, Kurnikova E, Boyakova E, Balashov D, et al. TCR-alpha/beta and CD19 depletion and treosulfan-based conditioning regimen in unrelated and haploidentical transplantation in children with acute myeloid leukemia. *Bone Marrow Transplant* (2016) 51(5):668–74. doi:10.1038/bmt.2015.343
9. Gratwohl A, Brand R, Apperley J, Biezen Av A, Bandini G, Devergie A, et al. Graft-versus-host disease and outcome in HLA-identical sibling transplantations for chronic myeloid leukemia. *Blood* (2002) 100(12):3877–86. doi:10.1182/blood.V100.12.3877
10. Kolb HJ, Schattenberg A, Goldman JM, Hertenstein B, Jacobsen N, Arcese W, et al. Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. *Blood* (1995) 86(5):2041–50.
11. Anguille S, Smits EL, Bryant C, Van Acker HH, Goossens H, Lion E, et al. Dendritic cells as pharmacological tools for cancer immunotherapy. *Pharmacol Rev* (2015) 67(4):731–53. doi:10.1124/pr.114.009456
12. Cruz CR, Bollard CM. T-cell and natural killer cell therapies for hematologic malignancies after hematopoietic stem cell transplantation: enhancing the graft-versus-leukemia effect. *Haematologica* (2015) 100(6):709–19. doi:10.3324/haematol.2014.113860
13. Trzonkowski P, Bacchetta R, Battaglia M, Berglund D, Bohnenkamp HR, ten Brinke A, et al. Hurdles in therapy with regulatory T cells. *Sci Transl Med* (2015) 7(304):304s18. doi:10.1126/scitranslmed.aaa7721
14. da Silva Meirelles L, Chagastelles PC, Nardi NB. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *J Cell Sci* (2006) 119(Pt 11):2204–13. doi:10.1242/jcs.02932
15. Zhao K, Lou R, Huang F, Peng Y, Jiang Z, Huang K, et al. Immunomodulation effects of mesenchymal stromal cells on acute graft-versus-host disease after hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant* (2015) 21(1):97–104. doi:10.1016/j.bbmt.2014.09.030
16. Jagasia M, Arora M, Flowers ME, Chao NJ, McCarthy PL, Cutler CS, et al. Risk factors for acute GVHD and survival after hematopoietic cell transplantation. *Blood* (2012) 119(1):296–307. doi:10.1182/blood-2011-06-364265
17. Le Blanc K, Frassoni F, Ball L, Locatelli F, Roelofs H, Lewis I, et al. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. *Lancet* (2008) 371(9624):1579–86. doi:10.1016/S0140-6736(08)60690-X
18. Arai S, Margolis J, Zahurak M, Anders V, Vogelsang GB. Poor outcome in steroid-refractory graft-versus-host disease with antithymocyte globulin treatment. *Biol Blood Marrow Transplant* (2002) 8(3):155–60. doi:10.1053/bbmt.2002.v8.pm11939605
19. Ferrara JL, Levine JE, Reddy P, Holler E. Graft-versus-host disease. *Lancet* (2009) 373(9674):1550–61. doi:10.1016/S0140-6736(09)60237-3
20. Martin PJ, Schoch G, Fisher L, Byers V, Anasetti C, Appelbaum FR, et al. A retrospective analysis of therapy for acute graft-versus-host disease: initial treatment. *Blood* (1990) 76(8):1464–72.
21. Brunstein CG, Miller JS, Cao Q, McKenna DH, Hippen KL, Curtsinger J, et al. Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. *Blood* (2011) 117(3):1061–70. doi:10.1182/blood-2010-07-293795
22. Di Ianni M, Falzetti F, Carotti A, Terenzi A, Castellino F, Bonifacio E, et al. Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. *Blood* (2011) 117(14):3921–8. doi:10.1182/blood-2010-10-311894
23. Le Blanc K, Rasmusson I, Sundberg B, Gotherstrom C, Hassan M, Uzunel M, et al. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet* (2004) 363(9419):1439–41. doi:10.1016/S0140-6736(04)16104-7
24. Ringden O, Uzunel M, Rasmusson I, Remberger M, Sundberg B, Lonnies H, et al. Mesenchymal stem cells for treatment of therapy-resistant graft-versus-host disease. *Transplantation* (2006) 81(10):1390–7. doi:10.1097/01.tp.0000214462.63943.14
25. von Bonin M, Stolzel F, Goedecke A, Richter K, Wuschek N, Holig K, et al. Treatment of refractory acute GVHD with third-party MSC expanded in platelet lysate-containing medium. *Bone Marrow Transplant* (2009) 43(3):245–51. doi:10.1038/bmt.2008.316
26. Wu Y, Wang Z, Cao Y, Xu L, Li X, Liu P, et al. Cotransplantation of haploidentical hematopoietic and umbilical cord mesenchymal stem cells with a myeloablative regimen for refractory/relapsed hematologic malignancy. *Ann Hematol* (2013) 92(12):1675–84. doi:10.1007/s00277-013-1831-0
27. Wu Y, Cao Y, Li X, Xu L, Wang Z, Liu P, et al. Cotransplantation of haploidentical hematopoietic and umbilical cord mesenchymal stem cells for severe aplastic anemia: successful engraftment and mild GVHD. *Stem Cell Res* (2014) 12(1):132–8. doi:10.1016/j.scr.2013.10.001
28. Kuzmina LA, Petinati NA, Parovichnikova EN, Lubimova LS, Gribanova EO, Gaponova TV, et al. Multipotent mesenchymal stromal cells for the prophylaxis of acute graft-versus-host disease – a phase II study. *Stem Cells Int* (2012) 2012:968213. doi:10.1155/2012/968213

29. Baron F, Lechanteur C, Willems E, Bruck F, Baudoux E, Seidel L, et al. Cotransplantation of mesenchymal stem cells might prevent death from graft-versus-host disease (GVHD) without abrogating graft-versus-tumor effects after HLA-mismatched allogeneic transplantation following nonmyeloablative conditioning. *Biol Blood Marrow Transplant* (2010) 16(6):838–47. doi:10.1016/j.bbmt.2010.01.011
30. Introna M, Lucchini G, Dander E, Galimberti S, Rovelli A, Balduzzi A, et al. Treatment of graft versus host disease with mesenchymal stromal cells: a phase I study on 40 adult and pediatric patients. *Biol Blood Marrow Transplant* (2014) 20(3):375–81. doi:10.1016/j.bbmt.2013.11.033
31. Sanchez-Guijo F, Caballero-Velazquez T, Lopez-Villar O, Redondo A, Parody R, Martinez C, et al. Sequential third-party mesenchymal stromal cell therapy for refractory acute graft-versus-host disease. *Biol Blood Marrow Transplant* (2014) 20(10):1580–5. doi:10.1016/j.bbmt.2014.06.015
32. Perez-Simon JA, Lopez-Villar O, Andreu EJ, Rifon J, Muntion S, Diez Campelo M, et al. Mesenchymal stem cells expanded in vitro with human serum for the treatment of acute and chronic graft-versus-host disease: results of a phase I/II clinical trial. *Haematologica* (2011) 96(7):1072–6. doi:10.3324/haematol.2010.038356
33. Lucchini G, Introna M, Dander E, Rovelli A, Balduzzi A, Bonanomi S, et al. Platelet-lysate-expanded mesenchymal stromal cells as a salvage therapy for severe resistant graft-versus-host disease in a pediatric population. *Biol Blood Marrow Transplant* (2010) 16(9):1293–301. doi:10.1016/j.bbmt.2010.03.017
34. Prasad VK, Lucas KG, Kleiner GI, Talano JA, Jacobsohn D, Broadwater G, et al. Efficacy and safety of ex vivo cultured adult human mesenchymal stem cells (Prochymal) in pediatric patients with severe refractory acute graft-versus-host disease in a compassionate use study. *Biol Blood Marrow Transplant* (2011) 17(4):534–41. doi:10.1016/j.bbmt.2010.04.014
35. Kurtzberg J, Prockop S, Teira P, Bittencourt H, Lewis V, Chan KW, et al. Allogeneic human mesenchymal stem cell therapy (remestemcel-L, Prochymal) as a rescue agent for severe refractory acute graft-versus-host disease in pediatric patients. *Biol Blood Marrow Transplant* (2014) 20(2):229–35. doi:10.1016/j.bbmt.2013.11.001
36. Ball LM, Bernardo ME, Roelofs H, van Tol MJ, Contoli B, Zwaginga JJ, et al. Multiple infusions of mesenchymal stromal cells induce sustained remission in children with steroid-refractory, grade III-IV acute graft-versus-host disease. *Br J Haematol* (2013) 163(4):501–9. doi:10.1111/bjh.12545
37. Resnick IB, Barkats C, Shapira MY, Stepensky P, Bloom AI, Shimoni A, et al. Treatment of severe steroid resistant acute GVHD with mesenchymal stromal cells (MSC). *Am J Blood Res* (2013) 3(3):225–38.
38. Herrmann R, Sturm M, Shaw K, Purtill D, Cooney J, Wright M, et al. Mesenchymal stromal cell therapy for steroid-refractory acute and chronic graft versus host disease: a phase 1 study. *Int J Hematol* (2012) 95(2):182–8. doi:10.1007/s12185-011-0989-2
39. von Bahr L, Sundberg B, Lonnies L, Sander B, Karbach H, Hagglund H, et al. Long-term complications, immunologic effects, and role of passage for outcome in mesenchymal stromal cell therapy. *Biol Blood Marrow Transplant* (2012) 18(4):557–64. doi:10.1016/j.bbmt.2011.07.023
40. Ringden O, Le Blanc K. Mesenchymal stem cells for treatment of acute and chronic graft-versus-host disease, tissue toxicity and hemorrhages. *Best Pract Res Clin Haematol* (2011) 24(1):65–72. doi:10.1016/j.beha.2011.01.003
41. Lalu MM, McIntyre L, Pugliese C, Fergusson D, Winston BW, Marshall JC, et al. Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials. *PLoS One* (2012) 7(10):e47559. doi:10.1371/journal.pone.0047559
42. Lucchini G, Dander E, Pavan F, Di Ceglie I, Balduzzi A, Perseghin P, et al. Mesenchymal stromal cells do not increase the risk of viral reactivation nor the severity of viral events in recipients of allogeneic stem cell transplantation. *Stem Cells Int* (2012) 2012:690236. doi:10.1155/2012/690236
43. Ning H, Yang F, Jiang M, Hu L, Feng K, Zhang J, et al. The correlation between cotransplantation of mesenchymal stem cells and higher recurrence rate in hematologic malignancy patients: outcome of a pilot clinical study. *Leukemia* (2008) 22(3):593–9. doi:10.1038/sj.leu.2405090
44. Battiwalla M, Barrett AJ. Bone marrow mesenchymal stromal cells to treat complications following allogeneic stem cell transplantation. *Tissue Eng B Rev* (2014) 20(3):211–7. doi:10.1089/ten.teb.2013.0566
45. Caplan AI, Correa D. The MSC: an injury drugstore. *Cell Stem Cell* (2011) 9(1):11–5. doi:10.1016/j.stem.2011.06.008
46. Sensebe L, Krampera M, Schrezenmeier H, Bourin P, Giordano R. Mesenchymal stem cells for clinical application. *Vox Sang* (2010) 98(2): 93–107. doi:10.1111/j.1423-0410.2009.01227.x
47. Vaes B, Van't Hof W, Deans R, Pinxteren J. Application of MultiStem® allogeneic cells for immunomodulatory therapy: clinical progress and pre-clinical challenges in prophylaxis for graft versus host disease. *Front Immunol* (2012) 3:345. doi:10.3389/fimmu.2012.00345
48. Wuchter P, Vetter M, Saffrich R, Diehlmann A, Bieback K, Ho AD, et al. Evaluation of GMP-compliant culture media for in vitro expansion of human bone marrow mesenchymal stromal cells. *Exp Hematol* (2016) 44(6):508–18. doi:10.1016/j.exphem.2016.02.004
49. Kinzebach S, Bieback K. Expansion of mesenchymal stem/stromal cells under xenogenic-free culture conditions. *Adv Biochem Eng Biotechnol* (2013) 129:33–57. doi:10.1007/10_2012_134
50. Brunner D, Frank J, Appl H, Schoffl H, Pfaller W, Gstraunthaler G. Serum-free cell culture: the serum-free media interactive online database. *ALTEX* (2010) 27(1):53–62.
51. Bieback K. Platelet lysate as replacement for fetal bovine serum in mesenchymal stromal cell cultures. *Transfus Med Hemother* (2013) 40(5):326–35. doi:10.1159/000354061
52. Gottipamula S, Ashwin KM, Muttigi MS, Kannan S, Kolkundkar U, Seetharam RN. Isolation, expansion and characterization of bone marrow-derived mesenchymal stromal cells in serum-free conditions. *Cell Tissue Res* (2014) 356(1):123–35. doi:10.1007/s00441-013-1783-7
53. Kobayashi T, Watanabe H, Yanagawa T, Tsutsumi S, Kayakabe M, Shinozaki T, et al. Motility and growth of human bone-marrow mesenchymal stem cells during ex vivo expansion in autologous serum. *J Bone Joint Surg Br* (2005) 87(10):1426–33. doi:10.1302/0301-620X.87B10.16160
54. Kocaoemer A, Kern S, Kluter H, Bieback K. Human AB serum and thrombin-activated platelet-rich plasma are suitable alternatives to fetal calf serum for the expansion of mesenchymal stem cells from adipose tissue. *Stem Cells* (2007) 25(5):1270–8. doi:10.1634/stemcells.2006-0627
55. Lin HT, Tarng YW, Chen YC, Kao CL, Hsu CJ, Shyr YM, et al. Using human plasma supplemented medium to cultivate human bone marrow-derived mesenchymal stem cell and evaluation of its multiple-lineage potential. *Transplant Proc* (2005) 37(10):4504–5. doi:10.1016/j.transproceed.2005.11.033
56. Burnouf T, Strunk D, Koh MB, Schallmoser K. Human platelet lysate: replacing fetal bovine serum as a gold standard for human cell propagation? *Biomaterials* (2016) 76:371–87. doi:10.1016/j.biomaterials.2015.10.065
57. Bernardo ME, Avanzini MA, Perotti C, Cometa AM, Moretta A, Lenta E, et al. Optimization of in vitro expansion of human multipotent mesenchymal stromal cells for cell-therapy approaches: further insights in the search for a fetal calf serum substitute. *J Cell Physiol* (2007) 211(1):121–30. doi:10.1002/jcp.20911
58. Crespo-Diaz R, Behfar A, Butler GW, Padley DJ, Sarr MG, Bartunek J, et al. Platelet lysate consisting of a natural repair proteome supports human mesenchymal stem cell proliferation and chromosomal stability. *Cell Transplant* (2011) 20(6):797–811. doi:10.3727/096368910X543376
59. Doucet C, Ernou I, Zhang Y, Llense JR, Begot L, Holy X, et al. Platelet lysates promote mesenchymal stem cell expansion: a safety substitute for animal serum in cell-based therapy applications. *J Cell Physiol* (2005) 205(2):228–36. doi:10.1002/jcp.20391
60. Apel M, Brüning M, Granzin M, Essl M, Stuth J, Blaschke J, et al. Integrated clinical scale manufacturing system for cellular products derived by magnetic cell separation, centrifugation and cell culture. *Chemie Ingenieur Technik* (2013) 85(1–2):103–10. doi:10.1002/cite.201200175
61. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* (2006) 8(4):315–7. doi:10.1080/14653240600855905
62. Wuchter P, Bieback K, Schrezenmeier H, Bornhauser M, Muller LP, Bonig H, et al. Standardization of good manufacturing practice-compliant production of bone marrow-derived human mesenchymal stromal cells for immunotherapeutic applications. *Cytotherapy* (2015) 17(2):128–39. doi:10.1016/j.jcyt.2014.04.002
63. Galipeau J, Krampera M, Barrett J, Dazzi F, Deans RJ, DeBruijn J, et al. International Society for Cellular Therapy perspective on immune functional assays for mesenchymal stromal cells as potency release criterion for

advanced phase clinical trials. *Cytotherapy* (2016) 18(2):151–9. doi:10.1016/j.jcyt.2015.11.008
64. Gyorgy B, Szabo TG, Pasztoi M, Pal Z, Misjak P, Aradi B, et al. Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. *Cell Mol Life Sci* (2011) 68(16):2667–88. doi:10.1007/s00018-011-0689-3
65. Raposo G, Nijman HW, Stoorvogel W, Liejendekker R, Harding CV, Melief CJ, et al. B lymphocytes secrete antigen-presenting vesicles. *J Exp Med* (1996) 183(3):1161–72. doi:10.1084/jem.183.3.1161
66. Zitvogel L, Regnault A, Lozier A, Wolfers J, Flament C, Tenza D, et al. Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nat Med* (1998) 4(5):594–600. doi:10.1038/nm0598-594
67. Raposo G, Tenza D, Mecheri S, Peronet R, Bonnerot C, Desaymard C. Accumulation of major histocompatibility complex class II molecules in mast cell secretory granules and their release upon degranulation. *Mol Biol Cell* (1997) 8(12):2631–45. doi:10.1091/mbc.8.12.2631
68. Peters PJ, Geuze HJ, Van der Donk HA, Slot JW, Griffith JM, Stam NJ, et al. Molecules relevant for T cell-target cell interaction are present in cytolytic granules of human T lymphocytes. *Eur J Immunol* (1989) 19(8):1469–75. doi:10.1002/eji.1830190819
69. Heijnen HF, Schiel AE, Fijnheer R, Geuze HJ, Sixma JJ. Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and alpha-granules. *Blood* (1999) 94(11):3791–9.
70. Fevrier B, Vilette D, Archer F, Loew D, Faigle W, Vidal M, et al. Cells release prions in association with exosomes. *Proc Natl Acad Sci U S A* (2004) 101(26):9683–8. doi:10.1073/pnas.0308413101
71. Wolfers J, Lozier A, Raposo G, Regnault A, Thery C, Masurier C, et al. Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. *Nat Med* (2001) 7(3):297–303. doi:10.1038/85438
72. Sullivan R, Saez F, Girouard J, Frenette G. Role of exosomes in sperm maturation during the transit along the male reproductive tract. *Blood Cells Mol Dis* (2005) 35(1):1–10. doi:10.1016/j.bcmd.2005.03.005
73. Pisitkun T, Shen RF, Knepper MA. Identification and proteomic profiling of exosomes in human urine. *Proc Natl Acad Sci U S A* (2004) 101(36):13368–73. doi:10.1073/pnas.0403453101
74. Caby MP, Lankar D, Vincendeau-Scherrer C, Raposo G, Bonnerot C. Exosomal-like vesicles are present in human blood plasma. *Int Immunol* (2005) 17(7):879–87. doi:10.1093/intimm/dxh267
75. Street JM, Barran PE, Mackay CL, Weidt S, Balmforth C, Walsh TS, et al. Identification and proteomic profiling of exosomes in human cerebrospinal fluid. *J Transl Med* (2012) 10:5. doi:10.1186/1479-5876-10-5
76. Colombo M, Raposo G, Thery C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu Rev Cell Dev Biol* (2014) 30:255–89. doi:10.1146/annurev-cellbio-101512-122326
77. Kim HS, Choi DY, Yun SJ, Choi SM, Kang JW, Jung JW, et al. Proteomic analysis of microvesicles derived from human mesenchymal stem cells. *J Proteome Res* (2012) 11(2):839–49. doi:10.1021/pr200682z
78. Tan SS, Yin Y, Lee T, Lai RC, Yeo RW, Zhang B, et al. Therapeutic MSC exosomes are derived from lipid raft microdomains in the plasma membrane. *J Extracell Vesicles* (2013) 2. doi:10.3402/jev.v2i0.22614
79. Baglio SR, Rooijers K, Koppers-Lalic D, Verweij FJ, Perez Lanzon M, Zini N, et al. Human bone marrow- and adipose-mesenchymal stem cells secrete exosomes enriched in distinctive miRNA and tRNA species. *Stem Cell Res Ther* (2015) 6:127. doi:10.1186/s13287-015-0116-z
80. Chen TS, Lai RC, Lee MM, Choo AB, Lee CN, Lim SK. Mesenchymal stem cell secretes microparticles enriched in pre-microRNAs. *Nucleic Acids Res* (2010) 38(1):215–24. doi:10.1093/nar/gkp857
81. Tomasoni S, Longaretti L, Rota C, Morigi M, Conti S, Gotti E, et al. Transfer of growth factor receptor mRNA via exosomes unravels the regenerative effect of mesenchymal stem cells. *Stem Cells Dev* (2013) 22(5):772–80. doi:10.1089/scd.2012.0266
82. Phinney DG, Di Giuseppe M, Njah J, Sala E, Shiva S, St Croix CM, et al. Mesenchymal stem cells use extracellular vesicles to outsource mitophagy and shuttle microRNAs. *Nat Commun* (2015) 6:8472. doi:10.1038/ncomms9472
83. De Luca L, Trino S, Laurenzana I, Simeon V, Calice G, Raimondo S, et al. MiRNAs and piRNAs from bone marrow mesenchymal stem cell extracellular vesicles induce cell survival and inhibit cell differentiation of cord blood hematopoietic stem cells: a new insight in transplantation. *Oncotarget* (2016) 7(6):6676–92. doi:10.18632/oncotarget.6791
84. Ti D, Hao H, Tong C, Liu J, Dong L, Zheng J, et al. LPS-preconditioned mesenchymal stromal cells modify macrophage polarization for resolution of chronic inflammation via exosome-shuttled let-7b. *J Transl Med* (2015) 13(1):308. doi:10.1186/s12967-015-0642-6
85. Nakamura Y, Miyaki S, Ishitobi H, Matsuyama S, Nakasa T, Kamei N, et al. Mesenchymal-stem-cell-derived exosomes accelerate skeletal muscle regeneration. *FEBS Lett* (2015) 589(11):1257–65. doi:10.1016/j.febslet.2015.03.031
86. Lai RC, Tan SS, Yeo RW, Choo AB, Reiner AT, Su Y, et al. MSC secretes at least 3 EV types each with a unique permutation of membrane lipid, protein and RNA. *J Extracell Vesicles* (2016) 5:29828. doi:10.3402/jev.v5.29828
87. Birge RB, Boeltz S, Kumar S, Carlson J, Wanderley J, Calianese D, et al. Phosphatidylserine is a global immunosuppressive signal in efferocytosis, infectious disease, and cancer. *Cell Death Differ* (2016) 23(6):962–78. doi:10.1038/cdd.2016.11
88. Wei X, Liu C, Wang H, Wang L, Xiao F, Guo Z, et al. Surface phosphatidylserine is responsible for the internalization on microvesicles derived from hypoxia-induced human bone marrow mesenchymal stem cells into human endothelial cells. *PLoS One* (2016) 11(1):e0147360. doi:10.1371/journal.pone.0147360
89. Thery C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol* (2006) Chapter 3:Unit 322. doi:10.1002/0471143030.cb0322s30
90. Momen-Heravi F, Balaj L, Alian S, Mantel PY, Halleck AE, Trachtenberg AJ, et al. Current methods for the isolation of extracellular vesicles. *Biol Chem* (2013) 394(10):1253–62. doi:10.1515/hsz-2013-0141
91. Eggenhofer E, Benseler V, Kroemer A, Popp FC, Geissler EK, Schlitt HJ, et al. Mesenchymal stem cells are short-lived and do not migrate beyond the lungs after intravenous infusion. *Front Immunol* (2012) 3:297. doi:10.3389/fimmu.2012.00297
92. Gatti S, Bruno S, Deregibus MC, Sordi A, Cantaluppi V, Tetta C, et al. Microvesicles derived from human adult mesenchymal stem cells protect against ischaemia-reperfusion-induced acute and chronic kidney injury. *Nephrol Dial Transplant* (2011) 26(5):1474–83. doi:10.1093/ndt/gfr015
93. Lai RC, Arslan F, Lee MM, Sze NS, Choo A, Chen TS, et al. Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. *Stem Cell Res* (2010) 4(3):214–22. doi:10.1016/j.scr.2009.12.003
94. Zhang Y, Chopp M, Meng Y, Katakowski M, Xin H, Mahmood A, et al. Effect of exosomes derived from multipluripotent mesenchymal stromal cells on functional recovery and neurovascular plasticity in rats after traumatic brain injury. *J Neurosurg* (2015) 122(4):856–67. doi:10.3171/2014.11.JNS14770
95. Budoni M, Fierabracci A, Luciano R, Petrini S, Di Ciommo V, Muraca M. The immunosuppressive effect of mesenchymal stromal cells on B lymphocytes is mediated by membrane vesicles. *Cell Transplant* (2013) 22(2):369–79. doi:10.3727/096368911X582769
96. Mokarizadeh A, Delirezh N, Morshedi A, Mosayebi G, Farshid AA, Mardani K. Microvesicles derived from mesenchymal stem cells: potent organelles for induction of tolerogenic signaling. *Immunol Lett* (2012) 147(1–2):47–54. doi:10.1016/j.imlet.2012.06.001
97. Blazquez R, Sanchez-Margallo FM, de la Rosa O, Dalemans W, Alvarez V, Tarazona R, et al. Immunomodulatory potential of human adipose mesenchymal stem cells derived exosomes on in vitro stimulated T cells. *Front Immunol* (2014) 5:556. doi:10.3389/fimmu.2014.00556
98. Favaro E, Carpanetto A, Lamorte S, Fusco A, Caorsi C, Deregibus MC, et al. Human mesenchymal stem cell-derived microvesicles modulate T cell response to islet antigen glutamic acid decarboxylase in patients with type 1 diabetes. *Diabetologia* (2014) 57(8):1664–73. doi:10.1007/s00125-014-3262-4
99. Liu M, Wang J, Liu M, Hu X, Xu J. [Study of immunomodulatory function of exosomes derived from human umbilical cord mesenchymal stem cells]. *Zhonghua Yi Xue Za Zhi* (2015) 95(32):2630–3. doi:10.3760/cma.j.issn.0376-2491.2015.32.014
100. Zhang B, Yin Y, Lai RC, Tan SS, Choo AB, Lim SK. Mesenchymal stem cells secrete immunologically active exosomes. *Stem Cells Dev* (2014) 23(11):1233–44. doi:10.1089/scd.2013.0479

101. Favaro E, Carpanetto A, Caorsi C, Giovarelli M, Angelini C, Cavallo-Perin P, et al. Human mesenchymal stem cells and derived extracellular vesicles induce regulatory dendritic cells in type 1 diabetic patients. *Diabetologia* (2016) 59(2):325–33. doi:10.1007/s00125-015-3808-0
102. Amarnath S, Foley JE, Farthing DE, Gress RE, Laurence A, Eckhaus MA, et al. Bone marrow-derived mesenchymal stromal cells harness purinergenic signaling to tolerize human Th1 cells in vivo. *Stem Cells* (2015) 33(4):1200–12. doi:10.1002/stem.1934
103. Kordelas L, Rebmann V, Ludwig AK, Radtke S, Ruesing J, Doeppner TR, et al. MSC-derived exosomes: a novel tool to treat therapy-refractory graft-versus-host disease. *Leukemia* (2014) 28(4):970–3. doi:10.1038/leu.2014.41
104. Galipeau J. Concerns arising from MSC retrieval from cryostorage and effect on immune suppressive function and pharmaceutical usage in clinical trials. *ISBT Sci Ser* (2013) 8(1):100–1. doi:10.1111/voxs.12022
105. Francois M, Copland IB, Yuan S, Romieu-Mourez R, Waller EK, Galipeau J. Cryopreserved mesenchymal stromal cells display impaired immunosuppressive properties as a result of heat-shock response and impaired interferon-gamma licensing. *Cytotherapy* (2012) 14(2):147–52. doi:10.3109/14653249.2011.623691
106. Moll G, Alm JJ, Davies LC, von Bahr L, Heldring N, Stenbeck-Funke L, et al. Do cryopreserved mesenchymal stromal cells display impaired immunomodulatory and therapeutic properties? *Stem Cells* (2014) 32(9):2430–42. doi:10.1002/stem.1729
107. Gramlich OW, Burand AJ, Brown AJ, Deutsch RJ, Kuehn MH, Ankrum JA. Cryopreserved mesenchymal stromal cells maintain potency in a retinal ischemia/reperfusion injury model: toward an off-the-shelf therapy. *Sci Rep* (2016) 6:26463. doi:10.1038/srep26463
108. Bennet W, Groth CG, Larsson R, Nilsson B, Korsgren O. Isolated human islets trigger an instant blood mediated inflammatory reaction: implications for intraportal islet transplantation as a treatment for patients with type 1 diabetes. *Ups J Med Sci* (2000) 105(2):125–33. doi:10.1517/03009734000000059
109. Moll G, Le Blanc K. Engineering more efficient multipotent mesenchymal stromal (stem) cells for systemic delivery as cellular therapy. *ISBT Sci Ser* (2015) 10(S1):357–65. doi:10.1111/voxs.12133
110. de Witte SFH, Franquesa M, Baan CC, Hoogduijn MJ. Toward development of iMesenchymal stem cells for immunomodulatory therapy. *Front Immunol* (2015) 6:648. doi:10.3389/fimmu.2015.00648
111. Liang X, Ding Y, Zhang Y, Tse HF, Lian Q. Paracrine mechanisms of mesenchymal stem cell-based therapy: current status and perspectives. *Cell Transplant* (2014) 23(9):1045–59. doi:10.3727/096368913X667709
112. Webber J, Clayton A. How pure are your vesicles? *J Extracell Vesicles* (2013) 2:19861. doi:10.3402/jev.v2i0.19861
113. Castro-Manrreza ME, Montesinos JJ. Immunoregulation by mesenchymal stem cells: biological aspects and clinical applications. *J Immunol Res* (2015) 2015:20. doi:10.1155/2015/394917
114. Siegel G, Kluba T, Hermanutz-Klein U, Bieback K, Northoff H, Schäfer R. Phenotype, donor age and gender affect function of human bone marrow-derived mesenchymal stromal cells. *BMC Med* (2013) 11:146. doi:10.1186/1741-7015-11-146
115. Conforti A, Scarsella M, Starc N, Giorda E, Biagini S, Proia A, et al. Microvescicles derived from mesenchymal stromal cells are not as effective as their cellular counterpart in the ability to modulate immune responses in vitro. *Stem Cells Dev* (2014) 23(21):2591–9. doi:10.1089/scd.2014.0091
116. Spring L, Li S, Soiffer RJ, Antin JH, Alyea EP III, Glotzbecker B. Risk factors for readmission after allogeneic hematopoietic stem cell transplantation and impact on overall survival. *Biol Blood Marrow Transplant* (2015) 21(3):509–16. doi:10.1016/j.bbmt.2014.11.682
117. Kedia S, Acharya PS, Mohammad F, Nguyen H, Asti D, Mehta S, et al. Infectious complications of hematopoietic stem cell transplantation. *J Stem Cell Res Ther* (2013) S3:002. doi:10.4172/2157-7633.S3-002
118. Junghanss C, Marr KA, Carter RA, Sandmaier BM, Maris MB, Maloney DG, et al. Incidence and outcome of bacterial and fungal infections following nonmyeloablative compared with myeloablative allogeneic hematopoietic stem cell transplantation: a matched control study. *Biol Blood Marrow Transplant* (2002) 8(9):512–20. doi:10.1053/bbmt.2002.v8.pm12374456
119. Marek A, Stern M, Chalandon Y, Ansari M, Ozsahin H, Gungor T, et al. The impact of T-cell depletion techniques on the outcome after haploidentical hematopoietic SCT. *Bone Marrow Transplant* (2014) 49(1):55–61. doi:10.1038/bmt.2013.132
120. Walls T, Shankar AG, Shingadia D. Adenovirus: an increasingly important pathogen in paediatric bone marrow transplant patients. *Lancet Infect Dis* (2003) 3(2):79–86. doi:10.1016/S1473-3099(03)00515-2
121. Papadopoulou A, Gerdemann U, Katari UL, Tzannou I, Liu H, Martinez C, et al. Activity of broad-spectrum T-cells as treatment for AdV, EBV, CMV, BKV and HHV6 infections after HSCT. *Sci Transl Med* (2014) 6(242):ra83–83. doi:10.1126/scitranslmed.3008825
122. La Rosa AM, Champlin RE, Mirza N, Gajewski J, Giralt S, Rolston KV, et al. Adenovirus infections in adult recipients of blood and marrow transplants. *Clin Infect Dis* (2001) 32(6):871–6. doi:10.1086/319352
123. Erice A. Resistance of human cytomegalovirus to antiviral drugs. *Clin Microbiol Rev* (1999) 12(2):286–97.
124. Winston DJ, Ho WG, Bartoni K, Du Mond C, Ebeling DF, Buhles WC, et al. Ganciclovir prophylaxis of cytomegalovirus infection and disease in allogeneic bone marrow transplant recipients: results of a placebo-controlled, double-blind trial. *Ann Intern Med* (1993) 118(3):179–84. doi:10.7326/0003-4819-118-3-199302010-00004
125. Salzberger B, Bowden RA, Hackman RC, Davis C, Boeckh M. Neutropenia in allogeneic marrow transplant recipients receiving ganciclovir for prevention of cytomegalovirus disease: risk factors and outcome. *Blood* (1997) 90(6):2502–8.
126. Staras SAS, Dollard SC, Radford KW, Flanders WD, Pass RF, Cannon MJ. Seroprevalence of cytomegalovirus infection in the United States, 1988-1994. *Clin Infect Dis* (2006) 43(9):1143–51. doi:10.1086/508173
127. Crough T, Khanna R. Immunobiology of human cytomegalovirus: from bench to bedside. *Clin Microbiol Rev* (2009) 22(1):76–98. doi:10.1128/CMR.00034-08
128. Emery V. Investigation of CMV disease in immunocompromised patients. *J Clin Pathol* (2001) 54(2):84–8. doi:10.1136/jcp.54.2.84
129. Cantoni N, Hirsch HH, Khanna N, Gerull S, Buser A, Bucher C, et al. Evidence for a bidirectional relationship between cytomegalovirus replication and acute graft-versus-host disease. *Biol Blood Marrow Transplant* (2010) 16(9):1309–14. doi:10.1016/j.bbmt.2010.03.020
130. Dunmire SK, Hogquist KA, Balfour HH. Infectious mononucleosis. *Curr Top Microbiol Immunol* (2015) 390:211–40. doi:10.1007/978-3-319-22822-8_9
131. Chiereghin A, Prete A, Belotti T, Gibertoni D, Piccirilli G, Gabrielli L, et al. Prospective Epstein-Barr virus-related post-transplant lymphoproliferative disorder prevention program in pediatric allogeneic hematopoietic stem cell transplant: virological monitoring and first-line treatment. *Transpl Infect Dis* (2016) 18(1):44–54. doi:10.1111/tid.12485
132. Petrara MR, Giunco S, Serraino D, Dolcetti R, De Rossi A. Post-transplant lymphoproliferative disorders: from epidemiology to pathogenesis-driven treatment. *Cancer Lett* (2015) 369(1):37–44. doi:10.1016/j.canlet.2015.08.007
133. Flomenberg P. Adenovirus infections. *Medicine* (2005) 33(5):128–30. doi:10.1383/medc.33.5.128.64961
134. Kontoyiannis DP, Marr KA, Park BJ, Alexander BD, Anaissie EJ, Walsh TJ, et al. Prospective surveillance for invasive fungal infections in hematopoietic stem cell transplant recipients, 2001-2006: overview of the transplant-associated infection surveillance network (TRANSNET) database. *Clin Infect Dis* (2010) 50:9. doi:10.1086/651263
135. Deo SS, Gottlieb DJ. Adoptive T-cell therapy for fungal infections in haematology patients. *Clin Transl Immunol* (2015) 4(8):e40. doi:10.1038/cti.2015.16
136. Mackinnon S, Thomson K, Verfuerth S, Peggs K, Lowdell M. Adoptive cellular therapy for cytomegalovirus infection following allogeneic stem cell transplantation using virus-specific T cells. *Blood Cells Mol Dis* (2008) 40(1):63–7. doi:10.1016/j.bcmd.2007.07.003
137. Peggs KS, Thomson K, Samuel E, Dyer G, Armoogum J, Chakraverty R, et al. Directly selected cytomegalovirus-reactive donor t cells confer rapid and safe systemic reconstitution of virus-specific immunity following stem cell transplantation. *Clin Infect Dis* (2011) 52(1):49–57. doi:10.1093/cid/ciq042
138. Feucht J, Opherk K, Lang P, Kayser S, Hartl L, Bethge W, et al. Adoptive T-cell therapy with hexon-specific Th1 cells as a treatment of refractory adenovirus infection after HSCT. *Blood* (2015) 125:1986–94. doi:10.1182/blood-2014-06-573725

139. Mackinnon S, Papadopoulos EB, Carabasi MH, Reich L, Collins NH, Boulad F, et al. Adoptive immunotherapy evaluating escalating doses of donor leukocytes for relapse of chronic myeloid leukemia after bone marrow transplantation: separation of graft-versus-leukemia responses from graft-versus-host disease. *Blood* (1995) 86(4):1261–8.
140. Dvorak CC, Gilman AL, Horn B, Jaroscak J, Dunn EA, Baxter-Lowe LA, et al. Clinical and immunologic outcomes following haplocompatible donor lymphocyte infusions. *Bone Marrow Transplant* (2009) 44(12):805–12. doi:10.1038/bmt.2009.87
141. Papadopoulos EB, Ladanyi M, Emanuel D, Mackinnon S, Boulad F, Carabasi MH, et al. Infusions of donor leukocytes to treat Epstein-Barr virus-associated lymphoproliferative disorders after allogeneic bone marrow transplantation. *N Engl J Med* (1994) 330(17):1185–91. doi:10.1056/NEJM199404283301703
142. Amrolia P, Rao K, Slater O, Ramsay A, Veys P, Webb D. Fatal graft-versus-host disease following HLA-mismatched donor lymphocyte infusion. *Bone Marrow Transplant* (2001) 28:3. doi:10.1038/sj.bmt.1703195
143. Riddell SR, Watanabe KS, Goodrich JM, Li CR, Agha ME, Greenberg PD. Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones. *Science* (1992) 257(5067):238–41. doi:10.1126/science.1352912
144. Rooney CM, Smith CA, Ng CY, Loftin S, Li C, Krance RA, et al. Use of gene-modified virus-specific T lymphocytes to control Epstein-Barr-virus-related lymphoproliferation. *Lancet* (1995) 345(8941):9–13. doi:10.1016/S0140-6736(95)91150-2
145. Gerdemann U, Keirnan JM, Katari UL, Yanagisawa R, Christin AS, Huye LE, et al. Rapidly generated multivirus-specific cytotoxic T lymphocytes for the prophylaxis and treatment of viral infections. *Mol Ther* (2012) 20(8):1622–32. doi:10.1038/mt.2012.130
146. Pourgheysari B, Piper KP, McLarnon A, Arrazi J, Bruton R, Clark F, et al. Early reconstitution of effector memory CD4+ CMV-specific T cells protects against CMV reactivation following allogeneic SCT. *Bone Marrow Transplant* (2008) 43(11):853–61. doi:10.1038/bmt.2008.403
147. Kumaresan P, Figliola M, Moyes JS, Huls MH, Tewari P, Shpall EJ, et al. Automated cell enrichment of cytomegalovirus-specific T cells for clinical applications using the cytokine-capture system. *J Vis Exp* (2015) 104. doi:10.3791/52808
148. Walter EA, Greenberg PD, Gilbert MJ, Finch RJ, Watanabe KS, Thomas ED, et al. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. *N Engl J Med* (1995) 333(16):1038–44. doi:10.1056/NEJM199510193331603
149. Einsele H, Roosnek E, Rufer N, Sinzger C, Riegler S, Löffler J, et al. Infusion of cytomegalovirus (CMV)-specific T cells for the treatment of CMV infection not responding to antiviral chemotherapy. *Blood* (2002) 99:3916–22. doi:10.1182/blood.V99.11.3916
150. Feuchtinger T, Matthes-Martin S, Richard C, Lion T, Fuhrer M, Hamprecht K, et al. Safe adoptive transfer of virus-specific T-cell immunity for the treatment of systemic adenovirus infection after allogeneic stem cell transplantation. *Br J Haematol* (2006) 134(1):64–76. doi:10.1111/j.1365-2141.2006.06108.x
151. Icheva V, Kayser S, Wolff D, Tuve S, Kyzirakos C, Bethge W, et al. Adoptive transfer of Epstein-Barr virus (EBV) nuclear antigen 1-specific t cells as treatment for EBV reactivation and lymphoproliferative disorders after allogeneic stem-cell transplantation. *J Clin Oncol* (2013) 31(1):39–48. doi:10.1200/JCO.2011.39.8495
152. Moosmann A, Bigalke I, Tischer J, Schirrmann L, Kasten J, Tippmer S, et al. Effective and long-term control of EBV PTLD after transfer of peptide-selected T cells. *Blood* (2010) 115(14):2960–70. doi:10.1182/blood-2009-08-236356
153. Qasim W, Gilmour K, Zhan H, Derniame S, McNicol AM, Ip W, et al. Interferon-gamma capture T cell therapy for persistent adenoviraemia following allogeneic haematopoietic stem cell transplantation. *Br J Haematol* (2013) 161(3):449–52. doi:10.1111/bjh.12251
154. Crompton JG, Sukumar M, Restifo NP. Uncoupling T-cell expansion from effector differentiation in cell-based immunotherapy. *Immunol Rev* (2014) 257(1):264–76. doi:10.1111/imr.12135
155. Schmitt A, Tonn T, Busch DH, Grigoleit GU, Einsele H, Odendahl M, et al. Adoptive transfer and selective reconstitution of streptamer-selected cytomegalovirus-specific CD8+ T cells leads to virus clearance in patients after allogeneic peripheral blood stem cell transplantation. *Transfusion* (2011) 51(3):591–9. doi:10.1111/j.1537-2995.2010.02940.x
156. Shedlock Devon J, Shen H. Requirement for CD4 T cell help in generating functional CD8 T cell memory. *Science* (2003) 300(5617):2. doi:10.1126/science.1082305
157. Sun JC, Williams MA, Bevan MJ. CD4(+) T cells are required for the maintenance, not programming, of memory CD8(+) T cells after acute infection. *Nat Immunol* (2004) 5(9):927–33. doi:10.1038/ni1105
158. Gamadia LERR, van Lier RA, ten Berge IJ. Properties of CD4(+) T cells in human cytomegalovirus infection. *Hum Immunol* (2004) 65(5):6. doi:10.1016/j.humimm.2004.02.020
159. Gerdemann U, Katari UL, Papadopoulou A, Keirnan JM, Craddock JA, Liu H, et al. Safety and clinical efficacy of rapidly-generated trivirus-directed T cells as treatment for adenovirus, EBV, and CMV infections after allogeneic hematopoietic stem cell transplant. *Mol Ther* (2013) 21(11):2113–21. doi:10.1038/mt.2013.151
160. Landais E, Morice A, Long HM, Haigh TA, Charreau B, Bonneville M, et al. EBV-specific CD4+ T cell clones exhibit vigorous allogeneic responses. *J Immunol* (2006) 177(3):1427–33. doi:10.4049/jimmunol.177.3.1427
161. Martins SLR, St. John LS, Champlin RE, Wieder ED, McMannis J, Molldrem JJ, et al. Functional assessment and specific depletion of alloreactive human T cells using flow cytometry. *Blood* (2004) 104:3429–36. doi:10.1182/blood-2004-05-1918
162. Burrows SR, Khanna R, Burrows JM, Moss DJ. An alloresponse in humans is dominated by cytotoxic T lymphocytes (CTL) cross-reactive with a single Epstein-Barr virus CTL epitope: implications for graft-versus-host disease. *J Exp Med* (1994) 179(4):1155–61. doi:10.1084/jem.179.4.1155
163. Melenhorst JJ, Leen AM, Bollard CM, Quigley MF, Price DA, Rooney CM, et al. Allogeneic virus-specific T cells with HLA alloreactivity do not produce GVHD in human subjects. *Blood* (2010) 116(22):4700–2. doi:10.1182/blood-2010-06-289991
164. Amir AL, D'Orsogna LJA, Roelen DL, van Loenen MM, Hagedoorn RS, de Boer R, et al. Allo-HLA reactivity of virus-specific memory T cells is common. *Blood* (2010) 115:3146–57. doi:10.1182/blood-2009-07-234906
165. Morice A, Charreau B, Neveu B, Brouard S, Soulillou J-P, Bonneville M, et al. Cross-reactivity of herpesvirus-specific CD8 T cell lines toward allogeneic class I MHC molecules. *PLoS One* (2010) 5(8):e12120. doi:10.1371/journal.pone.0012120
166. D'Orsogna LJA, Roelen DL, Doxiadis IIN, Claas FHJ. Alloreactivity from human viral specific memory T-cells. *Transpl Immunol* (2010) 23(4):149–55. doi:10.1016/j.trim.2010.06.008
167. Aissi-Rothe L, Decot V, Venard V, Jeulin H, Salmon A, Clement L, et al. Rapid generation of full clinical-grade human antiadenovirus cytotoxic T cells for adoptive immunotherapy. *J Immunother* (2010) 33(4):414–24. doi:10.1097/CJI.0b013e3181cc263b
168. Rauser G, Einsele H, Sinzger C, Wernet D, Kuntz G, Assenmacher M, et al. Rapid generation of combined CMV-specific CD4+ and CD8+ T-cell lines for adoptive transfer into recipients of allogeneic stem cell transplants. *Blood* (2004) 103(9):3565–72. doi:10.1182/blood-2003-09-3056
169. Feuchtinger T, Lang P, Hamprecht K, Schumm M, Greil J, Jahn G, et al. Isolation and expansion of human adenovirus-specific CD4+ and CD8+ T cells according to IFN-g secretion for adjuvant immunotherapy. *Exp Hematol* (2004) 32(3):282–9. doi:10.1016/j.exphem.2003.12.009
170. Qesari M, Richter A, Ogonek J, Mischak-Weissinger E, Wang X-n, Dickinson AM. Cytomegalovirus-specific T cells isolated by IFN-[gamma] secretion assay do not induce significant graft-versus-host reactions in vitro. *Transplantation* (2016) 100(11):2352–61. doi:10.1097/tp.0000000000001219
171. Feuchtinger T, Opherk K, Bethge WA, Topp MS, Schuster FR, Weissinger EM, et al. Adoptive transfer of pp65-specific T cells for the treatment of chemorefractory cytomegalovirus disease or reactivation after haploidentical and matched unrelated stem cell transplantation. *Blood* (2010) 116(20):4360–7. doi:10.1182/blood-2010-01-262089
172. Knabel M, Franz TJ, Schiemann M, Wulf A, Villmow B, Schmidt B, et al. Reversible MHC multimer staining for functional isolation of T-cell populations and effective adoptive transfer. *Nat Med* (2002) 8(6):631–7. doi:10.1038/nm0602-631
173. Neudorfer J, Schmidt B, Huster KM, Anderl F, Schiemann M, Holzapfel G, et al. Reversible HLA multimers (Streptamers) for the isolation of human

cytotoxic T lymphocytes functionally active against tumor- and virus-derived antigens. *J Immunol Methods* (2007) 320(1–2):119–31. doi:10.1016/j.jim.2007.01.001

174. Odendahl M, Grigoleit GU, Bonig H, Neuenhahn M, Albrecht J, Anderl F, et al. Clinical-scale isolation of 'minimally manipulated' cytomegalovirus-specific donor lymphocytes for the treatment of refractory cytomegalovirus disease. *Cytotherapy* (2014) 16(9):1245–56. doi:10.1016/j.jcyt.2014.05.023
175. Freimüller C, Stemberger J, Artwohl M, Germeroth L, Witt V, Fischer G, et al. Selection of adenovirus-specific and Epstein-Barr virus-specific T cells with major histocompatibility class I streptamers under good manufacturing practice (GMP)-compliant conditions. *Cytotherapy* (2015) 17(7):989–1007. doi:10.1016/j.jcyt.2015.03.613
176. Leen AM, Myers GD, Sili U, Huls MH, Weiss H, Leung KS, et al. Monoculture-derived T lymphocytes specific for multiple viruses expand and produce clinically relevant effects in immunocompromised individuals. *Nat Med* (2006) 12(10):1160–6. doi:10.1038/nm1475
177. Leen A, Gee AP, Leung K, Martinez C, Krance RA, Liu H, et al. Multi-virus-specific T-cell therapy for patients after hematopoietic stem cell and cord blood transplantation. *Blood* (2013) 122(21):140.
178. Leen AM, Christin A, Myers GD, Liu H, Cruz CR, Hanley PJ, et al. Cytotoxic T lymphocyte therapy with donor T cells prevents and treats adenovirus and Epstein-Barr virus infections after haploidentical and matched unrelated stem cell transplantation. *Blood* (2009) 114(19):4283–92. doi:10.1182/blood-2009-07-232454
179. Khanna N, Stuehler C, Conrad B, Lurati S, Krappmann S, Einsele H, et al. Generation of a multipathogen-specific T-cell product for adoptive immunotherapy based on activation-dependent expression of CD154. *Blood* (2011) 118(4):1121–31. doi:10.1182/blood-2010-12-322610
180. Barrett AJ, Bollard CM. The coming of age of adoptive T-cell therapy for viral infection after stem cell transplantation. *Ann Transl Med* (2015) 3(5):62. doi:10.3978/j.issn.2305-5839.2015.01.18
181. Schub A, Schuster IG, Hammerschmidt W, Moosmann A. CMV-specific TCR-transgenic T cells for immunotherapy. *J Immunol* (2009) 183(10):6819–30. doi:10.4049/jimmunol.0902233
182. Grigoleit GU, Kapp M, Hebart H, Fick K, Beck R, Jahn G, et al. Dendritic cell vaccination in allogeneic stem cell recipients: induction of human cytomegalovirus (HCMV)-specific cytotoxic T lymphocyte responses even in patients receiving a transplant from an HCMV-seronegative donor. *J Infect Dis* (2007) 196(5):699–704. doi:10.1086/520538
183. Haque T, Taylor C, Wilkie GM, Murad P, Amlot PL, Beath S, et al. Complete regression of posttransplant lymphoproliferative disease using partially HLA-matched Epstein Barr virus-specific cytotoxic T cells. *Transplantation* (2001) 72(8):1399–402. doi:10.1097/00007890-200110270-00012
184. Barker JN, Doubrovina E, Sauter C, Jaroscak JJ, Perales MA, Doubrovin M, et al. Successful treatment of EBV-associated posttransplantation lymphoma after cord blood transplantation using third-party EBV-specific cytotoxic T lymphocytes. *Blood* (2010) 116(23):5045–9. doi:10.1182/blood-2010-04-281873
185. O'Reilly RJ, Prockop S, Hasan AN, Koehne G, Doubrovina E. Virus-specific T-cell banks for 'off the shelf' adoptive therapy of refractory infections. *Bone Marrow Transplant* (2016) 51(9):1163–72. doi:10.1038/bmt.2016.17
186. Wy Ip W, Qasim W. Management of adenovirus in children after allogeneic hematopoietic stem cell transplantation. *Adv Hematol* (2013) 2013:176418. doi:10.1155/2013/176418
187. Ma CKK, Blyth E, Clancy L, Simms R, Burgess J, Brown R, et al. Addition of varicella zoster virus-specific T cells to cytomegalovirus, Epstein-Barr virus and adenovirus tri-specific T cells as adoptive immunotherapy in patients undergoing allogeneic hematopoietic stem cell transplantation. *Cytotherapy* (2015) 17(10):1406–20. doi:10.1016/j.jcyt.2015.07.005
188. Bhojwani D, Pui CH. Relapsed childhood acute lymphoblastic leukaemia. *Lancet Oncol* (2013) 14(6):e205–17. doi:10.1016/S1470-2045(12)70580-6
189. Dreger P, Schetelig J, Andersen N, Corradini P, van Gelder M, Gribben J, et al. Managing high-risk CLL during transition to a new treatment era: stem cell transplantation or novel agents? *Blood* (2014) 124(26):3841–9. doi:10.1182/blood-2014-07-586826
190. Brown JR. The treatment of relapsed refractory chronic lymphocytic leukemia. *Hematology Am Soc Hematol Educ Program* (2011) 2011:110–8. doi:10.1182/asheducation-2011.1.110
191. Loren AW, Porter DL. Donor leukocyte infusions for the treatment of relapsed acute leukemia after allogeneic stem cell transplantation. *Bone Marrow Transplant* (2008) 41(5):483–93. doi:10.1038/sj.bmt.1705898
192. Horowitz MM, Gale RP, Sondel PM, Goldman JM, Kersey J, Kolb HJ, et al. Graft-versus-leukemia reactions after bone marrow transplantation. *Blood* (1990) 75(3):555–62.
193. Fujiwara H. Adoptive immunotherapy for hematological malignancies using T cells gene-modified to express tumor antigen-specific receptors. *Pharmaceuticals* (2014) 7(12):1049. doi:10.3390/ph7121049
194. Maude SL, Frey N, Shaw PA, Aplenc R, Barrett DM, Bunin NJ, et al. Chimeric antigen receptor T cells for sustained remissions in leukemia. *N Engl J Med* (2014) 371(16):1507–17. doi:10.1056/NEJMoa1407222
195. Gill S, June CH. Going viral: chimeric antigen receptor T-cell therapy for hematological malignancies. *Immunol Rev* (2015) 263(1):68–89. doi:10.1111/imr.12243
196. Sadelain M. CAR therapy: the CD19 paradigm. *J Clin Invest* (2015) 125(9):3392–400. doi:10.1172/JCI80010
197. Maude SL, Teachey DT, Porter DL, Grupp SA. CD19-targeted chimeric antigen receptor T-cell therapy for acute lymphoblastic leukemia. *Blood* (2015) 125(26):4017–23. doi:10.1182/blood-2014-12-580068
198. Kowolik CM, Topp MS, Gonzalez S, Pfeiffer T, Olivares S, Gonzalez N, et al. CD28 costimulation provided through a CD19-specific chimeric antigen receptor enhances in vivo persistence and antitumor efficacy of adoptively transferred T cells. *Cancer Res* (2006) 66(22):10995–1004. doi:10.1158/0008-5472.CAN-06-0160
199. Carpenito C, Milone MC, Hassan R, Simonet JC, Lakhal M, Suhoski MM, et al. Control of large, established tumor xenografts with genetically retargeted human T cells containing CD28 and CD137 domains. *Proc Natl Acad Sci U S A* (2009) 106(9):3360–5. doi:10.1073/pnas.0813101106
200. Maher J, Brentjens RJ, Gunset G, Riviere I, Sadelain M. Human T-lymphocyte cytotoxicity and proliferation directed by a single chimeric TCR[zeta]/CD28 receptor. *Nat Biotech* (2002) 20(1):70–5. doi:10.1038/nbt0102-70
201. Savoldo B, Ramos CA, Liu E, Mims MP, Keating MJ, Carrum G, et al. CD28 costimulation improves expansion and persistence of chimeric antigen receptor-modified T cells in lymphoma patients. *J Clin Invest* (2011) 121(5):1822–6. doi:10.1172/JCI46110
202. Grupp SA, Kalos M, Barrett D, Aplenc R, Porter DL, Rheingold SR, et al. Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. *N Engl J Med* (2013) 368(16):1509–18. doi:10.1056/NEJMoa1215134
203. Chmielewski M, Kopecky C, Hombach AA, Abken H. IL-12 release by engineered T cells expressing chimeric antigen receptors can effectively muster an antigen-independent macrophage response on tumor cells that have shut down tumor antigen expression. *Cancer Res* (2011) 71(17):5697–706. doi:10.1158/0008-5472.CAN-11-0103
204. Pegram HJ, Lee JC, Hayman EG, Imperato GH, Tedder TF, Sadelain M, et al. Tumor-targeted T cells modified to secrete IL-12 eradicate systemic tumors without need for prior conditioning. *Blood* (2012) 119(18):4133–41. doi:10.1182/blood-2011-12-400044
205. Curran KJ, Seinstra BA, Nikhamin Y, Yeh R, Usachenko Y, van Leeuwen DG, et al. Enhancing antitumor efficacy of chimeric antigen receptor T cells through constitutive CD40L expression. *Mol Ther* (2015) 23(4):769–78. doi:10.1038/mt.2015.4
206. Hoyos V, Savoldo B, Quintarelli C, Mahendravada A, Zhang M, Vera J, et al. Engineering CD19-specific T lymphocytes with interleukin-15 and a suicide gene to enhance their anti-lymphoma/leukemia effects and safety. *Leukemia* (2010) 24(6):1160–70. doi:10.1038/leu.2010.75
207. Zhao Z, Condomines M, van der Stegen Sjoukje JC, Perna F, Kloss Christopher C, Gunset G, et al. Structural design of engineered costimulation determines tumor rejection kinetics and persistence of CAR T cells. *Cancer Cell* (2015) 28(4):415–28. doi:10.1016/j.ccell.2015.09.004
208. Dai H, Wang Y, Lu X, Han W. Chimeric antigen receptors modified T-cells for cancer therapy. *J Natl Cancer Inst* (2016) 108(7):djv439. doi:10.1093/jnci/djv439
209. Brentjens R, Davila ML, Riviere I, Park J, Wang X, Cowell LG, et al. CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia. *Sci Transl Med* (2013) 5(177):ra38–38. doi:10.1126/scitranslmed.3005930

210. Kochenderfer JN, Dudley ME, Feldman SA, Wilson WH, Spaner DE, Maric I, et al. B-cell depletion and remissions of malignancy along with cytokine-associated toxicity in a clinical trial of anti-CD19 chimeric-antigen-receptor-transduced T cells. *Blood* (2012) 119(12):2709–20. doi:10.1182/blood-2011-10-384388
211. Davila ML, Riviere I, Wang X, Bartido S, Park J, Curran K, et al. Efficacy and toxicity management of 19-28z CAR T cell therapy in B cell acute lymphoblastic leukemia. *Sci Transl Med* (2014) 6(224):ra25–25. doi:10.1126/scitranslmed.3008226
212. Lee DW, Gardner R, Porter DL, Louis CU, Ahmed N, Jensen M, et al. Current concepts in the diagnosis and management of cytokine release syndrome. *Blood* (2014) 124(2):188–95. doi:10.1182/blood-2014-05-552729
213. Maude SL, Barrett D, Teachey DT, Grupp SA. Managing cytokine release syndrome associated with novel T cell-engaging therapies. *Cancer J* (2014) 20(2):119–22. doi:10.1097/PPO.0000000000000035
214. Casucci M, Nicolis di Robilant B, Falcone L, Camisa B, Norelli M, Genovese P, et al. CD44v6-targeted T cells mediate potent antitumor effects against acute myeloid leukemia and multiple myeloma. *Blood* (2013) 122(20):3461–72. doi:10.1182/blood-2013-04-493361
215. Gill S, Tasian SK, Ruella M, Shestova O, Li Y, Porter DL, et al. Preclinical targeting of human acute myeloid leukemia and myeloablation using chimeric antigen receptor-modified T cells. *Blood* (2014) 123(15):2343–54. doi:10.1182/blood-2013-09-529537
216. Mardiros A, Dos Santos C, McDonald T, Brown CE, Wang X, Budde LE, et al. T cells expressing CD123-specific chimeric antigen receptors exhibit specific cytolytic effector functions and antitumor effects against human acute myeloid leukemia. *Blood* (2013) 122(18):3138–48. doi:10.1182/blood-2012-12-474056
217. Hombach A, Heuser C, Sircar R, Tillmann T, Diehl V, Kruis W, et al. T cell targeting of TAG72+ tumor cells by a chimeric receptor with antibody-like specificity for a carbohydrate epitope. *Gastroenterology* (1997) 113(4): 1163–70. doi:10.1053/gast.1997.v113.pm9322511
218. Mezzanzanica D, Canevari S, Mazzoni A, Figini M, Colnaghi MI, Waks T, et al. Transfer of chimeric receptor gene made of variable regions of tumor-specific antibody confers anticarbohydrate specificity on T cells. *Cancer Gene Ther* (1998) 5(6):401–7.
219. Westwood JA, Smyth MJ, Teng MW, Moeller M, Trapani JA, Scott AM, et al. Adoptive transfer of T cells modified with a humanized chimeric receptor gene inhibits growth of Lewis-Y-expressing tumors in mice. *Proc Natl Acad Sci U S A* (2005) 102(52):19051–6. doi:10.1073/pnas.0504312102
220. Stewart-Jones G, Wadle A, Hombach A, Shenderov E, Held G, Fischer E, et al. Rational development of high-affinity T-cell receptor-like antibodies. *Proc Natl Acad Sci U S A* (2009) 106(14):5784–8. doi:10.1073/pnas.0901425106
221. Kaiser AD, Assenmacher M, Schroder B, Meyer M, Orentas R, Bethke U, et al. Towards a commercial process for the manufacture of genetically modified T cells for therapy. *Cancer Gene Ther* (2015) 22(2):72–8. doi:10.1038/cgt.2014.78
222. Tumaini B, Lee DW, Lin T, Castiello L, Stroncek DF, Mackall C. Simplified process for the production of anti-CD19-CAR-engineered T cells. *Cytotherapy* (2013) 15:1406–15. doi:10.1016/j.jcyt.2013.06.003
223. Bellone G, Turletti A, Artusio E, Mareschi K, Carbone A, Tibaudi D, et al. Tumor-associated transforming growth factor-beta and interleukin-10 contribute to a systemic Th2 immune phenotype in pancreatic carcinoma patients. *Am J Pathol* (1999) 155:537–47. doi:10.1016/S0002-9440(10)65149-8
224. Decker T, Fischer G, Bucke W, Bucke P, Stotz F, Gruneberger A, et al. Increased number of regulatory T cells (T-regs) in the peripheral blood of patients with Her-2/neu-positive early breast cancer. *J Cancer Res Clin Oncol* (2012) 138:1945–50. doi:10.1007/s00432-012-1258-3
225. Karp JE, Ricklis RM, Balakrishnan K, Briel J, Greer J, Gore SD, et al. A phase 1 clinical-laboratory study of clofarabine followed by cyclophosphamide for adults with refractory acute leukemias. *Blood* (2007) 110:1762–9. doi:10.1182/blood-2007-03-081364
226. Goldstein MJ, Kohrt HE, Houot R, Varghese B, Lin JT, Swanson E, et al. Adoptive cell therapy for lymphoma with CD4 T cells depleted of CD137-expressing regulatory T cells. *Cancer Res* (2012) 72:1239–47. doi:10.1158/0008-5472.CAN-11-3375
227. Gomez-Eerland R, Nuijen B, Heemskerk B, van Rooij N, van den Berg JH, Beijnen JH. Manufacture of gene-modified human T cells with a memory stem/central memory phenotype. *Hum Gene Ther Methods* (2014) 25:277–87. doi:10.1089/hgtb.2014.004
228. van Loenen MM, de Boer R, van Liempt E, Meij P, Jedema I, Falkenburg JH, et al. A good manufacturing practice procedure to engineer donor virus-specific T cells into potent anti-leukemic effector cells. *Haematologica* (2014) 99:759–68. doi:10.3324/haematol.2013.093690
229. Louis CU, Savoldo B, Dotti G, Pule M, Yvon E, Myers GD, et al. Antitumor activity and long-term fate of chimeric antigen receptor-positive T cells in patients with neuroblastoma. *Blood* (2011) 118:6050–6. doi:10.1182/blood-2011-05-354449
230. Pule MA, Savoldo B, Myers GD, Rossig C, Russell HV, Dotti G, et al. Virus-specific T cells engineered to coexpress tumor-specific receptors: persistence and antitumor activity in individuals with neuroblastoma. *Nat Med* (2008) 14:1264–70. doi:10.1038/nm.1882
231. Berger C, Jensen MC, Lansdorp PM, Gough M, Elliott C, Riddell SR. Adoptive transfer of effector CD8+ T cells derived from central memory cells establishes persistent T cell memory in primates. *J Clin Invest* (2008) 118:294–305. doi:10.1172/JCI32103
232. Gattinoni L, Lugli E, Ji Y, Pos Z, Paulos CM, Quigley MF. A human memory T cell subset with stem cell-like properties. *Nat Med* (2011) 17:1290–7. doi:10.1038/nm.2446
233. Hinrichs CS, Borman ZA, Gattinoni L, Yu Z, Burns WR, Huang J, et al. Human effector CD8+ T cells derived from naive rather than memory subsets possess superior traits for adoptive immunotherapy. *Blood* (2011) 117:808–14. doi:10.1182/blood-2010-05-286286
234. Klebanoff CA, Gattinoni L, Restifo NP. Sorting through subsets: which T-cell populations mediate highly effective adoptive immunotherapy? *J Immunother* (2012) 35:651–60. doi:10.1097/CJI.0b013e31827806e6
235. Xu Y, Zhang M, Ramos CA, Durett A, Liu E, Dakhova O, et al. Closely related T-memory stem cells correlate with in vivo expansion of CAR.CD19-T cells and are preserved by IL-7 and IL-15. *Blood* (2014) 123:3750–9. doi:10.1182/blood-2014-01-552174
236. Porter DL, Levine BL, Bunin N, Stadtmauer EA, Luger SM, Goldstein S, et al. A phase 1 trial of donor lymphocyte infusions expanded and activated ex vivo via CD3/CD28 costimulation. *Blood* (2006) 107:1325–31. doi:10.1182/blood-2005-08-3373
237. Casati A, Varghaei-Nahvi A, Feldman SA, Assenmacher M, Rosenberg SA, Dudley ME. Clinical-scale selection and viral transduction of human naive and central memory CD8+ T cells for adoptive cell therapy of cancer patients. *Cancer Immunol Immunother* (2013) 62:1563–73. doi:10.1007/s00262-013-1459-x
238. Terakura S, Yamamoto TN, Gardner RA, Turtle CJ, Jensen MC, Riddell SR. Generation of CD19-chimeric antigen receptor modified CD8+ T cells derived from virus-specific central memory T cells. *Blood* (2012) 119:72–82. doi:10.1182/blood-2011-07-366419
239. Torikai H, Reik A, Liu P-Q, Zhou Y, Zhang L, Maiti S, et al. A foundation for universal T-cell based immunotherapy: T cells engineered to express a CD19-specific chimeric-antigen-receptor and eliminate expression of endogenous TCR. *Blood* (2012) 119(24):5697–705. doi:10.1182/blood-2012-01-405365
240. Poirot L, Philip B, Schiffer-Mannioui C, Le Clerre D, Chion-Sotinel I, Derniame S, et al. Multiplex genome-edited t-cell manufacturing platform for "Off-the-Shelf" adoptive T-cell immunotherapies. *Cancer Res* (2015) 75(18):3853–64. doi:10.1158/0008-5472.CAN-14-3321
241. English K, Barry FP, Field-Corbett CP, Mahon BP. IFN-gamma and TNF-alpha differentially regulate immunomodulation by murine mesenchymal stem cells. *Immunol Lett* (2007) 110(2):91–100. doi:10.1016/j.imlet.2007.04.001
242. Maccario R, Podesta M, Moretta A, Cometa A, Comoli P, Montagna D, et al. Interaction of human mesenchymal stem cells with cells involved in alloantigen-specific immune response favors the differentiation of CD4+ T-cell subsets expressing a regulatory/suppressive phenotype. *Haematologica* (2005) 90(4):516–25.
243. English K, Ryan JM, Tobin L, Murphy MJ, Barry FP, Mahon BP. Cell contact, prostaglandin E(2) and transforming growth factor beta 1 play non-redundant roles in human mesenchymal stem cell induction of CD4+CD25(High) forkhead box P3+ regulatory T cells. *Clin Exp Immunol* (2009) 156(1):149–60. doi:10.1111/j.1365-2249.2009.03874.x
244. Selmani Z, Naji A, Zidi I, Favier B, Gaiffe E, Obert L, et al. Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required

to suppress T lymphocyte and natural killer function and to induce CD4+CD25highFOXP3+ regulatory T cells. *Stem Cells* (2008) 26(1):212–22. doi:10.1634/stemcells.2007-0554
245. Nauta AJ, Kruisselbrink AB, Lurvink E, Willemze R, Fibbe WE. Mesenchymal stem cells inhibit generation and function of both CD34+-derived and monocyte-derived dendritic cells. *J Immunol* (2006) 177(4):2080–7. doi:10.4049/jimmunol.177.4.2080
246. Chen L, Zhang W, Yue H, Han Q, Chen B, Shi M, et al. Effects of human mesenchymal stem cells on the differentiation of dendritic cells from CD34+ cells. *Stem Cells Dev* (2007) 16(5):719–31. doi:10.1089/scd.2007.0065
247. Djouad F, Charbonnier LM, Bouffi C, Louis-Plence P, Bony C, Apparailly F, et al. Mesenchymal stem cells inhibit the differentiation of dendritic cells through an interleukin-6-dependent mechanism. *Stem Cells* (2007) 25(8):2025–32. doi:10.1634/stemcells.2006-0548
248. Li YP, Paczesny S, Lauret E, Poirault S, Bordigoni P, Mekhloufi F, et al. Human mesenchymal stem cells license adult CD34+ hemopoietic progenitor cells to differentiate into regulatory dendritic cells through activation of the Notch pathway. *J Immunol* (2008) 180(3):1598–608. doi:10.4049/jimmunol.180.3.1598
249. Zhang B, Liu R, Shi D, Liu X, Chen Y, Dou X, et al. Mesenchymal stem cells induce mature dendritic cells into a novel Jagged-2-dependent regulatory dendritic cell population. *Blood* (2009) 113(1):46–57. doi:10.1182/blood-2008-04-154138
250. Liu X, Qu X, Chen Y, Liao L, Cheng K, Shao C, et al. Mesenchymal stem/stromal cells induce the generation of novel IL-10-dependent regulatory dendritic cells by SOCS3 activation. *J Immunol* (2012) 189(3):1182–92. doi:10.4049/jimmunol.1102996
251. Kim J, Hematti P. Mesenchymal stem cell-educated macrophages: a novel type of alternatively activated macrophages. *Exp Hematol* (2009) 37(12):1445–53. doi:10.1016/j.exphem.2009.09.004
252. Corcione A, Benvenuto F, Ferretti E, Giunti D, Cappiello V, Cazzanti F, et al. Human mesenchymal stem cells modulate B-cell functions. *Blood* (2006) 107(1):367–72. doi:10.1182/blood-2005-07-2657
253. Rasmusson I, Le Blanc K, Sundberg B, Ringden O. Mesenchymal stem cells stimulate antibody secretion in human B cells. *Scand J Immunol* (2007) 65(4):336–43. doi:10.1111/j.1365-3083.2007.01905.x
254. Qin Y, Zhou Z, Zhang F, Wang Y, Shen B, Liu Y, et al. Induction of regulatory B-cells by mesenchymal stem cells is affected by SDF-1alpha-CXCR7. *Cell Physiol Biochem* (2015) 37(1):117–30. doi:10.1159/000430338
255. Raffaghello L, Bianchi G, Bertolotto M, Montecucco F, Busca A, Dallegri F, et al. Human mesenchymal stem cells inhibit neutrophil apoptosis: a model for neutrophil preservation in the bone marrow niche. *Stem Cells* (2008) 26(1):151–62. doi:10.1634/stemcells.2007-0416
256. Spaggiari GM, Capobianco A, Becchetti S, Mingari MC, Moretta L. Mesenchymal stem cell-natural killer cell interactions: evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation. *Blood* (2006) 107(4):1484–90. doi:10.1182/blood-2005-07-2775
257. Tu Z, Li Q, Bu H, Lin F. Mesenchymal stem cells inhibit complement activation by secreting factor H. *Stem Cells Dev* (2010) 19(11):1803–9. doi:10.1089/scd.2009.0418
258. Schraufstatter IU, Discipio RG, Zhao M, Khaldoyanidi SK. C3a and C5a are chemotactic factors for human mesenchymal stem cells, which cause prolonged ERK1/2 phosphorylation. *J Immunol* (2009) 182(6):3827–36. doi:10.4049/jimmunol.0803055
259. Rani S, Ryan AE, Griffin MD, Ritter T. Mesenchymal stem cell-derived extracellular vesicles: toward cell-free therapeutic applications. *Mol Ther* (2015) 23(5):812–23. doi:10.1038/mt.2015.44
260. Thery C, Ostrowski M, Segura E. Membrane vesicles as conveyors of immune responses. *Nat Rev Immunol* (2009) 9(8):581–93. doi:10.1038/nri2567
261. Yang J, Liu XX, Fan H, Tang Q, Shou ZX, Zuo DM, et al. Extracellular vesicles derived from bone marrow mesenchymal stem cells protect against experimental colitis via attenuating colon inflammation, oxidative stress and apoptosis. *PLoS One* (2015) 10(10):e0140551. doi:10.1371/journal.pone.0140551
262. Peggs KS, Verfuerth S, Pizzey A, Khan N, Guiver M, Moss PA, et al. Adoptive cellular therapy for early cytomegalovirus infection after allogeneic stem-cell transplantation with virus-specific T-cell lines. *Lancet* (2003) 362(9393):1375–7. doi:10.1016/S0140-6736(03)14634-X
263. Bao L, Cowan MJ, Dunham K, Horn B, McGuirk J, Gilman A, et al. Adoptive immunotherapy with CMV-specific cytotoxic T lymphocytes for stem cell transplant patients with refractory CMV infections. *J Immunother* (2012) 35(3):293–8. doi:10.1097/CJI.0b013e31824300a2
264. Blyth E, Clancy L, Simms R, Ma CK, Burgess J, Deo S, et al. Donor-derived CMV-specific T cells reduce the requirement for CMV-directed pharmacotherapy after allogeneic stem cell transplantation. *Blood* (2013) 121(18):3745–58. doi:10.1182/blood-2012-08-448977
265. Koehne G, Hasan A, Doubrovina E, Prockop S, Tyler E, Wasilewski G, et al. Immunotherapy with donor T cells sensitized with overlapping pentadecapeptides for treatment of persistent cytomegalovirus infection or viremia. *Biol Blood Marrow Transplant* (2015) 21(9):1663–78. doi:10.1016/j.bbmt.2015.05.015
266. Meij P, Jedema I, Zandvliet ML, van der Heiden PL, van de Meent M, van Egmond HM, et al. Effective treatment of refractory CMV reactivation after allogeneic stem cell transplantation with in vitro-generated CMV pp65-specific CD8+ T-cell lines. *J Immunother* (2012) 35(8):621–8. doi:10.1097/CJI.0b013e31826e35f6
267. Cobbold M, Khan N, Pourgheysari B, Tauro S, McDonald D, Osman H, et al. Adoptive transfer of cytomegalovirus-specific CTL to stem cell transplant patients after selection by HLA-peptide tetramers. *J Exp Med* (2005) 202(3):379–86. doi:10.1084/jem.20040613
268. Uhlin M, Gertow J, Uzunel M, Okas M, Berglund S, Watz E, et al. Rapid salvage treatment with virus-specific T cells for therapy-resistant disease. *Clin Infect Dis* (2012) 55(8):1064–73. doi:10.1093/cid/cis625
269. Haque T, Wilkie GM, Taylor C, Amlot PL, Murad P, Iley A, et al. Treatment of Epstein-Barr-virus-positive post-transplantation lymphoproliferative disease with partly HLA-matched allogeneic cytotoxic T cells. *Lancet* (2002) 360(9331):436–42. doi:10.1016/S0140-6736(02)09672-1
270. Haque T, Wilkie GM, Jones MM, Higgins CD, Urquhart G, Wingate P, et al. Allogeneic cytotoxic T-cell therapy for EBV-positive posttransplantation lymphoproliferative disease: results of a phase 2 multicenter clinical trial. *Blood* (2007) 110(4):1123–31. doi:10.1182/blood-2006-12-063008
271. Heslop HE, Slobod KS, Pule MA, Hale GA, Rousseau A, Smith CA, et al. Long-term outcome of EBV-specific T-cell infusions to prevent or treat EBV-related lymphoproliferative disease in transplant recipients. *Blood* (2009) 115(5):925–35. doi:10.1182/blood-2009-08-239186
272. Doubrovina E, Oflaz-Sozmen B, Prockop SE, Kernan NA, Abramson S, Teruya-Feldstein J, et al. Adoptive immunotherapy with unselected or EBV-specific T cells for biopsy-proven EBV+ lymphomas after allogeneic hematopoietic cell transplantation. *Blood* (2011) 119(11):2644–56. doi:10.1182/blood-2011-08-371971
273. Gallot G, Vollant S, Saïagh S, Clémenceau B, Vivien R, Cerato E, et al. T-cell therapy using a bank of EBV-specific cytotoxic T cells: lessons from a phase I/II feasibility and safety study. *J Immunother* (2014) 37(3):170–9. doi:10.1097/CJI.0000000000000031
274. Geyeregger R, Freimüller C, Stemberger J, Artwohl M, Witt V, Lion T, et al. First-in-man clinical results with good manufacturing practice (GMP)-compliant polypeptide-expanded adenovirus-specific T cells after haploidentical hematopoietic stem cell transplantation. *J Immunother* (2014) 37(4):245–9. doi:10.1097/CJI.0000000000000034

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