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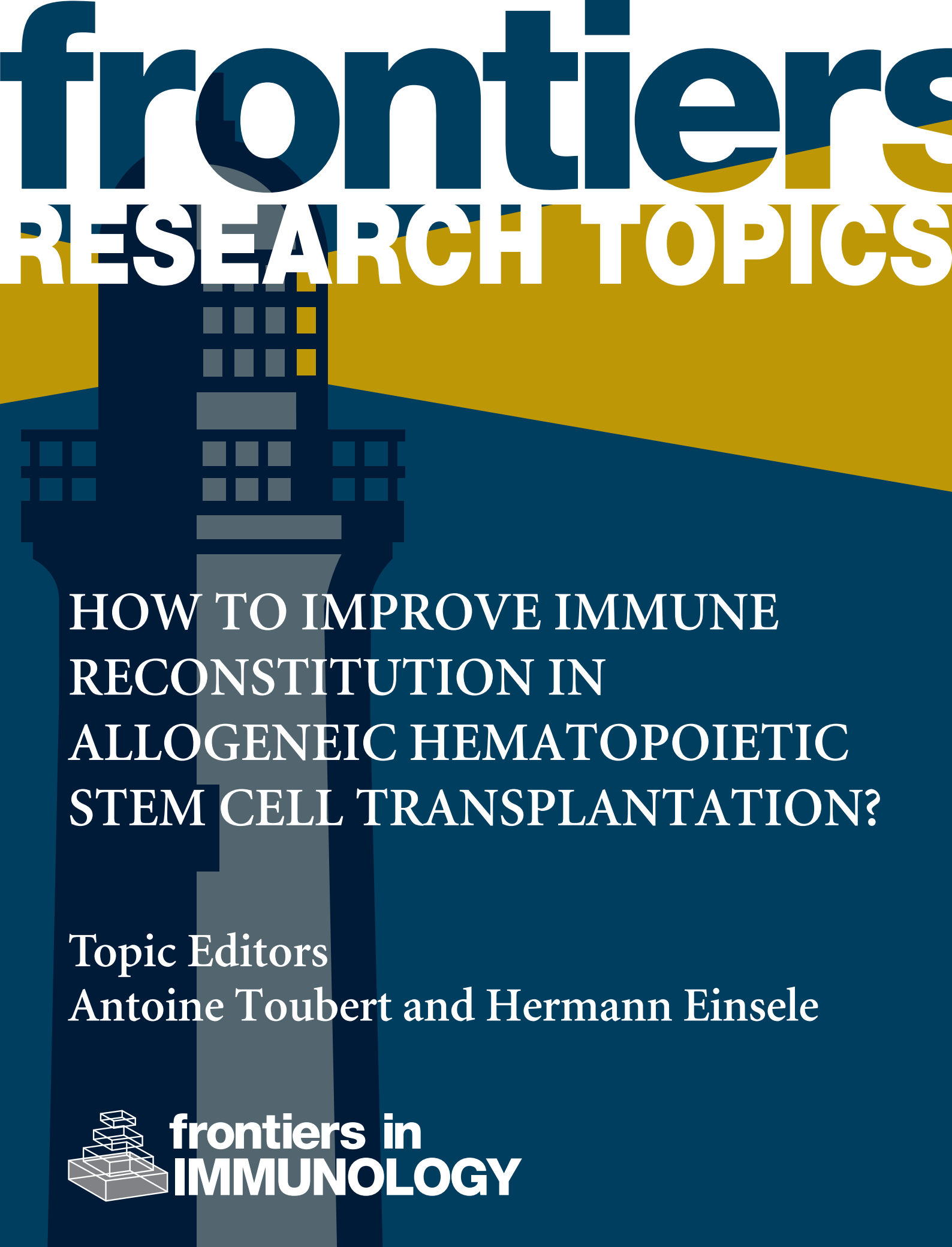

HOW TO IMPROVE IMMUNE RECONSTITUTION IN ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION?

Topic Editors
Antoine Toubert and Hermann Einsele

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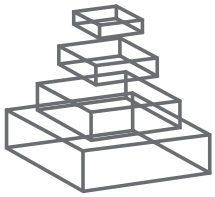

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HOW TO IMPROVE IMMUNE RECONSTITUTION IN ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION?

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Allogeneic haematopoietic stem cell transplantation (allo-HSCT) is widely used in the treatment of haematological malignancies as a form of immunotherapy acting through a graft-versus-leukemia (GvL) reaction. This curative allogeneic response can be associated with severe drawbacks, such as frequent and severe graft-versus-host disease (GvHD) and a long-lasting immunodeficiency, especially now with the development of innovative strategies such as umbilical cord blood transplantation or transplants from haplo-identical family donors (Haplo-HSCT). In the long-term follow-up of these patients, severe post-transplant infections, relapse or secondary malignancies may be directly related to persistent immune defects.

Reconstitution of the different lymphocyte populations (B, T, NK, NKT) and antigen presenting cells of myeloid origin (monocytes, macrophages and dendritic cells) should be considered not only quantitatively but especially qualitatively, in terms of functional subsets. Immune deficiency leading to an increased susceptibility to infections lasts for more than a year. Although infections that occur in the first month mostly result from a deficiency in both granulocytes and mononuclear cells (MNC), later post-engraftment infections are due to a deficiency in MNC subsets, primarily CD4 T-cells and B-cells. T-cell reconstitution has been extensively studied because of the central role of T-cells in mediating both GvHD, evidenced by the reduced incidence of this complication following T-Cell depletion, and a GvL effect as shown by DLI. In the recent years there has been renewed interest in the role of NK-cells, especially in the context of Haplo-HSCT, and in B-cell reconstitution.

This Frontiers Research Topic will provide state of the art knowledge of the mechanisms of immune reconstitution in an allogeneic environment, in order to improve monitoring and therapeutic intervention in allo-HSCT patients.

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EDITORIAL
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From immunomonitoring to immune intervention

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Keywords: HSCT, immune reconstitution, thymic function, cell therapy, Haplo-SCT

Host immune status is a key issue in allogeneic hematopoietic stem cell transplantation (allo-HSCT). In the long-term follow-up of these patients, severe post-transplant infections, relapse or secondary malignancies may be directly related to prolonged immune defects, especially in the context of innovative stem cell sources such as umbilical cord blood transplantation (CBT) (1) or transplants from HLA haplo-identical family donors (Haplo-HSCT) (2, 3).

This *Frontiers* Research Topic provides insights into mechanisms of immune reconstitution in an allogeneic environment, in order to improve monitoring and therapeutic intervention in allo-HSCT patients.

Forcina et al. (4) critically review T and NK biomarkers, their threshold and clinical relevance. Many factors from the host, transplant conditioning, stem cell source, and genetic disparity may impact immune recovery. Few biomarkers have reached a clinical consensus: a rapid and potent CD4 T-cell recovery is associated with a favorable clinical outcome, CMV-specific CD8 T-cell counts measured by tetramers can predict a lower incidence of CMV disease. Other phenotypic or molecular markers, still based on small-sized studies, will need further validation in large scale-multicentric cohorts. This is the case of molecular markers of lymphocyte generation, such as T-cells evaluated by the quantification of T-cell rearrangement excision circles (TREC) as a surrogate marker of thymic activity. Clave et al. (3) indicate an impact of thymic function recovery on relapse following CBT as well as Haplo-HSCT in children treated for an hematological malignancy. In order to reach the routine medical practice, immunomonitoring tests need also to be simple and fast. This practical issue is well taken into account by Fuji et al. (5) reviewing T-cell monitoring of viral and fungal infections. CMV is still the most intensively studied virus in immunocompromised hosts. CMV shapes T and NK cell responses in many ways and may escape immune response. Among still unanswered questions, is the role of viral-specific (CMV and EBV) T-cell cross-reactivity against allogeneic targets in graft-versus-host and graft-versus-leukemia (6).

Experimental models have proved their importance in allo-HSCT, especially deciphering mechanisms of allogeneicity in graft-versus-host disease (GVHD). Among rodents, rat models are relevant especially in some autoimmune conditions and solid organ transplantation, sometimes closer to the human disease than the "gold standard" murine models. Zinöcker et al. (7) emphasize the interest of rat model in experimental GVHD, especially the rat skin explant assay as a tool for functional evaluation of GVHD. Experimental models are also required to provide the basis and for preclinical evaluation of immune-based therapies.

This *Frontiers* Research Topic also reviews in some strategies already proposed in the clinics. Adoptive T-cell therapies have been conducted successfully by several groups to control life-threatening viral (EBV, CMV) reactivations. This leads to "off the shelf" strategies based on third party HLA-typed T-cell donors registries, which may have a major impact in transplant recipients (8). The development of therapeutic antibodies in cancer is one of the most active fields in clinical immunology. Many strategies are underway to improve their action, especially through their antibody-dependent cellular toxicity mediated by natural killer (NK) cells (9). One approach especially relevant in allo-HSCT would be to take advantage of the killer-immunoglobulin-like receptor (KIR) ligand incompatibility in addition to ADCC to boost NK functions.

The impact of these different concepts, from basic knowledge to translational medicine, is integrated in state-of-the art reviews of two main "success stories" in allo-HSCT: CBT (1) and Haplo-HSCT (2).

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MINI REVIEW ARTICLE
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Predicting the clinical outcome of allogeneic hematopoietic stem cell transplantation: the long and winding road toward validated immune biomarkers

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The clinical outcome of allogeneic hematopoietic stem cell transplantation (HSCT) is strongly influenced from the potential complications arising during the delicate phase of post-transplant immune restoration. The quantitative aspects of immune-cell repopulation after HSCT and the qualitative features their functional restitution have been extensively reported. Nevertheless, measurable immune biomarkers predicting the clinical outcome of HSCT await formal validation. The aim of this review is an appraisal of most studies published so far on the predictive value of different T and NK-cell biomarkers after HSCT with emphasis on defined thresholds endorsed by multivariate analysis.

Keywords: allogeneic hematopoietic stem cell transplantation, immune reconstitution, biomarkers, opportunistic infections, graft-versus-host disease

INTRODUCTION

The restoration of a functional immune system is one of the main factors influencing the clinical outcome of allogeneic hematopoietic stem cell transplantation (HSCT). The post-transplant period is characterized by multiple immune defects that expose the patient to a high risk of opportunistic infections and, eventually, disease relapse. The duration of this period may vary according to several variables, including patient age and immune status before transplant, the degree of donor compatibility, the intensity of the conditioning regimen, the source of stem cells, eventual graft manipulation, and pharmacological immune suppression. The normalization of granulocytes, monocytes, and NK-cell numbers usually occurs within the first weeks and, with the possible exception of NK-cells, coincides with their full competence. Conversely, the normalization of T and B cell numbers may take much longer and does not necessarily associate with their immediate functional restitution (Shiobara et al., 1982). Although often used interchangeably, it is therefore important to distinguish between *immune reconstitution*, which refers to quantitative immune-cell repopulation, and *immune recovery*, which pertains to their qualitative restitution.

The quantitative reconstitution of T cells post-transplant occurs through two main mechanisms: (i) the early peripheral expansion of donor-derived memory T cells present in the graft, which happens in weeks (ii) the late emergence of host-tolerant naïve T cells originating from donor stem cells after thymic education, which, depending on donor age, occurs in months (van den Brink et al., 2004). The qualitative recovery of T cells may follow their quantitative reconstitution with a delay of many years and implies *de novo* pathogen encounter, with ensuing differentiation into effector and memory T cells.

Although the quantitative aspects of immune reconstitution post-transplant and the qualitative features of immune recovery have been the subject of several studies, a tight association between measurable immune biomarkers and the clinical outcome of HSCT, is currently missing. So far, the specific issue of validating thresholds of immune measurements that may help predicting the incidence of major post-transplant events, such as opportunistic infections, graft-versus-host disease (GVHD), and disease relapse, has been approached by single-center, necessarily small-sized studies. While sometimes sufficiently powered to obtain statistically significant results, these studies often failed to draw definitive conclusions that may be relevant to daily clinical practice. Conversely, the lack of harmonized methods for immune biomarker measurements and the great heterogeneity of the transplant populations between the different studies have prevented from meaningful meta-analysis.

The aim of this review is an appraisal of the studies published so far on the predictive value of different T and NK-cell biomarkers after HSCT with emphasis on the thresholds chosen for statistical analysis. A comprehensive **Table 1** detailing the main results from the different studies has also been included. Descriptive studies based on the comparison between different groups as the only statistical approach, studies in the setting of autologous HSCT and immune biomarkers whose predictive value has not been endorsed by multivariate analysis, have been purposely excluded from this review.

T LYMPHOCYTES AND INVARIANT NKT CELLS

The absolute lymphocyte count (ALC) derived from routine blood-cell testing has been the first immune biomarker explored for predicting the clinical outcome of HSCT. In patients undergoing T cell-depleted HSCT from an HLA-identical sibling (HLA-sib), an ALC above the median [>690/μL (Montero et al., 2006) or >450/μL (Savani et al., 2007a)] at day 30 post-transplant was found to be independently associated with a lower

Table 1 | T- and NK-cell biomarkers, thresholds, and clinical outcome after HSCT.

Biomarker	Pts	Donor	Days	Statistics	Threshold	Outcome	Reference
ALC	138	HLA-sib	+30	Median	>690 cells/μL	↓TRM ↑LFS ↑OS	Montero et al. (2006)
	157	HLA-sib	+30	Median	>450 cells/μL	↓TRM ↓RI ↑OS	Savani et al. (2007a)
	102	MUD	+30	Arbitrary	>1000 cells/μL	↓TRM ↑LFS ↑OS	Le Blanc et al. (2009)
	65	Haplo UCB	+60	Arbitrary	>1000 cells/μL	↓TRM ↑OS	Ciurea et al. (2011)
	360	UCB	+30	From Savani et al. (2007a)	>200 cells/μL	↓TRM ↑LFS ↑OS	Burke et al. (2011)
$CD4^+$ T cells	69	HLA-sib MUD	+90	Arbitrary (HIV)	>200 cells/μL	↓TRM ↑OS ↓infect.	Kim et al. (2006)
	758	HLA-sib MUD	+35	Median	>86 cells/μL	↓TRM	Berger et al. (2008)
	345	HLA-sib MUD	+90	From Matthews et al. (2010)	>200 cells/μL	↓TRM	Buhlmann et al. (2011)
	99	HLA-sib MUD	+20	ROC	>115 cells/μL	↓TRM	Fedele et al. (2012)
$CD8^+$ T cells	32	HLA-sib MUD	+365	Arbitrary	>V percentile	↑OS	Koehl et al. (2007)
iNKT/T ratio	71	HLA-sib MUD	+15	ROC	$>0.58 \times 10^{-3}$	↓aGVHD ↓TRM ↑OS	Rubio et al. (2012)
	22	Haplo	+545	Median	$>10^{-4}$	No relapse	Casorati et al. (2012)
TREC values	102	HLA-sib	Pre-tx	Categories	172/150,000 T	↑OS ↓infections	Clave et al. (2005)
	33	Haplo	+180	Categories	sj < 0.1/150,000 T; β < 0.001/150,000 T	↑RI	Clave et al. (2012)
Tregs frequencies	60	HLA-sib MUD	aGVHD	Median	>0.5% over TNC	↓TRM ↑OS	Magenau et al. (2010)
	57	HLA-sib MUD	cGVHD	Categories	>3% over PBL	No cGVHD	Koreth et al. (2011)
CMV-specific $CD8^+$ T cells	24	HLA-sib MUD	+100	Arbitrary	>10 tet^+ cells/μL	No CMV disease	Cwynarski et al. (2001)
	83	HLA-sib MUD	+65	Categories	>7 tet^+ cells/μL	↓CMV disease	Gratama et al. (2010)
	133	HLA-sib MUD UCB	+120	Categories	>1 cyt^+ cells/μL	↓CMV DNAemia	Tormo et al. (2011)
	131	HLA-sib MUD Haplo	+365	ROC	>3 cyt^+ cells/μL	No CMV DNAemia	Lilleri et al. (2012)
CMV-specific	30	HLA-sib MUD	+120	Arbitrary	>2.5 S.I.	↓CMV disease	Krause et al. (1997)
$CD4^+$ T cells	32	HLA-sib MUD	+40	Median	>0.4 cyt^+ cells/μL	No CMV DNAemia	Pourgheysari et al. (2009)
	133	HLA-sib MUD UCB	+120	Categories	>1.2 cyt^+ cells/μL	↓CMV DNAemia	Tormo et al. (2011)
	117	UCB	+30	Arbitrary	>7 S.I.	No infections ↑ LFS	Parkman et al. (2006)
	131	HLA-sib MUD Haplo	+365	ROC	>1 cyt^+ cells/μL	No CMV DNA	Lilleri et al. (2012)
EBV-specific T cells	33	MUD	+56	Categories	>1 cyt^+ cells/μL	No relapse	Hoegh-Petersen et al. (2012)
NK-cells	43	Haplo	+15	Median	>9.27 cells/μL	↑LFS	Chang et al. (2008)
	54	HLA-sib	+30	Median	>150 cells/μL	↓TRM ↓RI ↑OS ↓aGVHD	Savani et al. (2007b)
	345	HLA-sib MUD	+365	From Ruggeri et al. (2002)	>150 cells/μL	↓TRM	Buhlmann et al. (2011)

ALC, absolute lymphocyte count; HLA-sib, HLA-identical sibling; TRM, transplant-related mortality; LFS, leukemia-free survival; OS, overall survival; RI, relapse incidence; MUD, matched unrelated donor; Haplo, HLA-haploidentical; UCB, umbilical cord-blood; ROC, receiver operating characteristic curve analysis; aGVHD, acute graft-versus-host-disease; TREC, T cell receptor excision circles; pre-tx, pre-transplant; TNC, total nucleated cells; cGVHD, chronic graft-versus-host-disease; PBL, peripheral blood lymphocytes; Tet^+, tetramer-positive cells; cyt^+, intracellular cytokine-positive; S.I., stimulation index.

transplant-related mortality (TRM) and longer leukemia-free survival (LFS) and overall survival (OS). The association between an higher ALC at early time points after transplant and a favorable clinical outcome was confirmed by taking an arbitrary threshold of 1000/μL in T cell-replete HSCT from matched unrelated donors (MUD) (Le Blanc et al., 2009) and CD34-selected HSCT form HLA-haploidentical donors (Ciurea et al., 2011), or by taking an arbitrary threshold of 200/μL in umbilical cord-blood (UCB) transplantation (Burke et al., 2011). Investigating the same issue in patients that received a reduced-intensity regimen, however, has found conflicting results (Matthews et al., 2010; Burke et al., 2011), suggesting that the type of conditioning may influence the predictive value of the ALC.

The predictive value of T lymphocyte subsets assessed by flow cytometry, rather than the simpler ALC, has been examined in more sophisticated studies. At day 90 after T cell-replete HSCT form a HLA-sib or a MUD, a $CD4^+$ T cell count above 200/μL, a threshold derived from the HIV field, was independently associated with a lower NRM, less opportunistic infections, and a longer OS (Kim et al., 2006). The role for a rapid reconstitution of $CD4^+$ T cells in protecting from transplant morbidity and mortality was confirmed in three subsequent studies using slightly different approaches for statistical analysis. The first study found that at day 30 post-transplant, a $CD4^+$ T cell count above the median (>86/μL) was associated with a lower TRM (Berger et al., 2008). The second study confirmed the association and observed no impact on relapse incidence (Buhlmann et al., 2011). The third study used receiver operator curve (ROC) analysis of $CD4^+$ T cell counts at day 20 post-transplant for determining a threshold of 115/μL, which was retrospectively found to be associated with a lower TRM (Fedele et al., 2012).

Differently from $CD4^+$ T cells, the predictive value of $CD8^+$ T cell biomarkers is less studied. In a combined series of HLA-sib, MUD, or HLA-haploidentical pediatric HSCT, reaching a $CD8^+$ T cell count above the fifth percentile of age-matched controls within the first year post-transplant was found to be independently associated with a longer OS and a trend toward a lower relapse incidence (Koehl et al., 2007).

The pattern of invariant natural killer T cells (iNKT) reconstitution has been explored for predicting the clinical outcome at earlier time points after HSCT and independently from conventional T cells. The reconstitution of iNKT cells after HLA-sib or MUD HSCT was found to precede that of T and NK-cells (Rubio et al., 2012). Accordingly, at day 15 post-transplant an iNKT/T cell ratio above 0.58×10^{-3}, a threshold identified after retrospective ROC analysis, was associated with a zero likelihood of GVHD. Moreover, reaching an iNKT/T cell ratio above 10^{-3} within the first 3 months after transplantation was independently associated with a lower NRM and a longer OS. In a concomitant study, reaching an NKT/T cell ratio above 10^{-4} within the first 18 months after CD34-selected HLA-haploidentical pediatric HSCT associated with the maintenance of disease remission in all children (de Lalla et al., 2011; Casorati et al., 2012).

TREC ANALYSIS

The molecular analysis of TCR excision circles (TRECs) in circulating T cells allows to quantitatively assess host thymic function, a parameter that has been shown to play a fundamental role in the rapidity of T cell immune reconstitution after HSCT (Talvensaari et al., 2002). After categorization of data from a retrospective cohort, the group of Antoine Toubert has prospectively shown that a pre-transplant TREC content above the threshold of 172 per 150,000 $CD3^+$ T cells is an independent factor associated with less infections, including Cytomegalovirus (CMV) reactivation, and a longer OS after T cell-replete HLA-sib HSCT (Clave et al., 2005). The same group has found that at 6 months after CD34-selected HLA-haploidentical pediatric HSCT, a TREC value below detection levels (<0.1 per 150,000 $CD3^+$ T cells for sjTREC and <0.001 per 150,000 $CD3^+$ T cells for βTREC) was associated with a higher relapse incidence (Clave et al., 2012).

NATURAL TREGS

In animal models of HSCT, natural regulatory T cells (Tregs) have a key role in promoting tolerance and, in particular, in protecting from GVHD (Nguyen et al., 2006). In humans, however, there are a number of controversial issues that so far have prevented from confirming the value of Tregs assessment for predicting the risk of GVHD, its grading and response to therapy. These include how to discriminate Tregs from activated T cells and what is the most appropriate way to express Tregs measurements.

In patients with acute GVHD after HLA-sib or MUD HSCT, Tregs frequencies measured at disease onset as the percentage of $CD4^+CD25^{bright}Foxp3^+$ T cells over total nucleated cells were reported to inversely correlate with acute GVHD grading (Magenau et al., 2010). Moreover, Tregs frequencies above the median, i.e., >0.5%, were associated with complete response to first-line therapy, resulting in a lower TRM and a longer OS. In another study considering patients with gastrointestinal GVHD, however, peripheral blood as well as mucosal Tregs frequencies, measured as the percentage of $CD4^+$ co-expressing Foxp3, were not found to correlate with disease severity (Lord et al., 2011).

The evaluation of Tregs biomarkers for predictive purposes has also yielded conflicting results in chronic GVHD. Some authors have found a paradoxical increase in Tregs measured both as the percentage and as the absolute count of $CD4^+CD25^{bright}$ (Clark et al., 2004). These T cells were later found to be suppressive *ex vivo*, ruling out that they were activated T cells in disguise. On the contrary, other authors have reported that Tregs frequencies measured as the percentage of $CD4^+CD25^{bright}$ T cells over peripheral blood lymphocytes below 3%, a threshold derived from linear and logistic regression, were associated with chronic GVHD. This threshold was derived from linear regression models based on data from healthy donors (Zorn et al., 2005). In a phase I/II trial investigating the administration of low dose IL-2 in chronic GVHD, the same group has found that changes in the median count of Tregs somewhat correlated with the probability of responding to the treatment (Koreth et al., 2011).

PATHOGEN-SPECIFIC T CELLS

The value of pathogen-specific T cell responses as an immune biomarker predictive of the risk and the severity of opportunistic infections after HSCT is still controversial. This is mostly due to the use of different methods for measurement (MHC-peptide tetramers, intracellular cytokine staining, ELISPOT assays) and

to the lack of harmonized protocols between the different studies. Other contentious issues are whether it is sufficient assessing either $CD8^+$ or $CD4^+$ responses or it is needed considering both, and whether complex and costly biomarkers are worthy compared with easier, cheaper, and already validated tests, such as serology (Ljungman et al., 2003).

Since CMV disease is a major complication after HSCT, the majority of the studies have focused their attention on CMV-specific responses. In an early study investigating the use of tetramers, it was found that reaching 10 CMV-specific $CD8^+$ T cells/μL within the first 100 days after HSCT from an HLA-id or a MUD with discordant serology associated with a zero likelihood of CMV disease (Cwynarski et al., 2001). The predictive value of the CMV-specific $CD8^+$ T cell count measured with tetramers was confirmed in a multicenter, prospective study including HLA-sib and MUD HSCT where, after categorization, it was found that a value above the threshold of 7 cells/μL associated with a lower incidence of CMV disease (Gratama et al., 2010).

Other studies have examined the predictive value of CMV-specific $CD4^+$ T cell responses showing comparable results. In a pioneering study by the group of Hermann Einsele, it was found that a positive $CD4^+$ T cell proliferative response defined as a stimulation index above the arbitrary value of 2.5 within the first 120 days after HLA-sib or MUD HSCT associated with a reduced incidence of CMV disease (Krause et al., 1997). The predictive value of studying $CD4^+$ T cell responses was confirmed by using intracellular cytokine staining. In a similar setting, reaching 0.4 CMV-specific $CD4^+$ T cells/μL within day 30 and 50, for example, was found to associate with complete prevention from subsequent CMV reactivation (Pourgheysari et al., 2009). In another study, a positive proliferative response to either CMV, HSV, or VZV defined as a stimulation index above 7 was associated not only with a zero probability of opportunistic infections, but also with the maintenance of disease remission, indicating that the recovery of pathogen-specific immunity may serve as a surrogate biomarker of immune restoration (Parkman et al., 2006).

In certain studies, the concomitant exploration of both $CD4^+$ and $CD8^+$ CMV-specific T cells allowed determining the predictive thresholds for the two subsets in a compared manner. In a pediatric population, after ROC analysis of intracellular cytokine staining data from a retrospective cohort (Lilleri et al., 2006), it was found that reaching a CMV-specific T cell count above the threshold of 1/μL for $CD4^+$ T cells and of 3/μL for $CD8^+$ T cells within the first year after HLA-sib, MUD, or HLA-haploidentical pediatric HSCT associated with a zero likelihood of CMV reactivation up to 2 years thereafter (Lilleri et al., 2012). These thresholds were found to be remarkably similar (1.2 and 1/μL for $CD4^+$ and $CD8^+$ CMV-specific T cells, respectively) in HLA-sib and MUD adult HSCT (Tormo et al., 2011) suggesting that measuring pathogen-specific responses in both subsets may be of predictive value, although with slightly different thresholds.

The results of the studies on Epstein-Barr virus (EBV)-specific responses are more controversial. Although measuring EBV-specific T cell responses by intracellular cytokine staining was found to have no value in predicting the likelihood of post-transplant lymphoproliferative disease (Hoegh-Petersen et al., 2011), finding at day 56 an EBV-specific T cell score above 1, a threshold found after categorization of a complex measure including CD4 and CD8 viral epitopes, was associated with a near-zero likelihood of leukemia relapse (Hoegh-Petersen et al., 2012).

NK-CELLS

The discovery that NK alloreactivity plays a major role in preventing disease relapse after CD34-selected HLA-haploidentical HSCT (Ruggeri et al., 2002), has fostered a number of studies investigating the predictive value of NK-cell biomarkers on clinical outcome. The reconstitution of NK-cells post-transplant is slightly slower compared to other cells of the innate immune system, but definitively faster than conventional T cells. At 30 days after T cell-depleted HLA-sib HSCT, an NK-cell count above the median (>150/μL) was found to associate with less acute GVHD, a lower relapse incidence, and a longer OS (Savani et al., 2007b). The association between higher NK-cell counts and lower relapse incidence was however restricted to patients with myeloid leukemia, a disease that is susceptible to NK lysis. The predictive value of NK-cell counts at early time points post-transplant was confirmed in the setting of unmanipulated HLA-haploidentical HSCT, where, after categorization, an NK-cell count above the threshold of 9.27/μL as early as 15 days post-transplant was associated with a longer LFS (Chang et al., 2008). The picture was shown to differ in the context of T cell-replete HSCT, where higher NK-cell counts (>150/μL, a threshold taken from previous studies (Savani et al., 2007b) were associated with a lower TRM at late time points, but not with a lower relapse incidence (Buhlmann et al., 2011).

CONCLUSION

In the era of predictive and molecular medicine, the practice of HSCT is still characterized by many prognostic uncertainties. Since many complications of HSCT derive from the state of temporary, although often prolonged, state of immunodeficiency post-transplant, it is clear that finding a tight correlation between certain immune system defects and the different complications may help predict the overall clinical outcome. This is important not only for improving the care of patients, who may expect benefits from ready and tailored strategies of intervention, such as intensification or discontinuation of antimicrobial and immune suppressive drugs, but also for establishing accepted surrogate markers of immune restoration that may accelerate the clinical development of novel transplant strategies, including the transfer of pathogen-specific T cells generated after *ex vivo* stimulation (Feuchtinger et al., 2010; Heslop et al., 2010).

The road leading to the validation of immune biomarkers answering to this crucial, unmet need is long and winding, and is possibly better traveled by joining forces in multicenter efforts. The recent launch of different, retrospective, and prospective studies coordinated by the Immunobiology Working Party of the European Bone Marrow Transplantation society goes exactly in this direction and is expected to contribute to filling this gap in the near future.

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Thymic function recovery after unrelated donor cord blood or T-cell depleted HLA-haploidentical stem cell transplantation correlates with leukemia relapse

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Use of alternative donors/sources of hematopoietic stem cells (HSC), such as cord blood (CB) or HLA-haploidentical (Haplo)-related donors, is associated with a significant delay in immune reconstitution after transplantation. Long-term T-cell immune reconstitution largely relies on the generation of new T cells in the recipient thymus, which can be evaluated through signal joint (sj) and beta T-cell-Receptor Excision Circles (TREC) quantification. We studied two groups of 33 and 24 children receiving, respectively, HSC Transplantation (HSCT) from an HLA-haploidentical family donor or an unrelated CB donor, for both malignant (46) and non-malignant disorders (11). Relative and absolute sj and beta-TREC values indicated comparable thymic function reconstitution at 3 and 6 months after the allograft in both groups. Compared to children with non-malignant disorders, those with hematological malignancies had significantly lower pre-transplantation TREC counts. Patients who relapsed after HSCT had a significantly less efficient thymic function both before and 6 months after HSCT with especially low beta-TREC values, this finding suggesting an impact of early intra-thymic T-cell differentiation on the occurrence of leukemia relapse.

Keywords: HSCT, thymic function, T cells, relapse, leukemia

INTRODUCTION

Whenever an HLA-identical related donor is not available, alternative sources of donors/stem cells are to be used for Allogeneic Hematopoietic Stem Cell Transplantation (HSCT) (Beatty et al., 1995). Both unrelated Cord Blood (CB) and HLA-haploidentical (Haplo) family donors provide an effective treatment for many malignant and non-malignant diseases in children (Rocha and Locatelli, 2008). For the latter, the HLA disparity in the donor/recipient pair may lead to a beneficial and selective graft-versus-leukemia (GVL) effect, associated with natural killer (NK)-cell mediated alloreactivity (Ruggeri et al., 2002). Despite improvement in the graft procedure such as use of T-cell depletion (TCD) and infusion of high doses of CD34+ cells, which allows engraftment and reduces graft-versus-host disease (GvHD) in Haplo-HSCT recipients (Aversa et al., 1994; Handgretinger et al., 2001), a significant delay in immune reconstitution still remains a relevant problem, leading to a high incidence of both opportunistic infection and, in the absence of NK alloreactivity, to relapse (Ball et al., 2005; Wils et al., 2011). In this respect, association between poor clinical outcome and low lymphocyte counts early after CB- or Haplo-HSCT (Ciurea et al., 2011) or at month 6 in case of T-cell depleted HSCT (Novitzky et al., 2002) has been described. Moreover, we showed recently, in 33 Haplo-HSCT patients an association between low thymic function and an increased risk of leukemia relapse (Clave et al., 2012).

Recently, tools have been developed to monitor thymic function. Signal-joint T-cell-Receptor (TCR) Excision Circles (sjTREC) are small episomal DNA that results from the deletion of the TCR δ region during TCR α locus rearrangement. They are not replicated during lymphocyte cell division and reflect, in the periphery, the number of new lymphocytes generated in the thymus (Douek et al., 1998; Dion et al., 2007). BetaTREC are produced during TCR Dβ–Jβ recombination, occurring before TCR α-chain recombination in αβ T-cell differentiation. BetaTREC counts may therefore reflect the early stages of intrathymic differentiation and sj/beta TREC ratios the T-cell proliferation rate between β- and α-chain recombination events. Quantification of sj and beta-TREC molecules in peripheral blood cells has provided useful information in studies addressing the issues of aging,

HIV infection, and HSCT (Dion et al., 2004, 2007), but have not yet been used in the context of studies on leukemia relapse after HSCT.

In view of these considerations, we decided to investigate thymic function recovery in patients given unrelated donor CB transplantation (UCBT) and to compare the results obtained in this cohort of patients with those of children given Haplo-HSCT. In detail, we retrospectively evaluated thymic function recovery through sj and beta TREC quantification in 57 pediatric patients, 33 receiving Haplo-HSCT previously reported and 24 given UCBT. Thymic function recovery was found to be similar in both groups. Moreover, correlation of TREC values with clinical parameters revealed a link between recovery of thymic function and specific disease. Indeed, we found that pre-transplantation sj and beta TREC were lower for patients with malignant disease. Moreover, there was a strong correlation between low thymic function persisting at 6 month after HSCT and a higher incidence of relapse in the Haplo-HSCT group.

PATIENTS

Two groups of pediatric patients, 24 receiving UCBT and 33 given Haplo-HSCT (**Table 1**) for either malignant or non-malignant diseases were studied, the last group being the same reported in our previously published study (Clave et al., 2012). In order to be included, patients had to be alive 6 months after transplantation (so, 3 patients that relapsed before 6 months were also included). All patients had been transplanted between October 2004 and February 2007 after having received a fully-myeloablative conditioning regimen. Details on patient and donor characteristics, as well as on transplantation outcome, are reported in **Table 1**.

Table 1 | Patient-, donor-characteristics, and transplant outcomes.

	Haplo-HSCT $N = 33$	UCBT $N = 24$	p
RECIPIENT			
Male, *N* (%)	23 (70%)	14 (58%)	0.411
Median age, years (range)	7.7 (3–17)	4.7 (1–16)	<0.001
Hematological malignancies	27 (82%)	19 (79%)	0.999
Acute lymphoblastic leukemia	20	11	
Acute myeloid leukemia	3	6	
Myelodysplastic syndromes	3	0	
Juvenile myelomonocytic leukemia	1	2	
Other diagnosis			
Hemophagocytic lymphohistiocytosis	0	5	
Fanconi anemia	3	0	
Congenital amegakaryocytic thrombocytopenia	2	0	
Blackfan-Diamond anemia	1	0	
TRANSPLANTATION			
Source of cells PB/CB	33/0	0/24	<0.001
Conditioning regimen[a]: TBI/chemo-based	24/9	9/15	0.014
GvHD prophylaxis[b]: TCD/CsA + steroids	33/0	0/24	<0.001
Infused CD34+ cells: Median (range) $\times 10^6$/Kg	22 (8.7–41)		
Infused nucleated cells: Median (range) $\times 10^7$/Kg		5.05 (1.4–12.5)	
CLINICAL OUTCOMES			
Acute GvHD			
Grade (I/II/III/IV)	6/4/1/0	4/8/1/0	
Grade II–IV	5 (15%)	9 (37%)	0.067
Chronic GvHD	6 (18%)	2 (8%)	0.446
Relapse	8 (24%)	3 (12%)	0.326
Serious infections[c]	20 (61%)	13 (54%)	0.786

Haplo-HSCT indicates HLA-Haploidentical Hematopoietic Stem Cell Transplantation; UCBT, Unrelated Cord Blood Transplantation; CsA, cyclosporin A; GvHD, graft-versus-host disease; PB, peripheral blood; TBI, total body irradiation.

[a] TBI-based conditioning regimen was employed in 33 children and consisted of: fractionated TBI (12 Gy over 6 fractions in 3 days), Thiotepa (10 mg/Kg in 2 doses) and fludarabine (160 mg/m^2 over 4 days). Chemotherapy-based conditioning regimen were as follows: 12 patients received Busulfan (16 mg/Kg in 16 doses over 4 days), Cyclophosphamide (120 mg/Kg in 2 days) and melphalan (140 mg/m^2 in single dose); 7 children received Busulfan (16 mg/Kg in 16 doses over 4 days), Thiotepa (10 mg/Kg in 2 doses) and fludarabine (160 mg/m^2 over 4 days); and 5 children were given Treosulfan (14 gr/m^2 for 3 consecutive days), Thiotepa (10 mg/Kg in 2 doses) and fludarabine (160 mg/m^2 over 4 days).

[b] Patients receiving Haplo-HSCT were transplanted with CD34+ selected cells and were not given any immune-suppressive drug after transplantation. Patients transplanted with cord blood cells received a combination of Cyclosporine-A (3 mg/Kg/day) and steroids [methylprednisolone (2 mg/Kg/day)] as GvHD prophylaxis.

[c] Reactivation of viral infections (i.e., cytomegalovirus and Epstein-Barr virus) and proven/probable invasive aspergillosis.

No patient given Haplo-HSCT received post-transplantation pharmacologic immune suppression, while those transplanted with CB cells received a combination of CsA and steroids (see also **Table 1** for details). For the purpose of the study, patients with acute leukemia transplanted in 1st complete remission (CR) or in 2nd CR after a relapse occurring more than 6 months after treatment discontinuation, as well as patients affected by refractory cytopenia, were assigned to the early disease group. All other patients were included in the advanced disease group. Acute and chronic GvHD (cGvHD) were diagnosed and graded according to the Seattle criteria (Glucksberg et al., 1974; Storb et al., 1983). Patients surviving more than 14 and 100 days post-transplantation were evaluated for acute and cGvHD, respectively. The study was approved by the Ethical committee of Policlinico San Matteo, Pavia, Italy (approval number 446/DG).

METHODS

DESIGN OF THE STUDY

DNA samples of patients enrolled in the study were collected before transplantation and at 3 and 6 months after the allograft. At the same time-points, we evaluated absolute number and percentage of the different T lymphocyte subsets.

sjTREC AND beta-TREC QUANTIFICATION

Genomic DNA was extracted from Peripheral Blood Mononuclear Cells (PBMC) using the QIAamp DNA Blood Minikit (Qiagen, Hilden, Germany). Quantification of sjTREC and beta-TREC was performed by real-time quantitative PCR (qPCR) (ABIPRISM7500, Applied Biosystems, Foster City, CA), as previously described (Clave et al., 2009). Briefly, a first PCR reaction was carried out in multiplex with 3 different outer primer mixes, 1–5 μg of genomic DNA, 200 μM each dNTP, 2.5 mM MgCl2, 1× buffer and 1.25 unit of *Platinum*® Taq polymerase (Invitrogen, Cergy-Pontoise, France) in 50 μL (10 min at 95°C, then 19 cycles of 95°C, 30 s; 60°C, 30 s and 72°C, 2 min). Final quantification was made on ABI PRISM 7700, in duplicate with a second multiplex reaction that contains 5 μL of a 1/100 or 1/1000 dilution of the first PCR product, inner primers, and probe for sjTREC or one of the Dβ–Jβ segments, inner primers and probe for albumin (alb) gene, 1.25 mM each dNTP, 3 mM MgCl2, 1× buffer and 1.25 unit of *Platinum*® Taq polymerase in 25 μL (5 min at 95°C then 40 cycles of 95°C, 15 s and 60°C, 1 min). The sum of the 10 Dβ–Jβ segments was finally multiplied by 1.3 to extrapolate for all the 13 existing Dβ–Jβ segments. All primers and probes have been obtained from Eurogentec (Seraing, Belgium) except the alb vic-labeled probe from Applied Biosystems.

TREC data were validated only if at least 50,000 genome equivalents were detected by alb qPCR; therefore, some time-points were missing for 5 patients but other negative TREC values were not due to absence of genomic DNA. Data were first expressed per 150,000 PBMC and the total number of TREC per μL of blood was calculated using the absolute white blood cells count at time of sample collection.

FLOW CYTOMETRY ANALYSIS

FITC, PE, PerCP, or APC monoclonal antibodies (MoAbs) specific for the following antigens were employed for the evaluation of lymphocyte subsets: CD45, CD3, CD4, CD8 (BD Biosciences, Mountain View, CA). Appropriate isotype-matched controls (BD Bioscience) were included. Three-color or four-color cytometry, through direct immune fluorescence and FACSCalibur or FACSCanto cytometer (BD Biosciences), was performed.

STATISTICAL ANALYSIS

Non-parametric Mann–Whitney or Kruskal–Wallis tests were used to correlate the effect of different clinical parameters on TREC counts. Since data did not have a normal distribution, a logarithmic transformation was performed. Difference in cumulative incidence of relapse was estimated using univariate Kaplan–Meier analysis. *P*-values <0.05 were considered to be statistically significant. All analyses were performed using SPSS Statistics 20 Software.

RESULTS

THYMIC FUNCTION RECOVERY IS SIMILAR AFTER HAPLO-HSCT AND UCBT IN PEDIATRIC PATIENTS

The distribution of circulating $CD3^+$, $CD4^+$, or $CD8^+$ T-lymphocyte subsets, 3 and 6 months after HSCT, was variable, especially for patients given Haplo-HSCT (**Table 2**). At month 3, CD3+, CD4+, and CD8+ cell counts/μL blood were lower in Haplo-HSCT than in UCBT patients ($p = 0.003$, 0.005, and 0.010, respectively). At month 6, only CD4+ cells remained significantly lower ($p = 0.022$) in the former group. These results confirm the detrimental role of TCD of the graft on early reconstitution of mature T cells and the role played by homeostatic expansion of T cells transferred with the graft in the case of UCBT (Roux et al., 1996).

Reconstitution of newly generated naïve T cells relies on thymic differentiation of donor-derived lymphoid progenitors. We, therefore, evaluated recovery of thymic function through the quantification of sjTREC and beta-TREC on the samples collected at the same points in which lymphocyte counts were investigated. Before the allograft, the median number of sjTREC per 150,000 PBMC were 1375 (range 0–27,612) and 2035 (range 0–16,626) for Haplo-HSCT and UCBT patients, respectively ($p =$ not significant, NS). Three months after the allograft the median number of sjTREC per 150,000 PBMC had dropped to 27 (0–1973) and 45 (0–1284) for Haplo-HSCT and UCBT patients, respectively. At 6 months, although there was a great inter-patient variability, the median number of sjTREC numbers almost returned to pre-graft levels with 281 (0–31,286) and 647 (0–16,395) sjTREC/150,000 PBMC, for Haplo-HSCT and UCBT patients (**Figure 1A**), respectively. The recovery of betaTREC paralleled that of sjTREC (**Figure 1B**) without any significant difference between the two groups of patients. Accordingly, at each time points, the number of sjTREC highly correlated with the number of beta-TREC ($r = 0.66$, $p < 0.001$, Pearson correlation).

Since proliferation of memory or effector T cells could affect TREC number per PBMC by dilution, we also expressed sjTREC and beta-TREC counts per microliter of patient blood using absolute cell counts. Absolute numbers of sjTREC (**Figure 1C**) and

Table 2 | Median number (range) of circulating T-lymphocyte subsets three (M3) and six (M6) months after HSCT (cells $\times 10^3/\mu l$).

		M3				M6			
		N	**CD3+**	**CD4+**	**CD8+**	***N***	**CD3+**	**CD4+**	**CD8+**
All patients	Haplo	33	264 (0–2666)	68 (0–1767)	80 (0–1666)	30	610 (140–2992)	242 (88–760)	270 (14–2596)
	UCBT	23	540 (180–4661)	190 (72–885)	280 (18–4092)	21	852 (352–6440)	336 (105–1288)	456 (80–5152)
	p		0.003	0.005	0.010		0.058	0.022	0.151
Malignant Haplo-HSCT	Relapse	8	279 (4–572)	29 (0–264)	84 (0–308)	7	867 (154–2460)	234 (98–369)	480 (14–2132)
	CR	19	252 (0–2666)	138 (0–1767)	63 (0–1666)	17	560 (140–2992)	273 (88–546)	248 (14–2596)
	p		0.894	0.300	0.831		0.193	0.975	0.075
Malignant UCBT	Relapse	3	1040 (266–4661)	234 (91–885)	819 (154–3540)	3	1250 (852–1288)	324 (250–529)	759 (516–1000)
	CR	15	715 (180–4465)	153 (72–616)	476 (18–4092)	14	811 (352–6440)	322 (105–1288)	442 (80–5152)
	p		0.441	0.594	0.441		0.314	0.801	0.313

M3 stands for Month 3; M6, Month 6, and CR, Complete remission.

beta-TREC (**Figure 1D**) followed the same kinetics as numbers of TREC/150,000 PBMC, without difference between Haplo-HSCT and UCBT patients, this ruling out an indirect, confounding effect of T-cell proliferation. Only absolute sj and beta-TREC numbers per μL will be used afterwards.

Since sjTREC value as a marker of thymic output is dependent on age (Douek et al., 1998), we also compared the median age of patients in the two groups. Haplo-HSCT patients were indeed significantly older ($p = 0.0008$) than UCBT patients (median age being 7.7 and 4.7 years, range 3–17 and 0.75–16 years, respectively). Thus, from the observed TREC values, we can conclude that, despite an older age, thymic reconstitution was equally effective in patients given either Haplo-HSCT or UCBT.

Since GvHD has a major impact on immune reconstitution after allogeneic HSCT, we studied its influence on both groups of patients. There were less cases of acute GvHD (aGvHD) in Haplo-HSCT patients (**Table 1**). We did not see any significant difference for sjTREC/μL of blood between patients without or with grade I aGvHD and patients with grade II and III aGvHD (**Figure 1E**), in both groups of patients. Incidence of cGvHD was low in both groups with 6 (18%) and 2 cases (8%) in the Haplo-HSCT and UCBT cohorts, respectively. TREC values were lower at month 6, in both groups, when cGvHD occurred, but the difference was not statistically significant (**Figure 1F**).

PATIENTS AFFECTED BY HEMATOLOGICAL MALIGNANCIES HAVE LOWER PRE-TRANSPLANT THYMIC FUNCTION

Diagnosis may also have an impact on recovery of thymic function (Petridou et al., 2002; Clave et al., 2005). Indeed, TREC values before transplantation were significantly lower in the 46 patients with hematological malignancies (median 10 sjTREC/μL blood, range 0–674) than in the 11 with non-malignant disorders (median, 235 sjTREC/μL blood, range 97–1340, $p = 0.0002$) (**Figure 2**). This finding was not influenced by the type of allograft that the patients received (data not shown). There was no significant difference in age between the two groups of patients, affected or not by hematological malignancy (median 6.6 vs. 5.0 years, range 1–9 vs. 1–17, $p = 0.18$) that could explain a lower thymic function in patients with malignancy. Before transplantation, patients with early disease had higher sj and beta-TREC than those with more advanced disease (**Figure 2**). Moreover, median numbers of betaTREC/μL of blood were also significantly lower in patients treated for malignancies (median 1.7 vs. 39, $p = 0.004$) (**Figure 2**), suggesting an effect of the disease itself or of previous treatment on the T-cell progenitor compartment or before β-chain recombination.

A LOW THYMIC FUNCTION IS ASSOCIATED WITH A HIGHER RELAPSE RISK

We have previously shown in the group of Haplo-HSCT patients that a low thymic function before transplantation or after 6 months, was associated with an increased risk of relapse (Clave et al., 2012). Here, thymic reconstitution appears similar after both UCBT and Haplo-HSCT. Thus, we decided to merge the 19 UCBT to the 27 Haplo-HSCT cases affected by malignancies. Among the 46 patients treated for malignancies, 11 relapsed: 8 after Haplo-HSCT and 3 after UCBT (see **Table 1**). The median time between transplantation and relapse was 10 months (range 1.6–18 months).

When considering both patients given UCBT and those receiving Haplo-HSCT, the median number of sjTREC/μL blood was low at 3 months and returned to the initial level at month 6. With a median follow-up of 43 months, patients who relapsed had lower values than the relapse-free patients both before transplantation ($p < 0.05$) and during follow-up ($p = 0.014$ and 0.006, at 3 and 6 months, respectively) (**Figure 3A**). Values of beta-TREC before transplantation and, even more significantly, after 6 months (**Figure 3B**) were also lower in patients who subsequently relapsed ($p = 0.037$ and 0.009, respectively).

We also carefully evaluated the occurrence of relapse in patients with a poor thymic function. We calculated the cumulative incidence of relapse in patients with a detectable thymic function compared to those without any thymic function (based on the detection sensitivity of the sj and beta TREC assays less than 0.1 and 0.01/150,000 PBMC, respectively). Because values at month 3 were too low to define this cut-off reliably, we studied only the values before transplantation and those at month 6 post transplantation. Absence of detectable sjTREC at each time

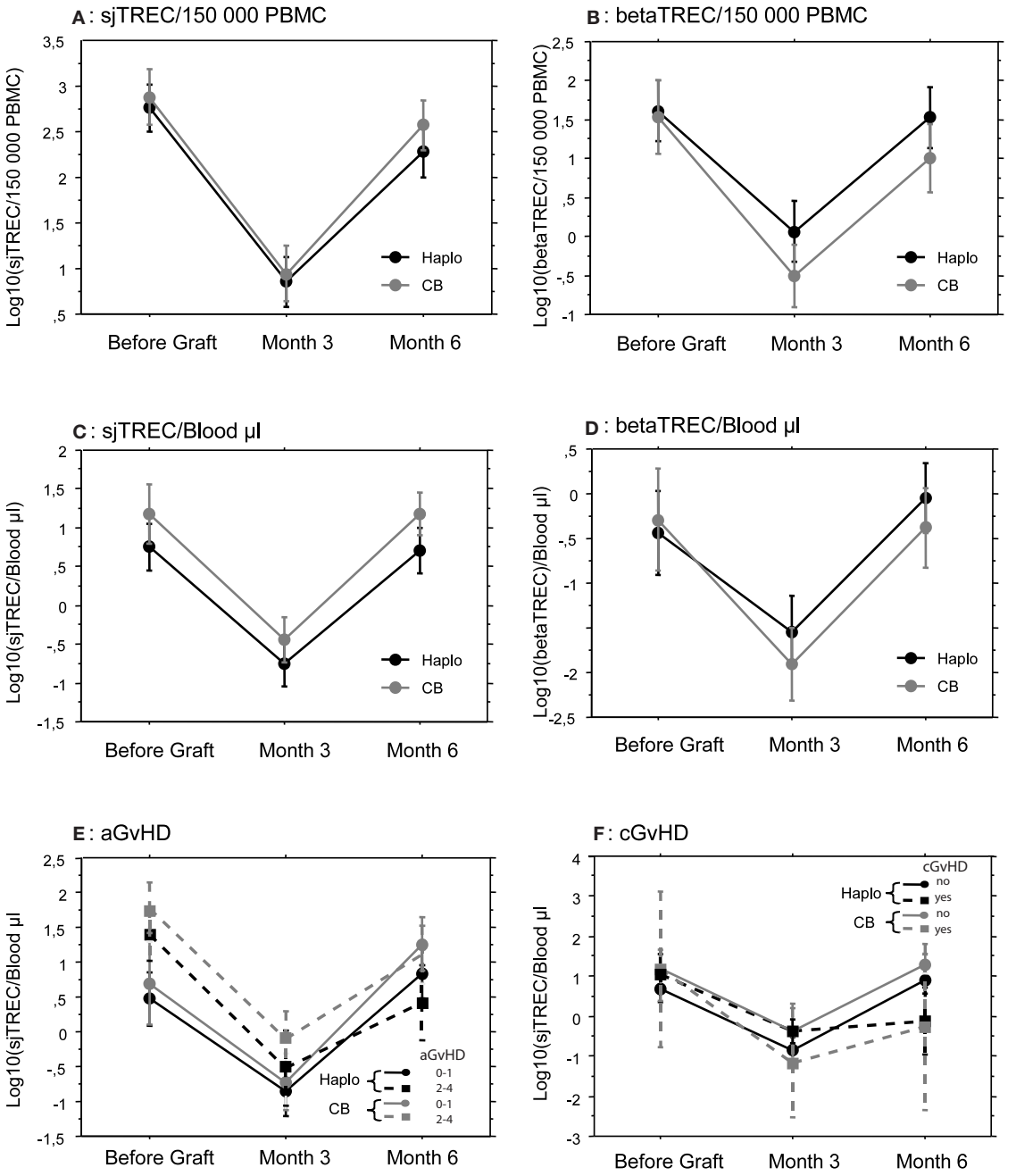

FIGURE 1 | Thymic reconstitution is not dependent on the stem cell source employed or GvHD occurrence. Mean (±SE) number of log10 TREC was measured by quantitative PCR, before, and 3 and 6 months after transplantation in patients that received either a haploidentical hematopoietic Stem Cell Transplantation (Haplo, $N = 33$) or a Cord Blood Unrelated Donor Graft (CB, $N = 24$). Signal Joint (sj) TREC were quantified in (**A,C,E,** and **F**) and betaTREC in (**B** and **D**). Results were expressed by 150,000 Peripheral Blood Mononuclear Cells in (**A** and **B**) and by µL of blood in (**C,D,E,** and **F**). Patients were subsequently subdivided according to GvHD occurrence (**E** and **F**).

point correlated with an increased incidence of relapse, although this correlation was not statistically significant, due to the limited number of events (data not shown). More notably, lack of detectable betaTREC before and at month 6 after the allograft was strongly associated to a higher incidence of relapse ($p = 0.03$ and 0.02, respectively) (**Figures 3C,D**). Use of median sj or beta-TREC value to split patient groups as low or high TREC gave similar results (data not shown). We also analyzed, in patients treated for malignancies, other parameters that have been shown to influence relapse rate, but neither age ($p = 0.28$), high-risk disease ($p = 0.73$), conditioning regimen ($p = 0.24$) nor number of CD34+ cells infused (for Haplo-HSCT patients, $p = 0.72$)

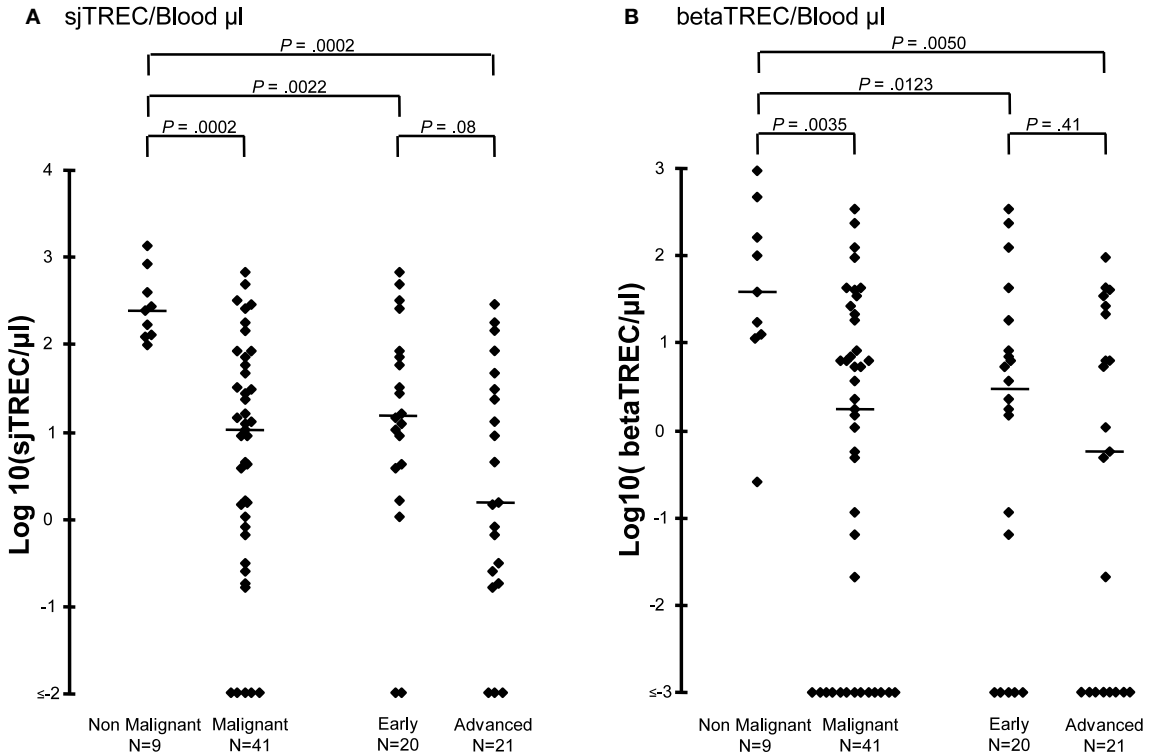

FIGURE 2 | Thymic function before transplantation is lower in patients affected by a hematological malignancy. Log10 sjTREC/blood µL **(A)** and Log10 betaTREC/blood µL **(B)** are shown before HSCT for patients diagnosed with either a malignant or a non-malignant hematological disease. Patients with malignancy were subsequently subdivided according to their disease status. Horizontal bars represent the median.

had a significant impact on relapse in univariate analysis in this relatively small cohort of patients.

DISCUSSION

Alternative sources of stem cells/donors are increasingly used in the treatment of children with both malignant and non-malignant diseases in need of an allograft (Copelan, 2006). However, their use remains associated with delayed immune reconstitution and a high rate of infectious complications (Aversa et al., 1998). Using sj and beta-TREC quantification, we found that thymic function had the same pattern of reconstitution in patients that had been transplanted from either an HLA haploidentical family donor or an unrelated CB donor despite the difference in stem cell source and transplant procedure. It is known that TCD of the graft (*in vivo* or *ex vivo*) has a huge impact on early reconstitution of the lymphocyte compartment, since patients cannot benefit from the homeostatic expansion of lymphocytes transferred with the graft (Roux et al., 1996). Accordingly, we found that reconstitution of T-cell subset numbers (i.e., CD4+ and CD8+ T cell subpopulations) was faster in UCBT patients than in those given Haplo-HSCT. However, median values of beta and sjTREC were similar in both groups, this indicating that, despite TCD of the graft, a larger use of total body irradiation (**Table 1**) and an older age for Haplo-HSCT recipients, recovery of thymic function was equally effective in patients given either Haplo-HSCT or UCBT. These results also illustrate that the TREC assay provides additional and more detailed information on T-cell reconstitution, in particular on that of newly re-generated T lymphocytes which mainly accounts for the medium and long-term patient's immune competence, than crude T cell subsets counts.

We found a very limited impact of GvHD (Hazenberg et al., 2002; Storek et al., 2002; Clave et al., 2009), and especially of aGvHD, on recovery of thymic function in both groups. This could be explained by both the limited incidence of this complication in the 2 groups and by the young age of our patients. Indeed, in a previous study we reported that young age at time of the allograft correlates with a better thymic function recovery (Clave et al., 2009).

In accordance with what we and others have previously shown (Petridou et al., 2002; Chen et al., 2005; Clave et al., 2005), patients with malignant diseases had lower TREC values already before the allograft than those with non-malignant disorders. However, in those previously published studies, the groups were not matched for age (Petridou et al., 2002; Clave et al., 2005) and only the relative number of TREC was assessed (Petridou et al., 2002; Chen et al., 2005), leaving the possibility of TREC dilution due to peripheral proliferation. Here, the median age in groups of patients affected by either a malignant or a non-malignant disease was similar and the number of TREC was given for unit of blood volume. Moreover, we specifically assessed beta-TREC,

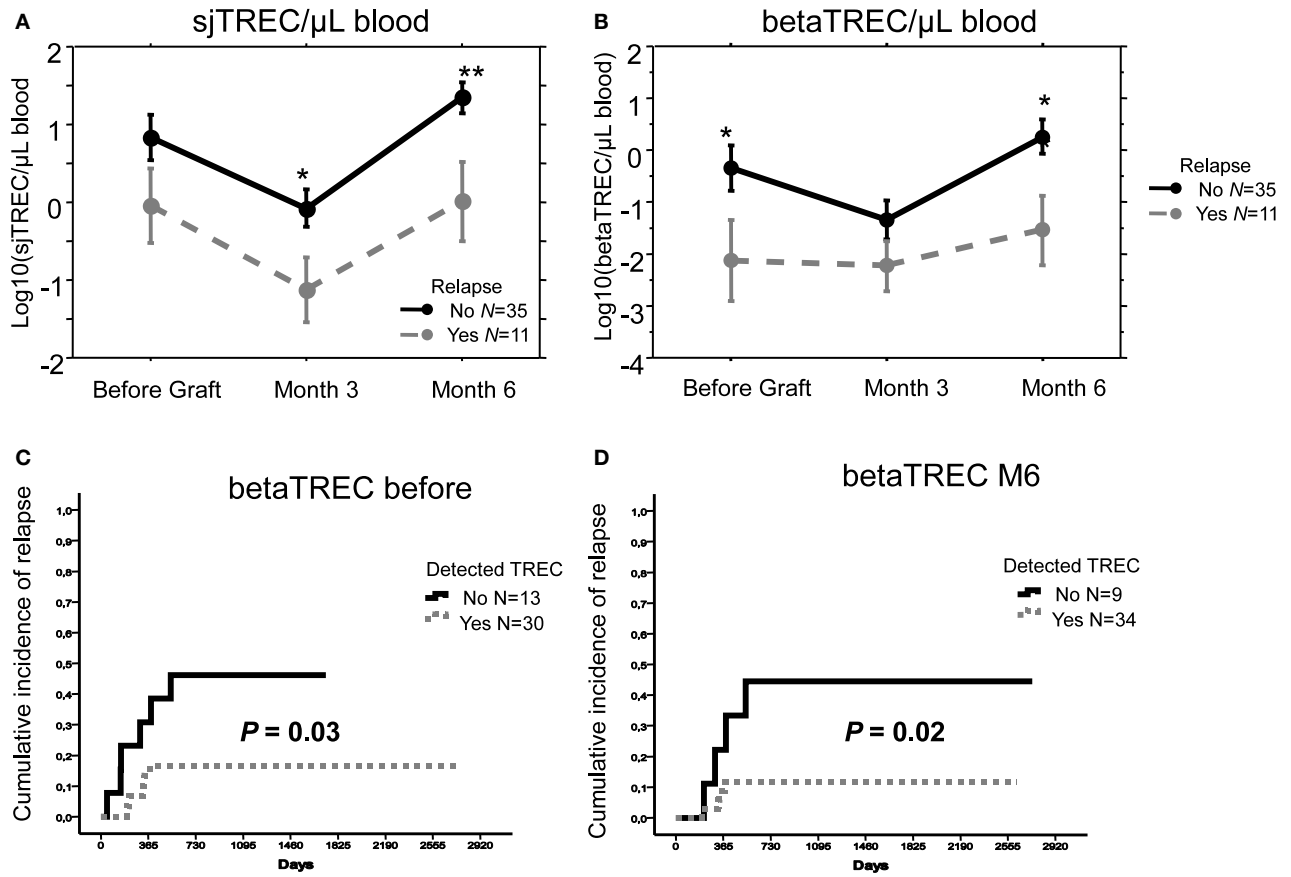

FIGURE 3 | A low thymic function is associated with a higher relapse risk. Mean (±SE) number of log10 sjTREC/ Blood µL **(A)** and betaTREC/ Blood µL **(B)**, before, 3 and 6 months after transplantation in patients with hematological malignancies that received either a T-cell depleted haploidentical Hematopoietic Stem Cell Transplantation or an Unrelated Donor Cord Blood Transplantation (Mal.) or the former type of allograft only (Haplo). Patients who experienced relapse were represented with gray dotted lines, while patients maintaining complete remission with black plain lines. $^{*}p < 0.05$, $^{**}p < 0.01$. Cumulative incidence of relapse in patients affected by malignant disorders who did or did not have detectable betaTREC before **(C)**, and at month 6 after graft **(D)** (p in Log Rank test).

a marker of intrathymic proliferation of T-cells undergoing differentiation. Beta-TREC values were also reduced in patients with malignancies, suggesting a mechanistic impact of the disease itself or its treatment on T-cell generation before beta chain TCR recombination, i.e., at the progenitor level and/or after homing to the thymus. In this respect, previous studies indicating the lack of association between TREC and chemotherapy (Petridou et al., 2002; Chen et al., 2005), could argue for a direct impact of hematological malignancy on thymic function rather than for a role of previous treatment.

In conclusion, we have previously shown an association between low levels of sj and beta-TREC and disease recurrence in the group of 27 haplo-HSCT patients with malignancies (Clave et al., 2012). Given the similarity in thymic function recovery of these patients with those treated with UCBT, by adding to the analysis the results obtained for these patients, we could increase to 46 the total number of patient treated for malignancies. With this addition, patients who relapsed still had a lower count of sj and beta TREC (**Figures 3A**,**B**) and patients with a low value of beta-TREC, both before and at month 6 after transplantation, had a significantly higher incidence of relapse (**Figures 3C**,**D**). Since the cohort size did not allow us to perform a multivariate analysis, we cannot exclude that the effect on relapse could be due to other disease variable. However, none of the different clinical parameters we tested such as disease risk or patient age, had a significant effect on relapse in univariate analysis. Therefore, the significant correlation of beta-TREC with relapse 6 months after transplantation suggest that a post-graft thymic differentiation of naïve T cells with anti-leukemia potential could play a crucial role in preventing relapse and mediating GVL.

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Monitoring of pathogen-specific T-cell immune reconstitution after allogeneic hematopoietic stem cell transplantation

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The clinical outcome after allogeneic hematopoietic stem cell transplantation (HSCT) has been significantly improved during the last decades with regard to the reduction in organ failure, infection, and severe acute graft-versus-host disease. However, severe complications due to infectious diseases are still one of the major causes of morbidity and mortality after allogeneic HSCT, in particular in patients receiving haploidentical HSCT or cord blood transplant due to a slow and often incomplete immune reconstitution. In order to improve the immune control of pathogens without an increased risk of alloreactivity, adoptive immunotherapy using highly enriched pathogen-specific T cells offers a promising approach. In order to identify patients who are at high risk for infectious diseases, several monitoring assays have been developed with potential for the guidance of immunosuppressive drugs and adoptive immunotherapy in clinical practice. In this article, we aim to give a comprehensive overview regarding current developments of T-cell monitoring techniques focusing on T cells against viruses and fungi. In particular, we will focus on rather simple, fast, non-labor-intensive, cellular assays which could be integrated in routine clinical screening approaches.

Keywords: virus, fungi, T cell, immune reconstitution, allogeneic stem cell transplantation

INTRODUCTION

The clinical outcome after allogeneic hematopoietic stem cell transplantation (HSCT) has been significantly improved during the last decades with regard to the reduction in organ failure, infection, and severe acute graft-versus-host disease (GVHD). These advances have rendered allogeneic HSCT to an integral part of treatment for hematological malignancies (1, 2). However, severe complications due to infectious diseases are still one of the major causes of morbidity and mortality after allogeneic HSCT, in particular in patients receiving haploidentical HSCT or cord blood transplant due to a slow and often incomplete immune reconstitution. The reduction of immunosuppressive drugs could pave the way to strengthen T-cell responses against pathogens after allogeneic HSCT. However, blind rapid tapering or cessation of immunosuppressive drugs is associated with an increased risk of alloreaction with subsequent clinical consequences such as increase of severe acute or chronic GVHD as demonstrated previously (3, 4). In order to improve the immune control of pathogens without an increased risk of alloreactivity, adoptive immunotherapy using highly enriched pathogen-specific T cells offers a promising approach. Adoptive immunotherapy against several pathogens has been already evaluated within clinical trials as reviewed previously (5).

In order to identify patients who are at high risk for infectious diseases, several monitoring assays have been developed with potential for the guidance of immunosuppressive drugs and adoptive immunotherapy in clinical practice. In this article, we aim to give a comprehensive overview regarding current developments of T-cell monitoring techniques focusing on T cells against viruses and fungi. In particular, we will focus on rather simple, fast, non-labor-intensive, cellular assays which could be integrated in routine clinical screening approaches.

THE ROLE OF PATHOGEN-SPECIFIC IMMUNITY IN PREVENTION AND CONTROL OF INFECTIOUS DISEASES

VIRUS-SPECIFIC T-CELL IMMUNITY

It is well-known that virus-specific T cells are important to prevent and/or control viral infection after allogeneic HSCT. Cytomegalovirus (CMV) is one of the most intensively investigated targets of immunotherapy after allogeneic HSCT (6). After allogeneic HSCT, the first emergence of CMV reactive antigenemia triggers the expansion of donor-derived CMV-specific T cells. These expanded cells usually have a phenotype of effector- or effector-memory type. The presence of CMV-specific T cells in patients after allogeneic HSCT was reported to be protective against the recurrence of CMV antigenemia (7–12). Especially, CMV seropositive patients with profound immunosuppression or CMV seropositive patients who received stem cells from a CMV-seronegative donor are at high risk for a significant delay in reconstitution of functional CMV-specific T cell which is associated with persistent CMV viremia and a higher risk of CMV disease (8–12). Furthermore, adoptive T-cell therapy

of CMV-specific T cells was demonstrated to be effective for the prophylaxis and treatment of CMV disease after allogeneic HSCT (13, 14).

The importance of virus-specific T cells has been also demonstrated with regard to other viruses such as adenovirus (15–18), EB virus (19–21), BK virus (22–25), and JC virus (26, 27). Recently, banked third party virus-specific T cells were reported to be safe and effective for the treatment of viral disease after allogeneic HSCT, which circumvents a major obstacle to the wider use of virus-specific T cells, in particular in patients after cord blood transplant (28).

The monitoring of T-cell immunity against these viruses can be useful to assess the risk of viral infections. The benefit of adoptive T-cell therapy as prophylaxis or as treatment should be ideally assessed in prospective clinical trials.

FUNGUS-SPECIFIC T-CELL IMMUNITY

For a long time fungus-specific T cells have not been regarded as important to control fungal diseases. However, there is growing evidence that $CD4^+$ T cells provide defense mechanisms against fungal infection (29–32). The majority of patients diagnosed with invasive aspergillosis after allogeneic HSCT are not neutropenic which for a long time was considered the only or at least the most important immune mechanism to prevent fungal disease (33, 34). Recent studies have shown that fungus-specific T cells are detectable in healthy individuals and patients with hematological malignancies (29, 32, 35). Due to the paucity of clinical studies which assessed the impact of presence of fungus-specific T cells compared to virus-specific T cells, further prospective studies which assess the importance of fungus-specific T cells on preventing/controlling fungal infection are urgently needed.

In addition, the improved outcome of invasive *Aspergillus* following adoptive T-cell therapy for invasive Aspergillosis demonstrates the clinical value of transfer of fungus-specific T cells from the stem cell donor (36). Furthermore, recent reports showed that the GMP-grade-Aspergillus-specific T cells could be produced for clinical trials using commercially available enrichment protocols (37–40).

HOW CAN PATHOGEN-SPECIFIC IMMUNITY BE MONITORED?

Up to date, various methods are available to assess T-cell immunity against specific antigens. However, some methods like limiting-dilution assays are not feasible due to the labor-intensive works which cannot be a part of routine clinical practice. Here we summarize three simple broadly available methods which can be performed using peripheral blood mononuclear cells (PBMC) or whole blood without long-term *ex vivo* culture, and using commercially available reagents. In addition, PBMC can be frozen without the loss of the function when tested in intracellular cytokine staining (ICS) or Enzyme-linked immunosorbent spot (ELISPOT), which is practically very important with regard to reproducibility and standardization with strict quality control (41). Combinations of these assays are needed for the confirmation of results and comprehensive measurement of different T-cell functions. The advantages and disadvantages of each method are summarized in **Table 1**.

ENZYME-LINKED IMMUNOSORBENT SPOT

Enzyme-linked immunosorbent spot is one of the most established methods to detect functional immunity (42–44). In brief, PBMC are cultured for 18–24 h on an anticytokine capture antibody-coated membrane in the presence of an antigen. Following culture, each antigen-specific T cells will release cytokines that will bind to the capture antibody on the membrane. The cells are then washed and the secreted cytokines can be detected on the membrane by use of an enzymatically labeled antibody and insoluble chromogenic substrate. In this assay, frequencies of cytokine-secreting T cells can be counted after *in vitro* stimulation of PBMC by defined antigens/peptides without previous *ex vivo* expansion. In addition, ELISPOT assays allow the size and intensity of the spots to be calculated, which correlated with the amount of cytokines secreted by each cell. As shown in **Figure 1A**, we are able to detect the induction of IFN-γ after the stimulation with CMV pp65 IE-derived peptides in patients after allogeneic HSCT.

Enzyme-linked immunosorbent spot offers several advantages: (1) many samples can be tested simultaneously using one plate; (2) the secretion of cytokines can be assessed in contrast to the

Table 1 | Comparison of three T-cell assays.

Assay	Advantage	Disadvantage
ELISPOT	No cell fixation The same cells can be retested Suitable to test many samples simultaneously Cytotoxicity assay can be induced A lower number of cells required for analysis	Cell of origin of cytokine production unclear No sorting of cytokine-secreting cells possible
Intracellular cytokine staining	Assessment of multiple cytokines at single cell level Combination with phenotyping and cytotoxicity assay	Cells have to be fixated and permeabilized No sorting of vital cell populations possible
MHC-multimer staining	Combination with phenotyping Sorting of antigen-specific T cells, which can be used for adoptive T-cell therapy Detection of dysfunction/non-functional antigen-specific T cells, e.g., naïve T cells	Each tetramer has to be produced for respective HLA typing and peptide Not suitable for the assessment of cytokine secretion (functionality)

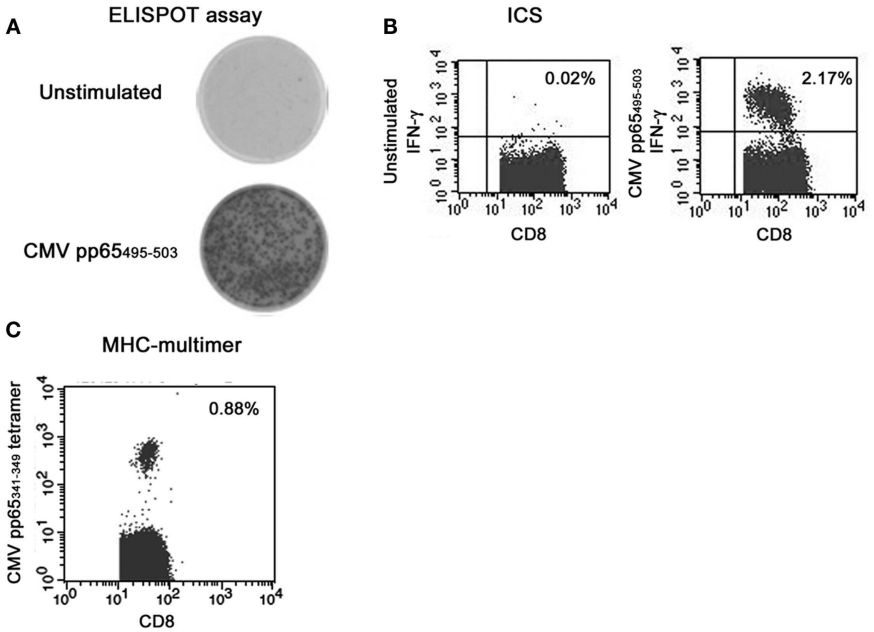

FIGURE 1 | Representative results of immune monitoring of CMV-specific T cells after allogeneic hematopoietic stem cell transplantation (A) ELISPOT assay, (B) intracellular cytokine staining, (C) tetramer.

artificially retained cytokines in ICS; (3) the cell numbers can be downscaled per well in comparison to flow cytometry-based methods.

Cytotoxic activity can be assessed using granzyme B ELISPOT. Granzyme B ELISPOT has been reported to have excellent correlation with the ^{51}Cr-release assay for measuring cytotoxic activity of T cells (45, 46). Furthermore, multiple-color fluorospot assays make the analysis of single cells secreting several cytokines possible (47, 48). Detecting each cytokine with a different fluorophore, polyfunctionality of T cells can be analyzed, suggested to be important to protect against various infectious diseases.

The disadvantages of ELISPOT are: (1) it is difficult to determine which immune cells secrete IFN-γ. This is critical to assess the immune status after allogeneic HSCT. As Wang and Colleagues reported, the response to 9-mer peptide, which is expected to induce cytokines in an HLA class I-restricted, can also be HLA class II-restricted (49, 50). Therefore, when IFN-γ induction in ELISPOT assay is detected using stimulation with lengths of peptides including peptide-pool, cell of origin of IFN-γ secretion has to be determined using CD4/CD8 depletion or HLA blocking assays; (2) sorting of cytokine-secreting cells is impossible.

INTRACELLULAR CYTOKINE STAINING

Intracellular cytokine staining is also one of the most established methods to detect functional immunity (51, 52). In brief, PBMC are cultured for 6–18 h in the presence of an antigen. To preserve the generated cytokines within the cytoplasm, a Golgi-blocking agent (e.g., Brefeldin A or Momensin) is added during the stimulation. After the stimulation, samples are collected, fixated and permeabilized. Consecutively, antibodies against intracellular cytokines are added. When surface markers whose binding is sensitive to fixation and permeabilization are stained in combination with ICS, they should be stained before fixation and permeabilization. Stained cells were analyzed using a flow cytometer. A representative result is shown in **Figure 1B**.

The advantages of ICS are as follows: (1) the phenotype of each cell which secretes the cytokine can be determined (53); (2) the cytolytic potential of the target cells can be assessed using CD107a degranulation assay in combination with the assessment of multiple cytokine induction.

The disadvantages of ICS are: (1) reagents such as Brefeldin A are required to retain cytokines in the cytoplasm; (2) the cells have to be permeabilized prior to the staining of the cells with antibodies against the cytokines, which makes it impossible to expand the sorted T cells.

MHC-MULTIMER STAINING

MHC-multimers are synthetic structures made from HLA molecules linked together to form a multimeric complex which are loaded with antigen-specific peptide. Cells stained with multimer and antibodies against surface markers can be analyzed using a flow cytometry. The fluorescence intensity using the tetramer loaded with a high-avidity peptide derived from virus is usually high enough to discriminate the positive population in contrast to the result using the tetramer loaded with a low-avidity peptide derived from autologous antigen (54). A representative result is shown in **Figure 1C**.

The advantages of multimer assays are as follows: (1) combined analysis of phenotyping and specificity can be performed using the antibodies against surface markers. MHC-multimer can detect T cells which do not secrete cytokines, for instance naïve T cells. In

combination with the phenotyping and MHC-multimer staining, we can assess the frequency of all antigen-specific T cells including dysfunctional/non-functional; (2) antigen-specific T cells can be sorted with a high purity. For this purpose, the streptamer technology is demonstrated to be useful (54, 55). Sorted cells can be used for adoptive T-cell therapy as a GMP-grade agent without regulatory issues (55).

The disadvantages of multimer staining are: (1) the multimer is not able to assess the functional status of antigen-specific T cells simultaneously. There can be a discrepancy in the frequency of antigen-specific T cells detected by multimer and by ELISPOT/ICS. Several papers reported that T cells detected by ICS were more important than those detected by multimer to control infectious diseases as demonstrated in the study of CMV infection (56–58). Multimer assays can be combined with functional assays, but it is well-known that the stimulation with a respective peptide leads to loss of multimer staining due to the downregulation of T-cell receptor, in particular when a high-avidity peptide is used (59); (2) MHC-multimer staining is HLA-specific and peptide-specific. Therefore the whole cell repertoire directed against a pathogen cannot yet be determined using MHC-multimer technology.

CONCLUSION

T-cell monitoring against specific targets including viruses and fungi is ready to be integrated in the clinical practice. The monitoring of pathogen-specific T cells may help to define the individual MPE of a patient to develop a certain infections complication and to assess the potential benefit of adoptive T-cell therapy against certain pathogens.

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Alloreactivity of virus-specific T cells: possible implication of graft-versus-host disease and graft-versus-leukemia effects

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Immune reconstitution of functional virus-specific T cells after allogeneic hematopoietic stem cell transplantation (HSCT) has been intensively investigated. However, the possible role of crossreactivity of these virus-specific T cells against allogeneic targets is still unclear. Theoretically, as in the field of organ transplantation, virus-specific T cells possess cross-reactivity potential after allogeneic HSCT. Such crossreactivity is assumed to play a role in graft-versus-host disease and graft-versus-leukemia effects. In this article, we aim to give a comprehensive overview of current understanding about crossreactivity of virus-specific T cells.

Keywords: allogeneic stem cell transplantation, virus-specific T-cell, GVHD, HLA antigens, GVL, mismatch

INTRODUCTION

The reconstitution of functional virus-specific T cells and the importance of these T cells in the control of viral diseases following allogeneic hematopoietic stem cell transplantation (HSCT) has been extensively investigated (1). This led to a successful transfer into the clinical setting within adoptive immunotherapeutic approaches (2).

Among various viruses, immune reconstitution against cytomegalovirus (CMV) has been most intensively studied. Regarding the recovery of CMV-specific T cells after allogeneic HSCT, several reports showed that subclinical CMV antigenemia drives the reconstitution of functional CMV-specific T cells (3–5). The rapid homeostatic expansion of CMV-specific T cells suggests that such T cells might be largely memory T cells, considering the insufficient regeneration of T cells due to the poor thymic function early after allogeneic HSCT. Similar to the immune response after primary CMV infection (6, 7), the proportion of virus-specific T cells including CMV-specific T cells can be high and in some cases CMV-specific T cells make up more than 10% of the circulating T cells after allogeneic HSCT (8, 9). If the number and/or functionality of CMV-specific T cells is insufficient in recipients of an allogeneic stem cell graft, they are at high risk of persistent viremia and CMV disease (10, 11).

Although immune reconstitution of virus-specific T cells has been intensively studied, the fact of possible alloreactivity of virus-specific T cells in the allograft recipient has only been evaluated in few trials (12, 13). However, as suggested in other fields like organ transplantation, virus-specific T cells are assumed to play a role in alloreactivity similarly in the field of allogeneic HSCT (14, 15).

In this review, we focus on crossreactivity of virus-specific T cells against allogeneic targets, and discuss the possible implication of such reactions on the allogeneic immune responses after allogeneic HSCT.

CROSSREACTIVITY OF VIRUS-SPECIFIC T CELLS

Virus-specific T cells, which dominate the memory pool in humans, have been reported to have the potential of crossreactivity (14). Crossreactivity of T cells is the ability to recognize several different peptide/human leukocyte antigen (HLA) complexes. $CD4^+/CD8^+$ and naïve/memory T cells were shown to mediate crossreactivity against allogeneic targets (16, 17). Upon activation, memory T cells proliferate more quickly and produce more cytokines than naïve T cells (18, 19). Considering these rapid and vigorous T-cell responses mediated by memory T cells when compared to naïve T cells, one could assume that such alloreactivity of virus-specific memory T cells could play a role in the pathogenesis of early-onset acute GVHD, in particular hyperacute GVHD, following HLA mismatched HSCT. The difference of these T cells in the ability to expand and express cytotoxic molecules might also contribute to the difference in the outcome in patients with acute GVHD. Virus-specific T cells might have much higher avidity against allogeneic targets after HLA mismatched HSCT when compared to that after HLA-matched HSCT, considering the mechanism of negative selection in thymus (20, 21). Virus-specific T cells possessing high avidity against autologous HLA molecules with a self-peptide should originally be deleted in thymus. However, if virus-specific T cells recognize the complex of a peptide and non-autologous HLA molecule via their T-cell receptor, the avidity of T cells against this complex can be high because this HLA molecule is not expressed in the thymus and thus cannot induce the negative selection in the thymus (22). One hypothesis could be that, if such strong peptide-specific crossreaction exists against tumor-associated antigen (TAA)-derived peptides expressed in leukemia cells accidentally, it should lead to a strong graft-versus-leukemia (GVL) effect. However, there is no data available so far which could support this idea. Furthermore, crossreactivity against non-self HLA presenting a non-polymorphic

hematopoietic cell-specific peptide or TAA-derived peptide might provide therapeutic tools for immunotherapy, similar to the concept for minor histocompatibility antigens like HA-1.

In addition, virus-specific T cells which have weak to moderate avidity against autologous HLA molecules with a self-peptide might theoretically remain *in vivo*, considering the mechanism of positive selection in thymus. Such virus-specific T cells might subsequently exert alloreaction in the setting of HLA-matched HSCT, only when the strong activating signals by various cytokines stimulate them (20, 21). However, such crossreactivity by virus-specific T cells against autologous HLA molecules has not yet been demonstrated so far.

Regarding crossreactivity of virus-specific T cells, Epstein–Barr virus (EBV)-specific T cells have been studied in detail (14). Burrows et al. demonstrated crossreactivity of EBV EBNA3A-specific T-cell clones for the immunodominant peptide FLRGRAYGL presented on HLA-B*08:01 against the alloantigen HLA-B*44:02 (23). This finding was reconfirmed by other researchers (24, 25). These reports did not demonstrate the requirement of a specific peptide presented on HLA molecule for crossreactivity. Later on, it has been demonstrated that crossreactivity of this EBV EBNA3A-specific T-cell clones is dependent on the presentation of the self-peptide derived from the ABCD3 gene (EEYLQAFTY) (26). Here, we have to point out the significant difference between the two peptides' sequences, suggesting that crossreactivity does not necessarily require a homology in sequences indicating promiscuity of the T-cell receptor. Another EBV EBNA3A-specific T-cell clone, recognizing the complex of HLA-B*08:01 and an EBNA3A-derived peptide FLRGRAYGL, has been shown to react with the complex of HLA-B*35:01 and the self-peptide derived from Cytochrome P450 (KPIVVLHGY) (22). This study demonstrated a similar avidity of the EBNA3A-specific T-cell clone against the complex of HLA-B*08:01 with EBNA3A-derived peptide and the complex of HLA-B*35:01 with Cytochrome P450-derived peptide.

Regarding other viruses, Amir et al. reported that crossreactivity of virus-specific T cells against mismatched foreign allogeneic HLA was common (25). They used expanded T cells which were isolated using various combinations of tetramers loaded with a virus-derived immunogenic peptide. The target cells were a panel of lymphoblastoid cell lines (LCL) expressing various combinations of HLA molecules. A major finding was that a large number of virus-specific T-cell clones have crossreactivity potential against various HLA molecules. It is also important that some CD8 T-cell clones showed crossreactivity against HLA class II, even though most CD8 and CD4 T cells were crossreactive against HLA class I and class II molecules, respectively (25). A similar phenomenon showing the recognition of HLA class II by CD8 T cells was also reported by Rist et al. (27).

However, in a clinical trial using expanded virus-specific cytotoxic T-cell lines for the treatment of viral diseases, GVHD was rarely (6.5%) observed even when crossreactivity of expanded virus-specific T cells was observed *in vitro* (13). The fact of rarely observing GVHD clinically in expanded virus-specific T cells in this study might be caused by the absence of the correct crossreactive HLA molecule in the mismatch combinations, the difference in homing capacities and the lack of respective target molecules on the GVHD-target organs. Therefore, the clinical relevance of crossreactivity which was detectable *in vitro* should be further clarified in clinical trials.

Previously, it has been assumed that crossreactivity against allogeneic HLA is independent of the peptides in the HLA groove but that the allogeneic mismatched HLA molecules are the target of this cross-reactivity. In contrast, recent reports support the idea that crossreactivity against allogeneic HLA is peptide-dependent as reviewed previously (28). Actually, it is still difficult to demonstrate non-peptide-dependency experimentally, because even transporter-associated with antigen processing (TAP) deficient cell lines, which were believed to be completely deficient in antigen processing- and antigen presenting-capability, are able to load endogenous peptides on HLA molecule (28). Weinzierl et al. have demonstrated the presentation of many peptides by HLA molecules on the TAP-deficient cell line (29).

Peptide-specificity of alloreactive T cells is also supported by tissue/cell type-specific alloreactivity of clinical samples. Various reports showed the presence of tissue-specific alloreactive T cells in patients with graft failure after organ transplant (30–33). Deckers et al. have reported that the cytotoxic potential of graft-infiltrating $CD8^+$ T cells against proximal tubular epithelial cells (PTEC), gonadal vein endothelial cells (GOVEC), and splenocytes depends on the clone of graft-infiltrating $CD8^+$ T cells in renal allografts (30, 31). In this report (30), 46 graft-infiltrating $CD8^+$ T cells were cloned. Out of 46 clones, 7 lines recognized PTEC but not splenocytes derived from the same donor. Thirty lines recognized PTEC and splenocytes equally. One line preferentially recognized splenocytes over PTEC. Eight lines were not cytotoxic either to PTEC or to splenocytes. Therefore, each clone recognized different targets of the recipient. Jutte et al. also showed the specific cytotoxicity against heart endothelial cells by expanded graft-infiltrating T cells in heart allografts (32, 33). Other reports using virus-specific T cells also support the idea of cell type-specific crossreactivity of virus-specific T cells. For example a VZV-IE62-specific HLA-A2 restricted T-cell clone recognizes allogeneic HLA-B*57:01-expressing LCLs, phytohemagglutinin (PHA) blasts, and monocyte-derived dendritic cells (DCs), but does not recognize HLA-B*57:01-expressing B-cells, T cells, monocytes nor fibroblasts in a standard ^{51}Cr release assay. Such tissue/cell type-specific crossreactivity has been also reported by D'Orgogna et al. showing that allogeneic HLA-B*44:02-positive PTECs and human umbilical vein endothelial cells (HUVECs) are poor targets for EBV EBNA3A-specific T cells due to the lack of EEYLQAFTY peptide presentation (34). Amir et al. also reported that certain cell types with the correct HLA mismatch were recognized by virus-specific T cells while other cell types were not (25).

Regarding T cells in a patient with acute GVHD after HLA mismatched HSCT, single-peptide specificity was documented using a single small hairpin RNA (shRNA) system (12). There is no data available for chronic GVHD. The concept of tissue-specific alloreactivity also might be applied to GVHD following allogeneic HSCT which is also restricted to a few organs (especially skin, gut, and liver), even though other factors such as proinflammatory environment caused by the conditioning regimen affect the specificity of target organs. Tissue damage could change the expression of

genes as well as the expression of HLA molecules. Furthermore, the effects of cytokine/chemokine are expected to differ among different organs.

CLINICAL DATA SUGGESTING ALLOREACTIVITY AND VIRAL INFECTION

Over the last decades, various retrospective studies have shown the possible association between viral infection and graft rejection/GVHD (15, 35). Although there is no published data assessing the impact of CMV prophylaxis on the incidence of GVHD, prospective studies assessing the impact of CMV prophylaxis using ganciclovir in organ transplantation have demonstrated a reduced risk of graft rejection in the group receiving prophylactic CMV therapy, which supports the idea that CMV infection can be associated with an increased risk of graft rejection (35).

One recent report evoked the attention of researchers on the importance of CMV infection regarding the effects on the GVL effect in allogeneic HSCT (36). In this study, Elmaagacli et al. demonstrated a significant association between early CMV reactivation and a reduced risk of relapse in acute myeloid leukemia (AML) patients after allogeneic HSCT. Patients who developed early CMV replication detected by pp65 antigenemia assay had a significantly lower risk of relapse compared with those without early CMV replication. Another group reported a similar finding in patients with chronic myeloid leukemia (CML) (37). A very recent report from Fred Hutchinson Cancer Research Center also supported the hypothesis that early CMV reactivation in AML but not in other diseases including CML may be associated with a reduced risk of relapse, even though the impact was much less in this study compared to the previous report (38). Regarding the difference among the diseases, it is potentially due to the difference in epitopes expressed on HLA molecules. The identification of target molecules recognized by virus-specific T cells might give us a clue to this issue. In contrast to these trials (36–38), persistent CMV antigenemia was associated with a poor clinical outcome possibly due to the fact that the impaired immune status is also associated with an insufficient GVL effects by functional T cells (39).

Previously, Parkman and colleagues reported that the presence of immune response to herpes viruses was associated with a reduced risk of relapse in patients with acute leukemia after cord blood transplantation, which led to a better progression-free survival (40). In this study, there was no association between the absolute count of lymphocytes and the presence of an antigen-specific immune response. Interestingly, neither acute nor chronic GVHD had any significant impact on the likelihood of leukemic relapse, suggesting that virus-specific T cells specifically induced GVL effects and graft-versus-host reaction. Hoegh-Petersen et al. also showed the significant impact of herpes virus-specific T cells at 56 days after HSCT on the incidence of subsequent relapse (41). In this study, in patients without relapse, functional T cells against various viral antigens including BZLF1 and EBNA3 were detected. There is a possibility that anti-viral immunity may be just a surrogate factor for the immunocompetence of the recipient after allogeneic HSCT and thus not have a direct causal relation with GVHD/GVL but being an epiphenomenon of other factors such as inflammation, cytokine storm, and so on. Thus, more detail about the crossreactivity of virus-specific T cells should be clarified in the setting of allogeneic HSCT. A better understanding of their role in alloreactivity will help to reduce acute and chronic GVHD but also to mediate the important GVL reactivity by more sophisticated immunosuppressive strategies, which makes allogeneic HSCT still the most effective form of immunotherapy – allowing to cure patients with hematological malignancies which are incurable by any other form of treatment.

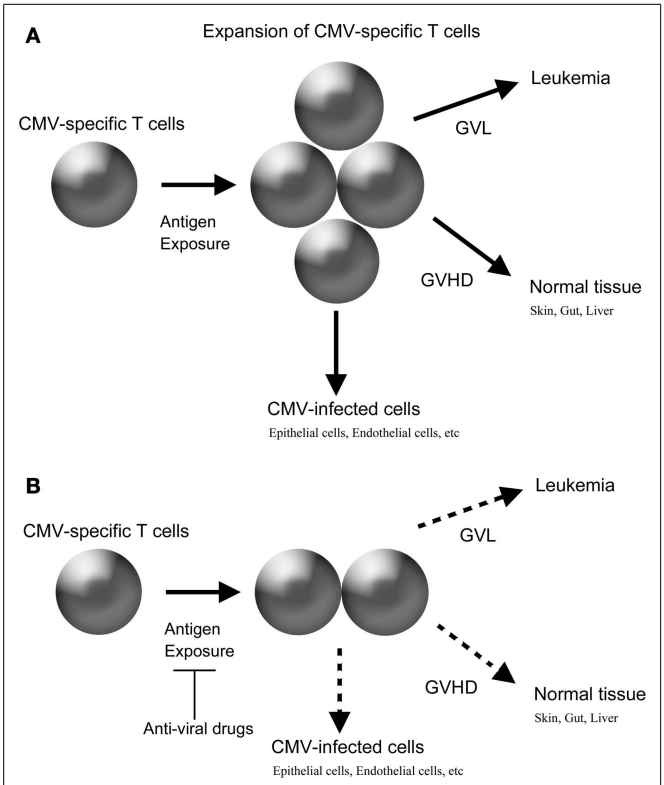

FIGURE 1 | Possible crossreactivity of CMV-specific T cells in allogeneic HSCT and the impact of anti-viral drugs. **(A)** Without anti-CMV drugs. **(B)** With anti-CMV drugs.

CONCLUSION

Virus-specific T cells can recognize and target allogeneic HLA in a peptide-dependent manner. In an HLA mismatched HSCT, the avidity of such crossreactivity can be theoretically high enough to exert clinically meaningful alloreaction. Furthermore, in an HLA-matched HSCT, there is a possibility that virus-specific T cells develop alloreaction, even if virus-specific T cells have only low to intermediate avidity against autologous targets, considering the high frequency of virus-specific T cells and the unique milieu of cytokine storm after a conditioning regimen. Furthermore, intensive prophylaxis of virus infection after allogeneic HSCT might be beneficial to reduce the incidence of GVHD similar to that after organ transplantation because such intervention could reduce the amount of antigen exposure, which is expected to decrease the expansion of donor-derived virus-specific T cells (**Figure 1**).

In conclusion, elaborate basic and clinical research to clarify the detail of crossreactivity of virus-specific T cells after allogeneic HSCT is warranted.

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Immune reconstitution and graft-versus-host reactions in rat models of allogeneic hematopoietic cell transplantation

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Allogeneic hematopoietic cell transplantation (alloHCT) extends the lives of thousands of patients who would otherwise succumb to hematopoietic malignancies such as leukemias and lymphomas, aplastic anemia, and disorders of the immune system. In alloHCT, different immune cell types mediate beneficial graft-versus-tumor (GvT) effects, regulate detrimental graft-versus-host disease (GvHD), and are required for protection against infections. Today, the "good" (GvT effector cells and memory cells conferring protection) cannot be easily separated from the "bad" (GvHD-causing cells), and alloHCT remains a hazardous medical modality. The transplantation of hematopoietic stem cells into an immunosuppressed patient creates a delicate environment for the reconstitution of donor blood and immune cells in co-existence with host cells. Immunological reconstitution determines to a large extent the immune status of the allo-transplanted host against infections and the recurrence of cancer, and is critical for long-term protection and survival after clinical alloHCT. Animal models continue to be extremely valuable experimental tools that widen our understanding of, for example, the dynamics of post-transplant hematopoiesis and the complexity of immune reconstitution with multiple ways of interaction between host and donor cells. In this review, we discuss the rat as an experimental model of HCT between allogeneic individuals. We summarize our findings on lymphocyte reconstitution in transplanted rats and illustrate the disease pathology of this particular model. We also introduce the rat skin explant assay, a feasible alternative to *in vivo* transplantation studies. The skin explant assay can be used to elucidate the biology of graft-versus-host reactions, which are known to have a major impact on immune reconstitution, and to perform genome-wide gene expression studies using controlled combinations of minor and major histocompatibility between the donor and the recipient.

Keywords: hematopoietic stem cell transplantation, graft-versus-host disease, rodentia, animal models, skin explant assay

HEMATOPOIETIC CELL TRANSPLANTATION IN HUMANS AND ANIMALS

At present, approximately 25,000 allogeneic hematopoietic cell transplantations (alloHCT) are performed each year worldwide, and the total number of such clinical procedures is predicted to rise in the future (Savani et al., 2011). AlloHCT can give rise to considerable toxicity and negative side-effects resulting in relatively high morbidity and mortality with poor quality of life and 5-year survival rates of 40–60% overall. It is however the only curative therapy in patients with relatively severe and advanced diseases for whom other treatment options are limited.

To date, alloHCT is complicated by opportunistic infections, disease relapse and graft-versus-host disease (GvHD). GvHD is an immunological condition that is caused by the reactivity of allogeneic T cells transferred in the stem cell graft from a genetically related or unrelated donor. After activation through recognition of disparate host antigens, alloreactive cells exert pathological damage via cytotoxic mechanisms and cytokine release on various organs and tissues, primarily the liver, gut, and skin, of the immunocompromised transplant recipient (Ferrara et al., 2008). In the clinical setting, relatives are favored over unrelated volunteer stem cell donors because of genetic similarity with the patient, reducing the overall risk of transplant failure and adverse graft-versus-host reactions (GvHR).

Immunity is deficient after alloHCT due to underlying disease, pre-transplant conditioning, or both, and it takes weeks to several months or years for the patient's immune system to recover (Storek, 2008; Seggewiss and Einsele, 2010; Bosch et al., 2012). Early after transplantation, the reconstitution of immune cells in lymphoid organs is inhibited: the primary sites of white and red blood cell generation, bone marrow and thymus, are damaged by chemotherapy and radiation conditioning, and they are targeted by GvHD (Krenger and Holländer, 2008; Shono et al., 2010). Furthermore, immune function is impaired in elderly patients (Krenger et al., 2011) as both the regeneration of hematopoietic cells in the bone marrow and the thymic

output of T lymphocytes decrease with age (Dorshkind et al., 2009).

Deficient and delayed restoration of immunity post-transplant contributes to the patient's susceptibility to opportunistic infections, recurrence of the original disease and occurrence of secondary malignancies (Toubert et al., 2012). The individual contributions of natural killer (NK) cells, $CD8^+$ cytotoxic T cells, $CD4^+$ helper T cells, B cells, and innate immune cells, and their combined effects on these complications are still not fully understood (Geddes and Storek, 2007; Bosch et al., 2012).

Moreover, T cell depletion of the graft, when applied to reduce the risk of GvHD and essential in haploidentical alloHCT between family members (Kolb, 2008), compromises immune reconstitution, illustrating that grafted "passenger" T cells are needed for successful engraftment. Depletion of T cells also abrogates the graft-versus-tumor (GvT) effect, as GvH and GvT effects are strongly correlated and transplanted donor T cells can either mediate beneficial GvT effects or cause GvHD (Appelbaum, 2001). The crucial question of how to avoid detrimental GvHR without compromising the curative potential of alloHCT through a strong GvT effect has yet to be solved by clinical hematologists and HCT researchers.

THE USEFULNESS OF ANIMAL MODELS IN STUDYING HEMATOPOIETIC CELL TRANSPLANTATION AND GRAFT-VERSUS-HOST DISEASE

The wish to better understand the dynamic biological processes between graft and host following transplantation, with the overall aim to improve the clinical management of alloHCT, has driven research in the development of adequate laboratory methods and animal models. The use of such models in transplantation research has contributed substantially to our current understanding of the nature of GvHR and its underlying principles in GvHD (Welniak et al., 2007; Socié and Blazar, 2009). Experimental alloHCT using mice and dogs have been particularly instrumental for the discovery of a variety of new therapies and for testing their feasibility, toxicity, and efficacy (Ferrara et al., 2008; Socié and Blazar, 2009).

Inbred rodent strains can be systematically established and maintained, yielding genetically homogeneous and biologically well-characterized strains for experimentation. Experiments in live animals facilitate and accelerate, for example, the study of genetic effects and mapping of gene associations with observable pathological phenotypes. An array of murine experimental models that employ donor and host strain combinations designed for mismatches only in the major histocompatibility complex (MHC) gene region (*H-2* in the mouse), only in minor histocompatibility antigens, or both, are available for the study of immune reconstitution and GvHD (Schroeder and DiPersio, 2011). Fully incompatible strain combinations, such as the popular [C57BL/6 ($H\text{-}2^b$) → Balb/C ($H\text{-}2^d$)] mouse model, are most often applied. Combinations of mice strains that are MHC matched but differ in minor histocompatibility antigens, for example [B10.D2 ($H\text{-}2^d$) → Balb/C ($H\text{-}2^d$)], [DBA/2($H\text{-}2^d$) → B10.D2 ($H\text{-}2^d$)], or [C3H.SW($H\text{-}2^b$) → C57BL/6 ($H\text{-}2^b$)] transplantation models (Korngold and Sprent, 1987), can mimic fully human leukocyte antigen (HLA) matched HCT between unrelated individuals, but not semi-HLA compatible HCT between sibling pairs. The latter scenario of haploidentical HCT between first-degree relatives can be modeled by inter-crossing two inbred strains and using the resulting hybrid progeny of the F_1 generation as recipients of a transfusion of parental leukocytes in so-called parent-into-F_1 models.

The mouse is historically and presently the most widely used model in biomedical research. However, other animal models have the potential to complement, validate, and extend the findings from experiments on mice. Studies of alloHCT in dogs make a case in point as they have added important knowledge in this field of research (Storb and Thomas, 1985), e.g., the use of delayed donor lymphocyte infusions to induce a GvT reaction and non-myeloablative ("reduced intensity") conditioning regimens to reduce toxicity and morbidity in patients. Murine models have been employed to test non-myeloablative conditioning regimens with reduced irradiation intensity and/or combination chemotherapy (fludarabine, busulfan, cyclophosphamide, etc.; Santos and Owens, 1969; Ruggeri et al., 2002; Sadeghi et al., 2008). Similarly, rat alloHCT models based on chemotherapeutic conditioning without the need for irradiation have been developed (Santos and Owens, 1966; Tutschka and Santos, 1975; Okayama et al., 2004).

Rats offer all the advantages of a rodent experimental model, such as short generation time and cost effectiveness. Rats are bigger than mice (about 10-fold in body weight) and thus yield more grams of biological material per animal and per cage unit that can be sampled in the course of experimentation. Their spleens, lymph nodes, efferent lymph, and peripheral blood contain lymphocytes that can be readily harvested and serve as organ sources of donor grafts for transplantation. Hematopoietic stem cells are commonly obtained from the marrow of large bones (e.g., femurs and tibiae). Cell grafts enriched in Sca-1^+ and $CD34^+$ hematopoietic stem cells that are being used in patients and experimentally in mice have not been available for rat models due to the lack of specific antibody markers for targeted cell purification.

Many genetically defined and well-characterized inbred rat strains are available for research in transplantation immunology, and a range of rat alloHCT models have been developed from these strains during the past decades. Especially the strains *Brown-Norway* (BN) and *Lewis* (LEW) are widely used for fully MHC mismatched alloHCT (Santos and Owens, 1966; Clancy et al., 1976; Pakkala et al., 2001; Okayama et al., 2004; Zhu et al., 2011; Lin et al., 2012). Also HCT between haploidentical parental and filial generations, e.g., transplantation of LEW or BN bone marrow into F_1 (BN × LEW) recipients, has been modeled in the rat (Clancy et al., 1983; Kimura et al., 1995; Ohajekwe et al., 1995; Peszkowski et al., 1996; Vaidya et al., 1996; Goral et al., 1998; Kobayashi et al., 1998; Sasatomi et al., 2005; Wolff et al., 2006; Kitazawa et al., 2012). In a number of these models, engraftment, reconstitution, chimerism, cell trafficking, and tolerance toward donor cells has been studied (Clancy et al., 1983; Oaks and Cramer, 1985; Ohajekwe et al., 1995; Engh et al., 2001; Foster et al., 2001; Okayama et al., 2004; Itakura et al., 2007; Klimczak et al., 2007; Nestvold et al., 2008; Zhou et al., 2008; Zhu et al., 2011; Zinöcker et al., 2011a;Lin et al., 2012). Furthermore, rat models have been employed to test prevention or treatment of GvHD by therapeutic regimens involving immunomodulatory drugs (Tutschka et al., 1979; Vogelsang et al., 1986; Vogelsang et al., 1988; Mrowka et al., 1994; Ohajekwe et al.,

1995; Pakkala et al., 2001; Okayama et al., 2006; Wolff et al., 2006; Jäger et al., 2007), infusion or induction of various suppressive cell types (Itakura et al., 2007; Aksu et al., 2008; Nestvold et al., 2008; Kitazawa et al., 2010; Zinöcker et al., 2011b; Kitazawa et al., 2012; Zinöcker et al., 2012), UV irradiation (Ohajekwe et al., 1995; Gowing et al., 1998), serum transfusion (Shimizu et al., 1997), surgical techniques (Kobayashi et al., 1998), and prolonged distribution of a chemical agent with subcutaneously implanted osmotic pumps (Fidler et al., 1993).

The MHC is the dominant genomic region that governs mutual tolerance, rejection, and GvHR between the donor and the host in alloHCT. The mouse and rat MHC regions are closely related and share overall similarity with the human MHC (*HLA*; Kelley et al., 2005a), however, some important structural differences exist. In particular, the rat MHC (*RT1*) differs from the mouse MHC region in that it encompasses a large number of polymorphic non-classical MHC class I genes which serve as potential ligands for NK cell receptors (Vance et al., 1998; Naper et al., 2002; Naper et al., 2005; Kveberg et al., 2010; Andrews et al., 2012). In addition, the rat genome encodes a broader spectrum of inhibitory and activating Ly49 receptors expressed by NK cells compared to the mouse (Kelley et al., 2005b). Therefore, alloreactive rat NK cells and NK cell subsets may behave differently than mouse NK cells in the context of transplantation. Transplanted donor-versus-recipient NK cells mediate both GvT and GvH effects in patients (Ruggeri et al., 2002). They are thus a key element in the risk of leukemia relapse after haploidentical alloHCT (Velardi, 2012) and could influence T cell alloreactivity and the development of GvHR. The considerable differences especially in the NK cell system even between closely related species like rat and mouse illustrate the importance of relying on more than one experimental animal model in HCT research.

The complete genome sequence of *Rattus norvegicus* has been resolved in 2004 (Rat Genome Sequencing Project Consortium, 2004). With the advent of commercial cloning technology for rats (Huang et al., 2011) this species will likely be applied more frequently as a study object in the future. In the following sections, we will discuss some contributions by which rat models have helped to advance our understanding of immune reconstitution and GvHR following alloHCT.

THE RAT AS AN *IN VIVO* MODEL OF IMMUNE RECONSTITUTION AND GRAFT-VERSUS-HOST REACTIONS AFTER HEMATOPOIETIC CELL TRANSPLANTATION

To study the role of GvHR on immune reconstitution *in vivo*, we employed experimental transplantation protocols using combinations of rat strains with defined genetic incompatibilities (Engh et al., 2001; Nestvold et al., 2008; Zinöcker et al., 2012). The genetic composition regarding the MHC region of relevant rat strains are listed in **Table 1**. For experiments on fully incompatible (mismatched at the MHC region as well as non-MHC genes) alloHCT, we employed a model using *Piebald Virol Glaxo* (PVG) rats as the donor strain and BN rats as recipients of transplants. Conveniently, the genetic make-up of the donor PVG.7B strain encodes an allele (the RT7.2 allotype) of the CD45 gene (Kampinga et al., 1990), which facilitates later detection of donor-derived cells distinguishable from host-derived cells by flow cytometry using allotype-specific antibody. The genetic background of the PVG.7B strain is identical with the original PVG strain; both carry the *c* haplotype of the rat MHC, i.e., $RT1^c$ (**Table 1**). The recipient BN strain has a different genetic background and carries the *n* haplotype of *RT1*, $RT1^n$.

Table 1 | MHC haplotypes of inbred congenic and recombinant rat strains.

Rat strain	MHC subregion		
	Classical class I ***RT1-A***	**Class II** ***RT1-B/D***	**Non-classical class I** ***RT1-CE/N/M***
BN	*n*	*n*	*n*
LEW	*l*	*l*	*l*
LEW.1N	*n*	*n*	*n*
PVG	*c*	*c*	*c*
PVG.7B	*c*	*c*	*c*
PVG.1N	*n*	*n*	*n*
PVG.1U	*u*	*u*	*u*
PVG.R23	*u*	*a*	*av1*

The reconstitution of blood and immune cells, especially of T lymphocytes, has been extensively studied in patients (Holland et al., 2008; Toubert et al., 2012), however, the mechanisms behind reconstitution and immunodeficiency and the complex interactions between donor and host cell types in this context remain incompletely understood. Many useful insights into the underlying cellular and molecular processes have been gained from experiments done mainly in mice. Immune reconstitution and post-transplant immune responses have been characterized also in the laboratory rat to some detail, facilitating the design of new transplantation protocols (Nestvold et al., 2008). The main rat blood cell types and cell subsets with relevance to HCT and their standard phenotypic characterization are summarized in **Table 2**.

One important aim of our studies was to describe in detail the reconstitution of the lymphocytes that mediate GvT and GvH effects, i.e., T cells and NK cells, in bone marrow recipients and in experimental GvHD. The experimental protocol used in these studies (Zinöcker et al., 2011a; adapted from Nestvold et al., 2008) is schematically outlined in **Figure 1**. Recipient rats received a lethal dose of 900 cGy total body irradiation from a Cesium-137 source emitting β and γ irradiation at an approximate rate of $4\,\mathrm{Gy\,min^{-1}}$. This conditioning regimen made recipient rats susceptible to donor engraftment. Donor bone marrow containing hematopoietic stem cells was obtained from leg bones (femurs and tibiae). The graft consisted of mononuclear bone marrow cells that were depleted of T cells by negative selection using magnetic beads with anti-αβTCR and anti-CD5 antibodies (cf. **Table 2**) prior to transplant (Nestvold et al., 2008). Thus, the primary graft served to establish stable donor engraftment. In this model, the transfer of donor-versus-recipient reactive NK cells (Løvik et al., 1995) contained in the graft should facilitate engraftment and donor reconstitution. GvT effects can be studied after injecting BN acute myelocytic leukemia cells, a model of human acute myeloid leukemia (Martens et al., 1990), into BN recipients (Nestvold et al., 2008). Transplanted rats were convalescent for 14 days before

Table 2 | Immunophenotype of rat leukocyte populations.

Cell type	Phenotype	Antibody	Reference
Leukocyte	CD45$^+$	OX-1	Sunderland et al. (1979)
	MHC-I$^+$	OX-18	Fukumoto et al. (1982)
T lymphocyte	CD2$^+$	OX-34	Jefferies et al. (1985)
	CD3$^+$	G4.18	Nicolls et al. (1993)
	CD5$^+$	OX-19	Dallman et al. (1984)
	CD6$^+$	OX-52	Castro et al. (2003)
αβ T cell	TCRαβ$^+$	R73	Hünig et al. (1989)
CD4 αβ T cell	CD4$^+$	W3/25	Williams et al. (1977)
		OX-38	Jefferies et al. (1985)
CD8 αβ T cell	CD8α$^+$	OX-8	Brideau et al. (1980)
Naive T cell	CD25$^-$	OX-39	Paterson et al. (1987)
	CD45RC^{hi}	OX-22	Spickett et al. (1983)
T blast	CD25$^+$	OX-39	Paterson et al. (1987)
CD4 T blast	CD134$^+$	OX-40	Paterson et al. (1987)
Memory T cell	CD62L$^-$	OX-85	Seddon et al. (1996)
	CD45RC^{lo}	OX-22	Spickett et al. (1983)
Regulatory CD4 T cell	CD45RC^{lo}	OX-22	Powrie and Mason (1990)
	CD25$^+$	OX-39	Stephens and Mason (2000)
	FoxP3$^+$	FJK-16s	Beyersdorf et al. (2005)
Regulatory CD8 T cell	CD45RC^{lo}	OX-22	Xystrakis et al. (2004b)
	FoxP3$^+$	FJK-16s	Han et al. (2007)
γδ T cell	TCRγδ$^+$	V65	Kühnlein et al. (1994)
B lymphocyte	CD45RA$^+$	OX-33	Woollett et al. (1985)
	MHC-II$^+$	OX-6	McMaster and Williams (1979)
Activated B cell	CD80$^+$	3H5	Maeda et al. (1997)
	CD86$^+$	24F	Maeda et al. (1997)
NK lymphocyte	CD3$^-$	G4.18	Nicolls et al. (1993)
	CD161a$^+$	3.2.3	Na et al. (1992)
	(NKR-P1A)	10/78	Chambers et al. (1992)
NKT lymphocyte	CD3$^+$	G4.18	Nicolls et al. (1993)
	CD161a$^+$	3.2.3	Na et al. (1992)
	(NKR-P1A)	10/78	Chambers et al. (1992)
Macrophage	MHC-II$^+$	OX-6	McMaster and Williams (1979)
	CD172a$^+$ (SIRPα)	OX-41	Robinson et al. (1986)
	CD11c$^+$	OX-42	Robinson et al. (1986)
	CD68$^+$	ED1	Dijkstra et al. (1985)
		ED2[a]	Dijkstra et al. (1985)
Dendritic cell	MHC-II$^+$	OX-6	McMaster and Williams (1979)
	CD172a$^+$ (SIRPα)	OX-41	Robinson et al. (1986)
	CD11c$^+$	OX-42	Robinson et al. (1986)
	α_{E2} integrin	OX-62	Brenan and Puklavec (1992)

Phenotypic markers of rat leukocyte populations and subpopulations are summarized together with available antibodies commonly used for their flow cytometric detection. Comprehensive lists of rat antigens and specific antibodies have been published by Puklavec and Barclay (2001) and van den Berg et al. (2001). A list of OX antibodies can be found online at http://users.path.ox.ac.uk/~ciu/mrc-mabs.html

[a]*No CD designation is assigned for the cognate antigen.*

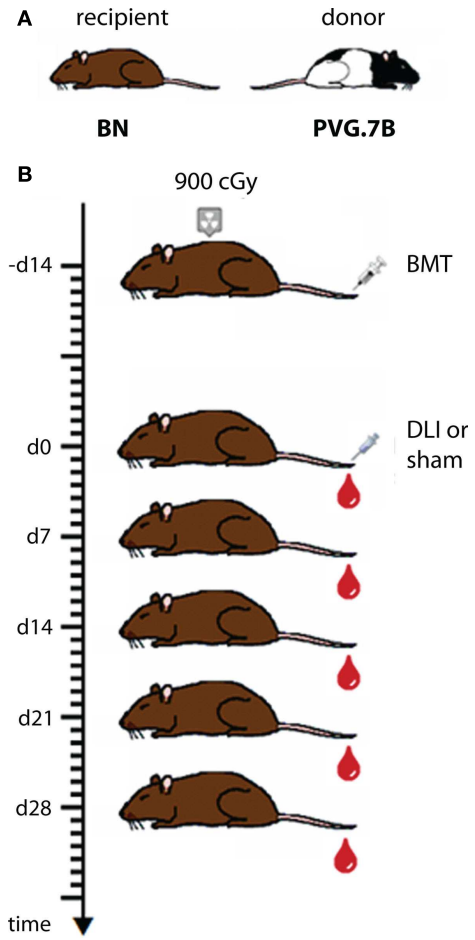

FIGURE 1 | Schematic outline of the transplantation protocol applied in the MHC mismatched rat model. (A) BN rats served as recipients and PVG.7B rats as bone marrow and lymph node donors for bone marrow transplantation (BMT) and donor lymphocyte infusion (DLI), respectively, in fully MHC-incompatible alloHCT performed as shown in **(B)**. Recipient rats were lethally irradiated (900 cGy), rescued by an intravenous injection of donor bone marrow cells and 14 days later received a graded dose of donor lymph node cells to invoke fatal GvHD. Controls received sham treatment (injection without cells) instead of DLI. Blood samples were collected in weekly intervals from the day of DLI for subsequent analysis of reconstitution and chimerism.

GvHD was induced in a cell-dose dependent manner by a single infusion of donor lymphocytes (DLI; cf. **Figure 1**) from lymph nodes. Alternatively, GvHD can be invoked by transfusion of donor cells derived from the spleen or peripheral blood, and the latter source is highly relevant with regard to clinical GvHD, which is commonly caused by the transfer of T cells contained in a donor blood graft or donor lymphocyte transfusion.

T cells, NK cells, and NKT cells

With respect to lymphocyte reconstitution in recipients of clinical HCT, NK cells recover relatively swiftly (weeks) and T cell reconstitution happens more slowly (months to years; Storek et al., 2008). In our hands, both NKT and NK cells (as defined by CD3 and NKR-P1 surface expression; cf. **Table 2**) of donor origin were rapidly generated in animals transplanted with T

cell-depleted bone marrow. NKT and NK cells of host origin were not detected in the circulation 2 weeks after HCT (Zinöcker et al., 2011a). Donor T cells showed a significantly slower pace of reconstitution in these animals, and reached approximately 90% donor chimerism in the blood only several months after transplant (Zinöcker et al., 2011a). Thus, the overall patterns of NK, NKT, and T lymphocyte reconstitution were similar to those observed in patients, albeit with accelerated kinetics as might be expected from a relatively short-lived rodent species.

Donor $CD4^+$ T cells showed a tendency to reconstitute slightly more slowly than did donor $CD8^+$ T cells (Zinöcker et al., 2011a), which is also comparable with known clinical data (Seggewiss and Einsele, 2010). Rats that received an infusion of donor lymph node cells and subsequently developed GvHD displayed full donor chimerism of $CD4^+$ and $CD8^+$ T cells shortly after DLI, coinciding with the onset of disease symptoms. This increase very likely reflected the activation by allo-antigens and rapid expansion of donor $CD4^+$ and $CD8^+$ T cell clones in the host. Any remaining host T cells were probably cleared by alloreactive donor cells.

Memory T cells

We also noted that donor-derived CD62L-expressing naive T cells were less frequent in the peripheral blood of rats that developed GvHD compared with healthy bone marrow-transplanted controls (Zinöcker et al., 2011a). This finding may likely be due to the loss of CD62L surface expression in the course of T cell activation as measured by the concomitant increase of CD25 and CD134 activation markers on $CD4^+$ and $CD8^+$ T cell populations (cf. **Table 2**). Although we did not specifically investigate a memory T cell phenotype in our study, we speculate that donor memory T cells may expand in the presence of host allo-antigens and allo-activation as $CD62L^-$ T cells were more frequent in the circulation in rats with GvHD. $CD44^+$ $CD62L^-$ memory T cells that are generated in response to pathogens during infection as well as other antigens have been reported not to cause GvHD (Anderson et al., 2003; Chen et al., 2004). In line with these studies, Xystrakis et al. (2004a) have employed a parent-into-F_1 HCT model and showed that $CD4^+$ $CD45RC^{lo}$ T cells containing mainly memory T cells, in contrast to $CD4^+$ $CD45RC^{hi}$ T cells containing mainly naive T cells, failed to induce acute or chronic GvHD in recipient rats. Conversely, transplanted memory T cells not specifically primed to allo-antigens can have a considerable potency to induce GvHD (Zheng et al., 2009). Therefore, the capacity of differentiated memory T cells to elicit a GvHR may depend on the nature of their primary antigen encounter.

Regulatory T cells

Furthermore, we found that host-derived FoxP3-expressing $CD4^+$ $CD25^+$ T cells, comprising a regulatory T cell subtype (Beyersdorf et al., 2005), reconstituted relatively quickly and were present at normal levels in peripheral blood at 4 weeks post-transplant (Zinöcker et al., 2011a). It has been shown previously in mice that recipient $CD4^+$ $CD25^+$ T cells can resist irradiation and regulate chronic GvHD (Anderson et al., 2004). Donor-type $FoxP3^+$ regulatory T cells, on the other hand, did not amount to significant numbers in the blood of transplanted rats at any time point post-transplant. Strikingly, and in contrast to transplanted controls which did not receive DLI, rats that suffered from GvHD failed to reconstitute $FoxP3^+$ regulatory T cells completely. We speculate that the absence of regulatory T cells is a major contributing factor in the lack of ability of these animals to avert alloreactivity as they uniformly developed lethal GvHD. Another regulatory T cell subtype that was defined as $CD8^+$ $CD45RC^{lo}$ $FoxP3^+$ in the rat (cf. **Table 2**) suppressed alloreactive donor $CD4^+$ T cells *in vitro* and prevented experimental GvHD *in vivo* (Xystrakis et al., 2004b). Decreased FoxP3 expression (Miura et al., 2004) and frequency of regulatory T cells (Zorn et al., 2005; Rieger et al., 2006; Magenau et al., 2010) has been associated with the incidence of GvHD in patients. Furthermore, $CD4^+$ $CD25^+$ regulatory T cells have been shown to suppress host-reactive T cells and reduce acute GvHD in murine models (Cohen et al., 2002; Hoffmann et al., 2002; Taylor et al., 2002; Edinger et al., 2003). Alleviation of GvHD after infusion of donor-derived $CD4^+$ $CD25^+$ regulatory T cells has recently been reported in some patients (Brunstein et al., 2011; Di Ianni et al., 2011), although these phase I clinical trials were not powered to assess the efficacy of this treatment modality and did not include control subjects. The therapeutic potential of regulatory T cell induction or adoptive transfer in GvHD prevention is currently under clinical exploration (Leventhal et al., 2012).

B cells and antibodies

In transplanted rats, B cells recover gradually in host lymphoid organs and peripheral blood with kinetics similar to that of T cells (Renkonen, 1986). This is consistent with what is known of B cell reconstitution in patients who receive an allogeneic donor transplant (Bosch et al., 2012), as is the finding that B cell reconstitution is delayed during acute GvHD (Renkonen, 1986; Bosch et al., 2012). B cells did not recover in the skin, lungs, or the gut of host rats that developed acute GvHD, but accumulation of immunoglobulin-producing cells was observed in the inflamed liver (Renkonen et al., 1986). B cells are thought to participate in the pathogenesis of GvHD (Shimabukuro-Vornhagen et al., 2009), possibly by presenting allo-antigens and by secreting auto- and alloreactive antibodies.

Disparate MHC molecules and minor histocompatibility (non-MHC) antigens of both donor and host type co-exist in radiation chimeras, by which B cells can be activated to produce allo-antibodies either against donor antigens (on hematopoietic cells) or against host antigens (mainly non-hematopoietic tissue). Putative donor-derived allo-antibodies will therefore bind mainly to tissue components of the host and may be difficult to detect in the serum unless circulating donor cells are screened for B cell receptor specificities. We know that the transfusion of PVG T cells into semi-allogeneic F_1 (PVG × DA) hybrid recipients induces auto-antibodies against basal membranes of the skin (Rolstad, 1985). Increased serum levels of antibodies against heat-shock proteins are associated with acute and chronic GvHD (Goral et al., 1995, 2002).

B cells play an important role in the etiology of chronic GvHD as increased auto-antibody titers and other autoimmune-typical symptoms are hallmarks of this disease (Ferrara et al., 2008). Laboratory rodents have been used to model chronic GvHD experimentally (Vogelsang et al., 1988; Schroeder and DiPersio, 2011).

Spleen cell transfer without bone marrow transplantation from either BN or LEW donor strains induced autoreactive antibodies that are typical of an autoimmune-like chronic GvHR in F_1 (LEW × BN) hybrid rats (Tournade et al., 1990; Xystrakis et al., 2004a). Interestingly, transfusions of LEW splenocytes resulted in acute GvHD and death of F_1 recipients, while transfusions of BN splenocytes caused no disease (Tournade et al., 1990; Xystrakis et al., 2004a). This may be due to different genetic factors in the respective donor strains and T cell responses that differ in the pattern of cytokine release upon allostimulation (Bernard et al., 2010). Studies done in our labs support the hypothesis that the quality of the allogeneic T cell response is important and depended on the tissue source: peripheral T cells typically induced acute GvHD (Nestvold et al., 2008) while bone marrow-derived T cells induced a more chronic form of the disease (Naper et al., 2010).

ALLO-TRANSPLANTATION IN A SEMI-COMPATIBLE RAT MODEL

Hematopoietic cell transplantations using fully MHC mismatched donors are not performed in the clinic because of the excessively high risk of GvHD. Previous efforts in our lab to generate a semi-compatible (haploidentical) HCT model by cross-breeding inbred PVG and BN rats failed because of what appeared to be a significant heterosis effect that rendered the F_1 (PVG × BN) offspring highly aggressive and impossible to handle. In another effort to design a model that reflects the clinical reality better than the completely mismatched [PVG.7B → BN] transplantation model, we selected rat strains that were partly matched at the MHC region. The PVG-*RT1*^{r23} strain (PVG.R23) and the PVG-*RT1*^{u}strain (PVG.1U) are partially compatible: PVG.1U rats express the *u-u-u* haplotype of *RT1* and PVG.R23 rats the intra-MHC recombinant *u-a-av1* haplotype, i.e., they are matched in the classical class I (*RT1-A*), but not the class II (*RT1-B/D*) and non-classical class I (*RT1-CE/N/M*) MHC regions (cf. **Table 1**). The strains are matched for minor histocompatibility antigens as both express the same class III MHC genes on the PVG genetic background. Disparity for either the class II locus *RT1-B/D* or non-MHC genes elicits fatal GvHD in the rat, while the class I loci *RT1-A* and *RT1-CE/N/M*, respectively, have little or no effect (Oaks and Cramer, 1985). Therefore, in the [PVG.1U → PVG.R23] combination, GvHD is primarily due to the mismatch of class II genes. Dahlke and coworkers (Jäger et al., 2007) have made use of a similar semi-allogeneic transplantation model, where a mismatch only in MHC class II genes induced lethal GvHD in [LEW.AR1 → LEW.AR2] transplanted rats. We used PVG.R23 as HCT recipients and PVG.1U rats as marrow donors, respectively, in our experiments (Zinöcker et al., 2012). This model was designed to better mimic a clinical setting where some degree of mismatch of the donor MHC is permissible to perform a HCT procedure on patients in need of a transplant (Petersdorf, 2007). It should not be inferred from this model, however, that it can replicate the setting of haploidentical HCT in humans, where siblings, who share half of their MHC genes and a range of minor histocompatibility antigens with the recipient, serve as stem cell donors. Rather, the semi-compatible rat HCT model reflects a transplantation scenario in which polymorphisms of both MHC and non-MHC genes contribute to the induction of acute GvHD.

The HCT protocols described above (cf. **Figure 1**) resulted in the onset of a rapid wasting disease with severe loss of weight that was typical of acute GvHD and was fatal in 90–100% of recipients (Zinöcker et al., 2011a,b, 2012). The kinetics of disease progression were similar in both the MHC mismatched [PVG.7B → BN] and the partially matched [PVG.1U → PVG.R23] model (Zinöcker et al., 2012). The titrated DLI dose required to reproducibly invoke lethal acute GvHD in the latter model (9×10^6 T cells from lymph nodes) was higher compared to the former (5×10^6 T cells from lymph nodes; Zinöcker et al., 2012). Recipients were monitored closely over time for GvHD-typical symptoms which were quantified using a scoring table (Zinöcker et al., 2011a) adapted from Cooke et al. (1996).

Acute GvHD primarily affects the liver, the intestine and the skin (Ferrara et al., 2008). In our models, severe GvHD symptoms were observed (Zinöcker et al., 2011a). The skin was targeted by GvHR; poor grooming, ruffling, fur loss, skin flaking, and occasionally, skin lesions were manifest. These observations were confirmed histologically, with GvHD symptoms present in the skin (**Figure 2**) and secondary lymphoid organs, spleen and lymph nodes (Zinöcker et al., 2011a). Histological evaluation of the liver and gastrointestinal tract revealed no significant pathological changes of these organs. Therefore, results from these models pertain primarily to cutaneous GvHD. The absence of observable pathology of the liver and the gut could simply depend on the rapid onset and progression of GvHD in host rats, marked by dehydration and severe weight loss, before histopathology of other organs became manifest. On the other hand, they may reflect significant differences in the underlying mechanisms of disease development affecting distinct target sites in these models.

Despite the considerable genetic barriers in the fully and partially mismatched alloHCT settings, we reproducibly established stable allogeneic rat chimeras by transplanting large numbers of bone marrow cells that were extensively depleted of T cells. These protocols demonstrate the central importance of T cells in GvHD as is the case in humans (Ferrara et al., 2008), and, as defined numbers of donor T cells were sufficient to subsequently induce severe acute GvHD, these models may serve to investigate the contributions of T cells and other cell types to GvHR *in vivo* and to manipulate such alloimmune responses with drugs or different modes of cellular therapy.

One important advantage of using the rat model, which we discuss further in the following section, is the availability of corresponding *in vitro* models of GvHR, especially the rat skin explant assay. This model resembles key features of clinical GvHR in a simple experimental setting that can be highly standardized and easily manipulated.

THE RAT SKIN EXPLANT ASSAY

Robust risk estimates of GvHD incidence and severity would allow clinicians to intervene at the stage of donor selection, pre-transplant conditioning, and choice of immunosuppressive treatment in the course of transplantation. It is well established that disparities in minor and major human leukocyte antigens between patients and donors can cause GvHD. A number of human biomarkers of acute and chronic GvHD for prediction of treatment outcome and mortality have been identified recently

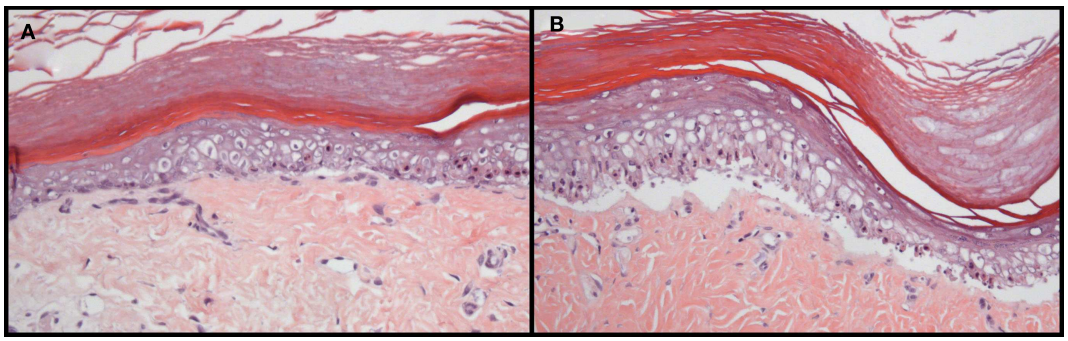

FIGURE 2 | Histopathology of GvHD in rats transplanted with allogeneic, fully MHC mismatched bone marrow. Grade II **(A)** and III–IV **(B)** pathology is manifest in the skin of BN rats that suffered from acute GvHD after bone marrow transplantation and donor lymphocyte infusion from PVG donors. Pathological grading was performed based on the GvHD classification originally described by Lerner et al. (1974) for human skin GvHD. Skin was removed from the paws at autopsy and tissue sections were stained with hematoxylin and eosin. These photographs are reprinted from Novota et al. (2008) with permission from the publisher.

(Paczesny et al., 2009; Levine et al., 2012b) and their reliability was prospectively evaluated in patients (Levine et al., 2012a).

The skin explant assay is an *in vitro* model which can predict GvHD in patients with high sensitivity and specificity. It was prospectively tested as a method to predict GvHD incidence and severity in a donor/patient cohort by Vogelsang et al. (1985). Several studies used the skin explant assay to predict the likelihood of acquiring GvHD and reported correlation rates of 80–90% with clinical outcome, which proved the skin explant assay superior to other *in vitro* models that had been designed for this purpose (Sviland and Dickinson, 1999; Dickinson et al., 2001; Hromadnikova et al., 2001; Sviland et al., 2001; Wang et al., 2006). The accuracy of the prediction by skin explant, however, was dependent on the type of GvHD prophylaxis and conditioning regimens used. Matched unrelated donor transplants, including T cell depletion protocols, gave understandably low prediction rates (Wang et al., 2006).

The skin explant closely resembles the histopathology of cutaneous GvHD and allows the study of pathophysiological processes related to GvHR (Sviland and Dickinson, 1999; Dickinson et al., 2001; Dickinson et al., 2002). Histological grading of the skin explant, from grade I to grade IV with increasing severity (Lerner et al., 1974), represents a standardized method to evaluate the risk for selected donor/recipient combinations before transplantation. One problem for a broad application of the assay in experimental studies is the shortage and genetic heterogeneity of skin biopsies from healthy donors. To overcome this problem and to reduce the need for animal experimentation, we developed a rat skin explant assay that can complement and in part replace transplantations in rodents. This assay can be highly standardized and results are not hampered by undefined genetic differences between tissue samples which cannot be avoided in human studies. The experimental procedure is described in detail elsewhere (Novota et al., 2008). Briefly, lymphocytes from the spleens of donors are stimulated with irradiated lymphocytes from the spleens of recipients in a mixed lymphocyte reaction for 7 days, then transferred to a cultured skin biopsy of the recipient and co-incubated for three more days. The GvHR is then evaluated and scored by histology using the same criteria as for grading GvHR in human skin explants (Sviland and Dickinson, 1999). Using genetically well-defined inbred strains, we showed that combinations with differences in major histocompatibility antigens usually led to grade III or IV GvHR (Novota et al., 2008). GvHR was on average less severe (grade II) in MHC congenic strain combinations which are only mismatched in minor histocompatibility antigens than in combinations with major or minor plus major histoincompatibility (Novota et al., 2008). The histopathology of cutaneous GvHR in these skin explants closely resembled the histopathology of GvHR in human skin explants (**Figure 3**). We were also interested to determine whether the rat skin explant assay could resemble, in addition to histological features of GvHD, the molecular profile of GvHD in the patient. In our initial study (Novota et al., 2008), we focused on MHC-linked *Hsp70* genes because the expression of stress-inducible heat shock protein 70 has been correlated with the GvHR grading in human skin explants (Jarvis et al., 2003). Indeed, the relative expression of the *Hsp70-1* (*Hspa1b*) and *Hsp70-2* (*Hspa1a*) genes correlated with the grade of GvHR in rat skin explants (Novota et al., 2008), indicating that certain molecular patterns of GvHR are conserved between human and rat skin explants.

This result encouraged us to use the rat skin explant assay for searching new genetic biomarkers of GvHR. In an expression profiling study with special emphasis on MHC and NK gene complex (NKC) genes, 11 MHC, 6 NKC, and 168 genes in other genomic regions were identified to be regulated during GvHR in rat skin explants (Novota et al., 2011). For MHC and NKC genes, the results were verified by real time PCR experiments on samples from other skin explant assays using different strain combinations, on skin with cutaneous GvHD from transplanted rats, and on samples from human skin explant assays. The genes that were confirmed to be regulated also in human skin explant samples with GvHR included *TAP1*, *PSMB8*, *C2*, *UBD*, and *OLR1* (Novota et al., 2011). These genes have known polymorphisms in humans and are therefore potential candidates for diagnostic biomarkers in the clinic.

The rat skin explant assay is a promising tool not only for the identification of candidate genes regulated in GvHR but also for

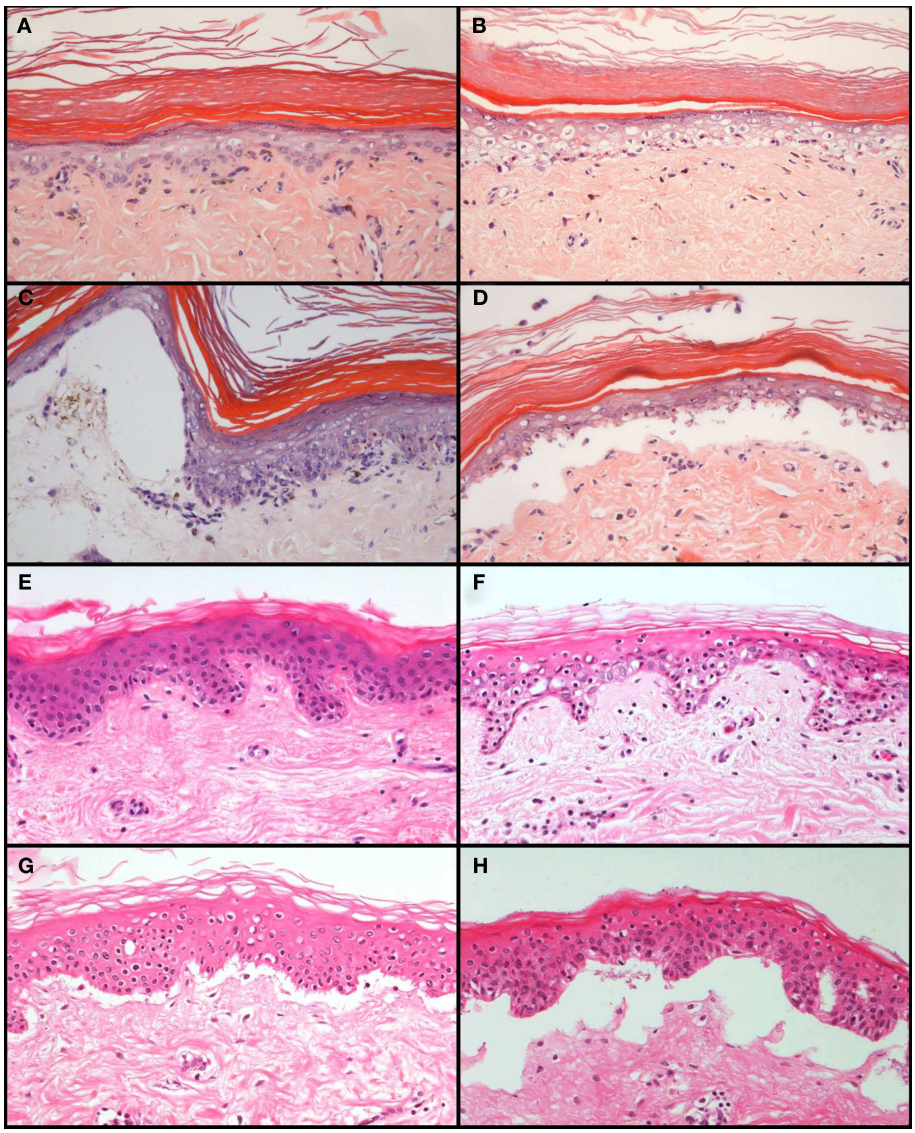

FIGURE 3 | Comparison of the histopathology of GvHR in human skin explants and rat skin explants. GvHR of increasing severity occur during co-incubation of pre-stimulated, allogeneic lymphocytes with explants of rat skin **(A–D)** or human skin biopsies **(E–H)**. Grade I [mild vacuolization of epidermal cells with occasional dyskeratotic bodies; **(A,E)**], grade II [diffuse vacuolization of basal cells with scattered dyskeratotic bodies; **(B,F)**], grade III [formation of subepidermal clefts; **(C,G)**], and grade IV [complete separation of the epidermis from the dermis; **(D,H)**] pathological changes are similar in human and rat skin explants. The photographs of the rat skin explants depicted in **(A–D)** were reprinted from Novota et al. (2008) with permission from the publisher.

the functional evaluation of GvHR in an experimental system. As with the human skin explant assay, it can be used to investigate the role of cytokines (Dickinson et al., 1991; Dickinson et al., 2002; Wang et al., 2002) and innate immunity in GvHR, e.g., heat-shock protein 70 (Jarvis et al., 2002, 2003), the roles of NK cells, dendritic cells (Wilson et al., 2009), regulatory T cells (Wang et al., 2009; Mavin et al., 2012), and subsets of T cells (Dickinson et al., 2002) as well as Fas expression (Ruffin et al., 2011).

CONCLUSION

The laboratory rat is a useful biological model organism for the study of alloHCT, immune reconstitution, and transplant-related complications. While the mouse has found its place in the limelight of comparative and translational immunology, a steadily increasing number of insightful investigations in the rat have been undertaken. When compared to mice and other animal models, experiments in rats can add instrumental insights into the consistency or divergence of findings made in related mammalian species. We found that after transplantation, host rats reconstituted peripheral regulatory T cells of host but not donor origin, and were deficient of any such cells when suffering from severe acute GvHD.

Important aspects of alloHCT, such as GvHR and tolerance mechanisms that are highly relevant for immune reconstitution

can now be addressed *ex vivo* in the rat skin explant model. Therefore, this assay represents a valuable and accessible research tool that complements the *in vivo* model. These research tools currently available in the rat model might also prove to be important for other areas of research.

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Adoptive T-cell immunotherapy from third-party donors: characterization of donors and set up of a T-cell donor registry

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Infection with and reactivation of human cytomegalovirus (CMV), Epstein-Barr virus (EBV), and adenovirus (ADV) are frequent and severe complications in immunocompromised recipients after hematopoietic stem cell transplantation (HSCT) or solid organ transplantation (SOT). These serious adverse events are associated with significant morbidity and mortality. Donor lymphocyte infusions (DLIs) are often used to treat both viral infections and leukemia relapses after transplantation but are associated with potentially life-threatening graft-versus-host disease (GvHD). Adoptive immunotherapy with virus-specific cytotoxic effector T cells (CTLs) derived from seropositive donors can rapidly reconstitute antiviral immunity after HSCT and organ transplantation. Therefore, it can effectively prevent the clinical manifestation of these viruses with no significant acute toxicity or increased risk of GvHD. In conditions, where patients receiving an allogeneic cord blood (CB) transplant or a transplant from a virus-seronegative donor and since donor blood is generally not available for solid organ recipients, allogeneic third party T-cell donors would offer an alternative option. Recent studies showed that during granulocyte colony-stimulating factor (G-CSF) mobilization, the functional activity of antiviral memory T cells is impaired for a long period. This finding suggests that even stem cell donors may not be the best source of T cells. Under these circumstances, partially human leukocyte antigen (HLA)-matched virus-specific CTLs from healthy seropositive individuals may be a promising option. Therefore, frequency assessments of virus-specific memory T cells in HLA-typed healthy donors as well as in HSCT/SOT donors using a high throughput T-cell assay were performed over a period of 4 years at Hannover Medical School. This chapter will address the relevance and potential of a third-party T-cell donor registry and will discuss its clinical implication for adoptive T-cell immunotherapy.

Keywords: adoptive immunotherapy, T-cell therapy, antiviral T lymphocytes, cytomegalovirus, Epstein–Barr virus, adenovirus

INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) is used to cure many malignant, benign and genetic disorders of the bone marrow, solid tumors, immunodeficiencies, metabolic, and autoimmune disorders (Ljungman et al., 2010). HSCT is generally performed after administration of sublethal doses of chemotherapy or chemoradiotherapy to achieve myeloablation, immunosuppression and eradication of abnormal cells. Intensive immunosuppressive therapy for prevention of graft rejection and graft-versus-host disease (GvHD) and for treatment of GvHD puts the patients at risk of opportunistic infections due to an ablated or severely compromised T-cell immune response. Such invasive conditioning procedures lead to a lack of immunological competence, which results mainly in a decrease in the number of CD3+ T lymphocytes in the patient's peripheral blood. Lymphopenia increases the patient's risk of *de novo* infection or reactivation of a latent virus. This mainly occurs during the early post-transplantation period and usually leads to a disseminated disease. The immune reconstitution period following HSCT (as long as 3–6 months) is therefore accompanied by a high incidence of infections with various pathogens that are normally controlled by T-cell immunity.

ROLE OF T CELLS IN TRANSPLANTATION

In allogeneic HSCT, the presence of a defined number of donor-derived T cells in the stem cell graft may prevent graft failures, infections or reactions caused by different pathogens (graft-versus-infection effect, GvI) as well as disease relapses (graft-versus-leukemia/graft-versus-tumor effect, GvL/GvT). On the other hand, an excessive number of T cells may increase the risk of developing GvHD. Major complications of stem cell and organ transplantation, such as graft rejection and GvHD, are countered by suppressing the host immune system via chemotherapy and radiation, immunosuppressive drugs, or conditioning regimens such as *in vivo* or *in vitro* T-cell depletion (Gooley et al., 2010). While immunocompromised, the patient is rendered susceptible

to a number of viral infections mainly caused by endogenous herpes viruses like cytomegalovirus (CMV) and Epstein-Barr virus (EBV) and by lytic agents such as adenovirus (ADV). Infections by several other viruses such as polyoma virus BK (BKV) and human herpesvirus 6 (HHV-6) as well as by invasive fungal pathogens such as *Aspergillus* are also reported to cause significant complications after stem cell and solid organ transplantation (SOT) (Marr et al., 2002; Garcia-Vidal et al., 2008; Pappas et al., 2010; Amir et al., 2011; Breuer et al., 2012).

VIRAL COMPLICATIONS AFTER ALLOGENEIC STEM CELL TRANSPLANTATION AND ORGAN TRANSPLANTATION

CMV INFECTION

Human CMV is a persistent β-herpesvirus that infects most healthy individuals during the first years of life (Khan, 2007). Healthy CMV-seropositive individuals have a high number of CMV-specific CD8+ and CD4+ T lymphocytes, which are essential to control viral reactivation without clinical symptoms (Rauser et al., 2004). Immunocompromised CMV-seropositive patients (R+) receiving a graft from a seronegative donor (D−) have a high risk of CMV disease (Zhou et al., 2009; Borchers et al., 2011; Ugarte-Torres et al., 2011). Additionally, it was reported that CMV reactivation developed in 96% of D+R+ patients but in less than 50% of D+R– patients (Lilleri et al., 2008, 2012). Reactivation of CMV results in significant morbidity and mortality; clinical manifestations include interstitial pneumonitis, gastroenteritis, fever, hepatitis, encephalitis, and retinitis (Einsele et al., 2008; Fujita et al., 2008a,b). While ganciclovir, valganciclovir, foscarnet, and cidofovir may help to prevent or treat CMV infection, they are associated with late-onset disease and serious side-effects, such as nephrotoxicity, myelosuppression, and impaired immune reconstitution, leading to an increase in invasive fungal infections and bacterial sepsis (Broers et al., 2000; Battiwalla et al., 2007; Fujita et al., 2008a,b; Boeckh and Ljungman, 2009). Furthermore, these drugs are often ineffective due to primary or secondary resistance, and patients still develop CMV disease in spite of antiviral treatment (Einsele et al., 2000; Mori et al., 2000; Fuji et al., 2011). Hence, cellular immunity is important for the control of CMV infection, and CMV-specific CD8+ and CD4+ T cells are essential for efficient immune protection after both primary activation and reactivation of latent CMV disease (Fujita et al., 2008a,b; Feuchtinger et al., 2010; Fuji et al., 2011).

EBV INFECTION

EBV-associated post-transplant lymphoproliferative disease (PTLD) occurring after HSCT or SOT is a potentially life-threatening condition (Cohen, 2000; Gottschalk et al., 2005). The overall incidence of PTLD after allogeneic HSCT is less than 1%, but was reported to be increased after transplantation with human leukocyte antigen (HLA)-mismatched or T-cell-depleted grafts (Landgren et al., 2009). Further risk factors for the development of PTLD include the degree and duration of immunosuppressive treatment and the use of antithymocyte globulin (ATG) with reduced-intensity transplant conditioning (Landgren et al., 2009; Pidala et al., 2011). PTLD developing after hematopoietic SCT usually results from donor B cells and appears within the first 6–12 months post-transplant, when profound deficiencies of EBV-specific cytotoxic effector T cells (CTLs) (EBV-CTLs) occur (Meij et al., 2003). In SOT, the incidence varies with the type of organ (1–2% after kidney transplantation and up to 20% after thoracic organ transplantation) (Trappe et al., 2012). Eighty five percentage of pediatric PTLDs and 60–70% of adult PTLDs in Europe are EBV-associated. Insufficient EBV-specific T-cell responses have been linked to a higher risk of PTLD development (Guppy et al., 2007). PTLD in SOT recipients usually originates from recipient B cells; however, a significant percentage (10–15%) of predominantly early PTLD in kidney or liver graft recipients restricted to the organ graft displays donor origin (Olagne et al., 2011). Treatment includes reduction of immunosuppressive drugs as far as tolerated, immunotherapy (monoclonal antibodies like Rituximab), and cytotoxic chemotherapy. Preemptive therapy with CD20 monoclonal antibodies (Rituximab) has been attempted and may control EBV-associated lymphoproliferation (Kuehnle et al., 2000; van Esser et al., 2002; Trappe et al., 2012). Treatment is often complicated by side effects, and severe complications are foreseeable in patients with pre-existing organ dysfunction. Second line treatment options are scarce and have not been tested in clinical studies. Adoptive T-cell therapy using EBV-CTLs has been successfully employed for prophylaxis and treatment of PTLD in high-risk patients (Haque et al., 2007; Heslop et al., 2010; Shen et al., 2011).

ADV INFECTION

The incidence of ADV infection ranges from 3 to 20%, and is significantly higher in pediatric patients (Feuchtinger et al., 2005; Fowler et al., 2010). Overall ADV-associated mortality ranges from 18 to 26%. ADV infection may involve the respiratory, gastrointestinal and/or urinary tract. The diagnosis of ADV infection can be difficult due to its complexity. The most common cause of adenoviral infection after HSCT is reactivation of a latent virus persisting, for example, in intestinal mucosa. Furthermore, there are reports indicating a more than 4-fold increased risk of ADV infection in patients with grafts from seropositive donors (Runde et al., 2001; Walls et al., 2003; Fowler et al., 2010). Risk factors underlying this increase are: T cell-depleted grafts (Chakrabarti et al., 2002; Lion et al., 2003), allogeneic graft from matched unrelated donors (MUD) (Ebner et al., 2005), acute GvHD (Bruno et al., 2003), cytotoxic and immunosuppressive therapy (Watcharananan et al., 2010), and lymphocytopenia (Feuchtinger et al., 2008; Watcharananan et al., 2010). Currently, 53 different human serotypes (ADV1 to ADV53) are classified into seven species (A to G). The most prevalent serotypes in transplant patients are ADV 1, 2, 5, 31, and 41. In 49 pediatric patients who received a stem cell transplant at Hannover Medical School (MHH) from 2003 to 2011, sequence analysis revealed ADV species A (1% ADV18, 20.5% ADV31), B (4% ADV3), C (20.5% ADV1, 32% ADV2, 4% ADV6), E (8% ADV4), and F (10% ADV41) (Mynarek et al., submitted). Incidence of ADV viremia was high (50%) with mostly asymptomatic patients, who developed only low viral loads. Despite a low ADV-related mortality rate of 0.84% in this cohort, high peak ADV blood loads were a significant

and independent risk factor for survival after HSCT. Cidofovir and Ribavirin have been used to treat immunocompromised patients suffering from ADV infection (Lankester et al., 2004; Lindemans et al., 2010). However, these antiviral agents were shown to limit but not clear the infection and are associated with severe side effects. Recent studies indicate that the elimination of ADV is only achieved by recovery of cellular immunity (Feuchtinger et al., 2006). Therefore, the adoptive transfer of antigen-specific T cells could be an effective and non-toxic alternative strategy.

ADOPTIVE T-CELL THERAPY USING ANTIVIRAL T CELLS

Although donor lymphocyte infusions (DLIs) can be used after transplantation to treat both viral infections and leukemia relapses, they are associated with potentially life-threatening GvHD (Collins et al., 2000; Choi et al., 2005). The shortcomings of conventional therapies have increased the interest in an immunotherapeutic approach to treat viral disorders. It was recently shown that the adoptive transfer of antiviral cytotoxic T lymphocytes directed against CMV (Einsele et al., 2008; Mackinnon et al., 2008; Brestrich et al., 2009; Feuchtinger et al., 2010; Peggs et al., 2011), EBV (Haque et al., 2010; Heslop et al., 2010; Moosmann et al., 2010; Doubrovina et al., 2012), and ADV (Feuchtinger et al., 2008; Hoffman, 2009; Zandvliet et al., 2010; Qasim et al., 2011) isolated from seropositive donors can rapidly reconstitute antiviral immunity after stem cell and organ transplantation without significant toxicity and with limited increase in GvHD. Infusions of peripheral blood-derived T-lymphocyte lines enriched in multivirus (CMV, EBV, and ADV)-specific T cells reproducibly controlled infections by all three viruses after allogeneic HSCT and may form the basis of future adoptive immunotherapy trials in patients at risk of multiple infections (Leen et al., 2006; Fujita et al., 2008a,b; Khanna et al., 2011; Zandvliet et al., 2011; Gerdemann et al., 2012).

Although the minimal frequency of antigen-specific T cells required to mediate an antiviral effect in patients is not known it is likely to vary widely depending on the target antigen and other factors, including quantitative and even more qualitative properties of the effector T cells as well as the host environment. The importance of the host environment to facilitate persistence and function of transferred T cells has recently been elucidated (Berger et al., 2009).

ADOPTIVE T-CELL THERAPY FOR CMV INFECTION

The presence of CD8+ and CD4+ antiviral T cells was reported to be essential in controlling viral infection and reactivation by restoring cellular immunity. Since the first promising results began to emerge in the early 1990s (Greenberg et al., 1991; Riddell et al., 1991), different strategies to generate virus-specific T lymphocytes for clinical use have been described. In 1995, Walter and colleagues demonstrated that CMV reactivation after HLA-identical allogeneic HSCT can be prevented by adoptive transfer of CMV-specific cytotoxic T cells, which were generated *in vitro* from the transplant donor and transferred to the patient (Walter et al., 1995). To be suitable for clinical applications, the cells used for adoptive T-cell transfer must be virus-specific T cells generated by *in vitro* induction and expansion from a small number of precursor cells, over a short period of culture, under highly reproducible conditions, and in accordance with good manufacturing practice (GMP). CMV-specific memory T cells are present at high frequencies in the blood of healthy CMV-seropositive donors. Typically, they represent 0.5% to 4% of the CD8+ T-cell pool and 0.05% to 1.6% of the CD4+ T helper (Th) cell pool (Rentenaar et al., 2000; Cwynarski et al., 2001). Most protocols for the generation of virus-specific T cells use peptide-loaded monocyte-derived dendritic cells (DCs), artificial antigen-presenting cells (aAPCs), or CMV-infected immature dendritic cells as stimulator cells (Sun et al., 1999; Peggs et al., 2001; Carlsson et al., 2003; Oelke et al., 2003; Lozza et al., 2005; Paine et al., 2007, 2010; Lilleri et al., 2008). However, these protocols are difficult to standardize and often laborious to adapt to GMP conditions. Furthermore, previous works have defined CD4+ and/or CD8+ T-cell responses to whole viral lysates, virally infected cells, recombinant proteins, and various HLA-restricted viral peptides.

The majority of studies have focused on the 65 kDa matrix phosphoprotein (pp65, also known as glycoprotein 64 and UL83) and the immediate-early protein 1 (IE1) of CMV as immunodominant targets of CMV-specific T-cell responses (Solache et al., 1999; Elkington et al., 2003; Sylwester et al., 2005). Regarding the induction of antiviral T-cell responses, pp65 has been recognized as a source of immunodominant epitopes that stimulate both CTLs and T helper cells. Most pp65-specific T cells predominantly produce effector cytokines like interferon-gamma (IFN-γ), interleukin-2 (IL-2) and tumor necrosis factor-alpha (TNF-α). The secretion of these cytokines is used for the detection and enrichment of antiviral T cells (Rauser et al., 2004). HLA class I-restricted peptides derived from CMV pp65 protein (e.g., the HLA-A*0201-restricted CMV$pp65_{495-503}$ peptide) are known to be potent inducers of CTLs (Oelke et al., 2003; Paine et al., 2007). Because the known peptide epitopes are restricted to certain HLA alleles, the use of HLA-restricted peptides cannot exploit the full range of HLA diversity present in the patient. Furthermore, the use of HLA class I immunogenic peptides mainly leads to the generation of CD8+ T cells, resulting in the generation of an immune response restricted to cytotoxic T cells.

ADOPTIVE T-CELL THERAPY FOR EBV INFECTION

Adoptive T-cell therapy using EBV-CTLs has been successfully employed for prophylaxis and treatment of PTLD in high-risk patients (Haque et al., 2007; Heslop et al., 2010; Shen et al., 2011). EBV-transformed B-lymphoblastoid cell lines (B-LCLs) are established as antigen-presenting cells (APCs) for the generation of EBV-specific T cells. Following this approach, Tanzina Haque and colleagues (University of Edinburgh, UK) established and used a bank of frozen EBV-specific CTLs generated from the peripheral blood of Scottish blood donors to treat patients with progressive PTLD with CTLs selected on the basis of the best HLA-matches between the CTL donor and PTLD patient (Wilkie et al., 2004; Haque et al., 2007). In this multicenter clinical phase II trial, CTLs showed high efficacy varying according to the degree of HLA-match (at least 3/6) and did not induce any GvHD. Haque and colleagues demonstrated that the transfer of partially HLA-matched EBV-CTLs grown from healthy donors by repetitive antigenic stimulation is safe and results in

tumor regression in about 60% of PTLD patients unresponsive to at least one prior treatment (Haque et al., 2007, 2010). In a study by Doubrovina et al., 49 HSCT patients with biopsy-proven EBV-lymphoproliferative disease (EBV-LPD) were treated with either HLA-compatible DLIs or HLA-compatible or HLA-disparate EBV-specific CTLs (Doubrovina et al., 2012). Acute GvHD was observed in 17% of all DLI recipients but in no EBV-CTL recipients. The data further supports the findings of Haque et al. indicating that EBV-CTLs from healthy, partially HLA-matched third-party donors provide an easily accessible source of effector T cells for the treatment of EBV-associated PTLD (Bollard et al., 2012; Doubrovina et al., 2012).

Nevertheless, the use of EBV-transformed cells as APCs to generate EBV-specific T cells for clinical use has three major limitations: (1) The manufacturing process for the B-LCL-based generation of sufficient numbers of EBV-CTLs for clinical use takes approximately 3 months (half for the generation of B-LCLs and half for T-cell expansion). Consequently, the production of EBV-CTLs for the individual patient with PTLD required diligent identification of patients at risk. Furthermore, the process is difficult to standardize and poses a potential biohazard due to the presence of live viruses. (2) So far, EBV-CTLs have mostly been manufactured from autologous peripheral blood mononuclear cells by repetitive *in vitro* stimulation with EBV antigens presented by APCs. In children, the lack of EBV infection prior to organ transplantation is an additional obstacle to the generation of sufficient numbers of EBV-CTLs. (3) The coverage of latency types is incomplete. Understanding the latency types of EBV is important for the effective design of adoptive T-cell strategies. While EBV-transformed B-LCLs express viral antigens representing latency type III (10 viral proteins), these cells might not be useful for generating specific T cells that effectively target late PTLDs expressing latency type II [only three viral proteins EBV nuclear antigen-1 (EBNA-1), late membrane proteins (LMP) LMP1 and LMP2 expressed] or latency type I tumors (e.g., Burkitt's lymphoma) [see review in (Bollard et al., 2012)]. The antigenic specificity of T cells generated by this method is further limited by the set of EBV proteins available in the B-LCLs—they contain mostly proteins from the early replication cycle (e.g., EBNA1-3) and no lytic proteins like BZLF. Unfortunately, EBNA-1, which is expressed in all three latency types, is poorly immunogenic (Thorley-Lawson and Allday, 2008). This was confirmed in studies analyzing the frequency of EBV-specific memory T cells in response to three commercially available peptide pools (EBNA-1, BZLF1, and LMP2A) in 195 healthy EBV-seropositive blood and platelet donors. It has been found that T-cell populations against the BZLF1-derived peptide pool were the most frequent in seropositive donors, as reflected by a high number of responders: 112 (57%) vs. 90 (49%) for EBNA-1 and 64 (33%) for LMP2A (Sukdolak et al., submitted).

ADOPTIVE T-CELL THERAPY FOR ADV INFECTION

Feuchtinger et al. clearly demonstrated that children with ADV-associated mortality had no ADV-specific T cells, whereas patients who cleared ADV infection had normal frequencies of antiviral T cells (Feuchtinger et al., 2006). Since an increased risk of adenoviral infection in immunocompromised patients has been shown to correlate with low numbers of T cells, efforts have been made over the past years to identify immunogenic ADV-derived epitopes. As of now, the 53 known human serotypes (ADV1 to ADV53) are classified into seven species (A to G). The most prevalent serotypes in transplant patients are ADV1, 2, 4, 5, 41, and 31. Hexon, the major capsid protein, serves as the immunodominant target antigen across the different serotypes of ADV. A few hexon-derived $CD8^+$ T-cell epitopes for ADV species C have been identified and, for the most of them their clinical relevance remains unclear. This makes diagnosis and treatment very challenging. These epitopes are highly conserved, suggesting that ADV-specific T cells can cross-react with ADV serotypes and may therefore provide protection against a wide range of ADV strains (Zandvliet et al., 2010). Feuchtinger et al. tested the specific T-cell response to both hexon protein and whole ADV in HSCT donors and found that 10.5% of donors had a detectable T-cell response to whole ADV but no response to hexon protein, and 17% of donors had no detectable T-cell response to ADV (Feuchtinger et al., 2008). Zandvliet et al. were able to detect specific $CD8^+$ T cells in 6/16 healthy donors after stimulation with 15-mer hexon peptide pool, while stimulation with peptides corresponding to known $CD8^+$ hexon epitopes induced responses in 3/16 donors (Zandvliet et al., 2010). These studies clearly indicate the need to identify more immunodominant ADV epitopes.

STRATEGIES FOR ISOLATION OF ANTIGEN-SPECIFIC T CELLS FOR ADOPTIVE T-CELL THERAPY

Direct selection of virus-specific T cells without long-term *ex vivo* stimulation and manipulation is an attractive way to generate clinical-grade antiviral T cells. The two main approaches are separation by the use of cytokine secretion assays [e.g., interferon-gamma (IFN-γ) secretion assay (Rauser et al., 2004; Feuchtinger et al., 2008, 2010; Mackinnon et al., 2008; Moosmann et al., 2010; Peggs et al., 2011)] and isolation by the use of peptide-MHC (pMHC) multimers (Cobbold et al., 2005; Yao et al., 2008; Casalegno-Garduno et al., 2010; Schmitt et al., 2011). Direct isolation of antigen-specific T-cells by stimulation with antigenic peptides, proteins, or peptide-pools followed by cytokine capture and magnetic isolation is a rapid method of producing antiviral T-cells according to GMP guidelines (Rauser et al., 2004). It avoids safety and regulatory issues associated with prolonged T-cell culture and potential viral biohazards. Cytokine secretion assays using recombinant proteins or synthetic peptide pools consisting of overlapping peptides spanning an entire immunodominant protein are not restricted by HLA variations, and they enable the generation of CD4+ and/or CD8+ T-cell responses to multiple epitopes (Rauser et al., 2004). In the case of CMV 2, EBV 3, and ADV 1, GMP-grade peptide pools covering the viral proteins pp65 and IE-1 (CMV); LMP-2A, EBNA-1, and BZLF-1 (EBV); and the hexon (ADV) are now available for the generation of clinical-grade antiviral CD4+ and CD8+ T cells, irrespective of the HLA-type. It is known that specific CD4+ T-cell help is required to elicit and promote an efficient CD8+-restricted CTL response to viral antigens. CD4+ T cells secrete various cytokines to regulate and coordinate the function of T cells and other immune cells. They are also known to be the most effective cell population in clearing infections, such as ADV

(Feuchtinger et al., 2006). Unfortunately, compared to the isolation of T cells by pMHC multimer technologies the purity is lower and alloreactivity of T cells might result in undesirable immune responses especially in HLA-mismatched or haploidentical settings. Nevertheless, clinical trials showed no increase in GvHD or graft rejection after adoptive immunotherapy using IFN-γ-isolated antiviral T cells (Feuchtinger et al., 2006; Peggs et al., 2011; Doubrovina et al., 2012).

The pMHC multimer technology requires knowledge of immunodominant HLA-restricted peptide epitopes and enables the isolation of either antigen-specific CD8+ T cells (pMHC class I multimers) or CD4+ T cells (pMHC class II multimers) of high purity. It is still difficult to generate the respective multimers needed for CD4+ T-cell isolation. Cobbold and colleagues, the first investigators to use tetramer-purified CMV-specific CD8+ T cells for adoptive transfer, were able to detect functionally active antiviral T cells within 10 days (Cobbold et al., 2005). In a study by Uhlin et al., tetramers corresponding to two EBV antigens were used to treat a patient suffering from PTLD after cord blood (CB) transplantation (Uhlin et al., 2010). Reversible pMHC multimers (streptamers, histamers), the latest generation of pMHC multimer technologies, were developed in order to isolate antigen-specific T cells without altering their functional status (Knabel et al., 2002; Tischer et al., 2012) and are already used clinical applications (Schmitt et al., 2011). Although the results are promising, this technology is limited to those donors who express an HLA allele with known viral epitopes and have sufficient numbers of memory T cells present in the peripheral blood.

T cells for adoptive immunotherapy could potentially be isolated from a T lymphocyte pool phenotypically identified as CD45RA+ CD62L+ naïve (N), CD45RO+ CD62L+ central memory (TCM), and CD62L- effector memory (TEM) T-cells subsets. These cells differ in phenotype, function, and homing (Sallusto et al., 2004). Recently it was shown, that although TEM have proliferative potential *in vitro*, these cells fail to survive in primates *in vivo* (Berger et al., 2008). These results most likely have implications for the types of T cells that should be selected for adoptive transfer.

DO WE NEED THIRD-PARTY T-CELL DONORS?

The first clinical trials showed that T cells generated by the above-described procedures can be successfully used to treat viral infection, reactivation, or virus-induced malignancies after stem cell and SOT. It was also shown that adoptive immunotherapy with donor-derived virus-specific CTLs generated *in vitro* can effectively prevent the clinical manifestation of these viruses with no acute toxicity or increased risk of GvHD. In allogeneic stem cell transplantation, seropositive stem cell donors can usually serve as T cell donors and are available for T-cell donation. However, some seropositive donors may not consent, may be unavailable to provide T cells, or may not have enough antiviral memory T cells in their blood despite seropositivity. Recent studies have also shown that granulocyte colony-stimulating factor (G-CSF) mobilization has a long-term negative effect on the functional activity of T cells (Franzke et al., 2003; Toh et al., 2009). Bunse et al. (in preparation), suggesting that antiviral memory T cells from stem cell donors might not be the best source. Furthermore, delayed hematologic engraftment and immune reconstitution are a major problem in patients receiving CB transplants. These problems may be attributable to a low stem cell dose, small numbers of transferred T cells, the absence of memory T cells within the CB grafts, or the hyporesponsiveness of CB APCs. Therefore, these patients are at high risk of developing viral complications as are patients receiving transplants from seronegative donors or SOT patients receiving organ grafts from deceased donors. CMV-seropositive immunocompromised patients (R+) with transplants from seronegative donors (D−) were shown to have a high risk of CMV disease (Zhou et al., 2009; Ugarte-Torres et al., 2011). It was reported that CMV reactivation occurs in 96% of D+R+ patients but in less than 50% of D+R– patients (Lilleri et al., 2008; Borchers et al., 2011). Therefore, adoptive transfer of virus-specific CTL is not a viable option for high-risk patients (R+) with seronegative donors (D−).

Under these conditions, partially HLA-matched virus-specific T cells from healthy seropositive individuals could be a successful alternative and could play a significant role in the prevention and treatment of viral infections in transplant recipients. Studies on the use of HLA-matched T-cells from third-party donors for the treatment of stem cell and organ recipients are currently in progress.

The third-party approach was first successfully tested in SOT and HSCT patients with EBV-associated PTLD at the University of Edinburgh (Haque et al., 2002, 2007; Wilkie et al., 2004). As mentioned in chapter 4.2 Haque and colleagues showed that partially HLA-matched EBV-specific T cells (at least 3/6) produce a 65% response rate and a 42% complete response rate in PTLD patients after SOT, indicating that the transferred EBV-specific T cells were highly effective and did not induce any GvHD (Haque et al., 2007, 2010). Later studies (Barker et al., 2010; Uhlin et al., 2010; Doubrovina et al., 2012) including CB transplant patients confirmed these results. O'Reilly and colleagues used partially HLA-matched EBV-specific T cells to treat EBV lymphoproliferative disease in allogeneic HSCT recipients and achieved complete or partial remission in 68% (Barker et al., 2010; Doubrovina et al., 2012).

The effectiveness of third-party donor-derived T cells in treating CMV and ADV is now being investigated in various clinical trials (e.g., in Memorial Sloan-Kettering Cancer Center's phase II trial on the treatment of CMV). Feuchtinger et al. reported their results obtained with T cells from two third-party, partially HLA-matched, unrelated CMV-specific T-cell donors to treat CB transplanted patients (Feuchtinger et al., 2010). The cells were rapidly isolated from the donors using an IFN-γ cytokine secretion assay after brief stimulation of peripheral blood mononuclear cells with CMV pp65. *In vivo* expansion of CMV-specific T cells and clearance of CMV infection was observed in one patient (Schottker et al., 2008; Feuchtinger et al., 2010). Third-party virus-specific T cells directed against ADV were also shown to be effective for the eradication of ADV (Qasim et al., 2011). Rooney and Leen recently investigated the use of banks of third-party T cells specific for CMV, EBV, and ADV in HSCT patients and observed a high (>70%) response rate to all three viruses, even in case of only one HLA allele match between the CTL line and the recipient (Gerdemann et al., 2012).

THE ALLOGENEIC T-CELL DONOR REGISTRY

First results using T cells from partially HLA-matched third-party donors are promising. The data indicate that allogeneic T-cell therapy is an attractive option for patients suffering from viral infections after allogeneic HSCT or organ transplantation. Therefore, we hypothesize that a registry of HLA-typed allogeneic T-cell donors typed for virus-specific T cells would enable rapid availability of T cells for adoptive immunotherapy of virus-associated diseases in transplant recipients without an adequate T-cell donor. This registry might provide a stand-alone off-the-shelf product.

To gain more insight into virus-specific memory T-cell pools in healthy donors and to identify the most efficient antigens for adoptive immunotherapy, we determined the frequencies of virus-specific memory T cells in healthy donors. To date, T-cell frequencies have been determined in more than 300 HLA high-resolution typed donors at Hannover Medical School's Institute for Transfusion Medicine by INF-γ enzyme-linked immunospot (ELISpot) assay and flow cytometry using pMHC multimers (Sukdolak et al., submitted). Using these well-established methods of T-cell monitoring (Cox et al., 2006; Hadrup and Schumacher, 2010), we assessed the frequencies of T cells against GMP-quality peptides and peptide pools derived from viral proteins known to be immunodominant or subdominant. For example, phosphoprotein 65 (pp65) and immediate early (IE)−1 were used for CMV (Wills et al., 1996), BZLF1, nuclear antigen (EBNA)−1 and latent membrane protein 2A (LMP2A) for EBV (Houssaint et al., 2001), and hexon, the major capsid protein of ADV, for ADV (Leen et al., 2008). For optimal T-cell help and cytotoxic response, the T-cell population should consist of CD4+ and CD8+ virus-specific T cells. For high efficiency, these cells should also target various viral epitopes. For each virus studied, we identified at least 61% potential CTL donors with highly significant differences in frequencies of T cells against each of the six viral antigens tested: pp65 and IE-1 (CMV), BZLF1, LMP2A, and EBNA1 (EBV), and hexon (ADV).

All CMV-seropositive donors were reactive to the CMV pp65 peptide pool, whereas only 79% reacted with IE-1. One hundred and seventy three of the EBV-seropositive donors had antigen-specific T cells that reacted with at least one of three EBV peptide pools, showing highest frequencies for BZLF1, and 73% of the ADV-seropositive donors reacted with the hexon peptide pool. Interestingly we found that in short-term *in vitro* peptide stimulation assays for ADV and EBV a donor response to a certain peptide may not be determined on day 0. Peptide-specific T cells were detected by multimer staining, but overall frequencies were lower than those obtained for the corresponding peptide pools. The results of our study demonstrate that, depending on the antigen used, no antiviral T cells can be detected in approximately one-third of donors despite seropositivity, and that serological testing for CMV by the standard ELISA technique gives false-positive results in approximately 10% of donors. It is important to remember that GvHD remains a dreaded side effect and there is a particularly risk of alloreactivity, especially in partially-HLA-matched settings (Amir et al., 2010; D'Orsogna et al., 2010; Qasim et al., 2011). Therefore, we developed a modified granzyme B ELISpot assay to detect T-cell specificity and alloreactivity against patient cells and used it to test T-cell effector function against unloaded PBMCs, (HLA class I-negative) K562 cells and "patient unloaded and antigen-loaded PBMCs." This method can also be used to identify the best HLA-matched allogeneic antiviral CTL donor. The HLA-types of CTLs with the highest specific killing of "patient antigen-loaded PBMCs" were identified and considered in partially HLA-matched allogeneic T-cell therapy.

The results were used to establish a registry of potential T-cell donors (allogeneic T-cell donor registry, alloTCDR) with highly virus-specific T-cell precursors. The alloTCDR database will document the donors' HLA-type (class I and II high resolution), virus serology (ADV, CMV, and EBV), virus-specific T-cell frequencies, best T-cell detection method, and results of functional and alloreactivity assays. This registry of HLA-typed allogeneic T-cell donors profiled for virus-specific T cells will ensure the rapid availability of T cells for adoptive immunotherapy of virus-associated diseases in transplant recipients without an adequate T-cell donor.

SUMMARY

Antigen-specific T cells can be effectively used in the treatment of viral infection or reactivation after stem cell and SOT. So far most studies did not show significant increase in the development of acute toxicity or increased risk of GvHD following T-cell transfer. Unfortunately, for patients receiving an allogeneic CB transplant, a transplant from a virus-naïve donor or a transplant from a cadaveric donor no T-cell donor will be available. Furthermore, it was shown that in some cases no antiviral memory T cells are present in the donor despite seropositivity, and that G-CSF treatment has a negative effect on antiviral cell function. Third-party partially HLA-matched virus-specific T cells from healthy seropositive individuals may be an option, which can be successfully employed under these circumstances. In future, we will extend the typing and profiling of potential third-party donors to include the T-cell frequencies of other viruses, such as polyoma virus BK, human herpesvirus 6, and invasive fungal pathogens such as *Aspergillus.* The registry of unrelated HLA-typed allogeneic T-cell donors profiled for virus-specific T cells will ensure the rapid availability of T cells for adoptive immunotherapy of pathogen-associated diseases in transplant recipients.

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Natural killer cell mediated antibody-dependent cellular cytotoxicity in tumor immunotherapy with therapeutic antibodies

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In the last decade several therapeutic antibodies have been Federal Drug Administration (FDA) and European Medicines Agency (EMEA) approved. Although their mechanisms of action *in vivo* is not fully elucidated, antibody-dependent cellular cytotoxicity (ADCC) mediated by natural killer (NK) cells is presumed to be a key effector function. A substantial role of ADCC has been demonstrated *in vitro* and in mouse tumor models. However, a direct *in vivo* effect of ADCC in tumor reactivity in humans remains to be shown. Several studies revealed a predictive value of FcγRIIIa-V158F polymorphism in monoclonal antibody treatment, indicating a potential effect of ADCC on outcome for certain indications. Furthermore, the use of therapeutic antibodies after allogeneic hematopoietic stem cell transplantation is an interesting option. Studying the role of the FcγRIIIa-V158F polymorphism and the influence of Killer-cell Immunoglobuline-like Receptor (KIR) receptor ligand incompatibility on ADCC in this approach may contribute to future transplantation strategies. Despite the success of approved second-generation antibodies in the treatment of several malignancies, efforts are made to further augment ADCC *in vivo* by antibody engineering. Here, we review currently used therapeutic antibodies for which ADCC has been suggested as effector function.

Keywords: natural killer cells, ADCC, tumor immunotherapy, therapeutic antibodies, allogeneic stem cell transplantation

INTRODUCTION

Naturally cytotoxic cells against tumor cells were first described in humans and mice in the 1970s of the last century (Rosenberg et al., 1972; Oldham and Herberman, 1973; Herberman et al., 1975a,b; Kiessling et al., 1975a,b). Initially regarded as artifacts, these cells were recognized eventually as a novel lymphocyte population and named natural killer (NK) cells after their natural occurrence and spontaneous capacity to kill lymphomas and leukemic cells in non-immunized animals (Kiessling et al., 1975a,b). Nowadays, NK cells are recognized as a subset of cytotoxic innate lymphoid cells (ILCs) which are able to directly kill virus-infected cells and tumor cells and participate in shaping the adaptive immunity by secretion of cytokines (e.g., IFN-γ) (Vivier et al., 2011). The role of NK cells in human cancer is highlighted by a study linking low peripheral blood NK cell activity with increased cancer risk (Imai et al., 2000). Furthermore, an association of NK cell infiltration into the tumor site with better disease prognosis has been shown for several malignancies (Carrega et al., 2008; Halama et al., 2011; Platonova et al., 2011; Eckl et al., 2012).

Human NK cells are defined by the phenotype $CD3^-$ $CD56^+$; additionally they are CD19 and CD14 negative. The only marker that is specific for NK cells is NKp46. NK cells comprise 5–15% of all circulating lymphocytes (Lanier et al., 1986; Walzer et al., 2007). They are commonly divided into two major subpopulations, $CD56^{dim}$ $CD16^+$ and $CD56^{bright}$ $CD16^-$, with each of those possessing distinct effector functions. The $CD56^{dim}$ $CD16^+$ subset comprises 90% of all peripheral blood NK cells and mediates an early response via direct cellular cytotoxicity induced by perforin and granzyme, FasL, and TRAIL interactions as well as cytokine production (De Maria et al., 2011). One major characteristic of NK cells is their constant state of readiness to respond immediately. In contrast to T cells, NK cells constitutively express perforin. This facilitates the instant polarized delivery of apoptosis-inducing granzymes after formation of a lytic synapse between the NK cell and a target cell (Shresta et al., 1995). The $CD56^{bright}$ $CD16^-$ subset mediates a late but sustained effector function via potent pro-inflammatory cytokine and chemokine release of mainly IFN-γ, but is poorly cytotoxic (De Maria et al., 2011). NK cell activation and cytotoxicity is controlled by a complex balance between activating receptors, inhibitory receptors and co-receptors (Lanier, 2003; Leung, 2011). Positive and negative downstream signals of these receptors are integrated and decisive for NK cell activation (**Figure 1**). Hence, absence of inhibitory signals on target cells together with engagement of activating receptors as NKG2D, DNAM-1, and 2B4 as well as the natural cytotoxicity receptors (NCRs), including NKp46, NKp44, and NKp30 mediate triggering of resting NK cells (Moretta et al., 2001; Bryceson and Long, 2008; Lanier, 2008). Whereas for NKG2D, DNAM-1, and 2B4 multiple ligands are known (Bottino et al., 2005), despite their involvement in tumor cell lysis the NCR ligands have remained rather elusive (Moretta et al., 2006). Nevertheless, NKp30 has been shown to recognize a tumor cell ligand of the B7 family, B7-H6 (Brandt et al., 2009) and NKp44 is suggested to recognize proliferating cell nuclear antigen (PCNA), which surprisingly triggers inhibition of

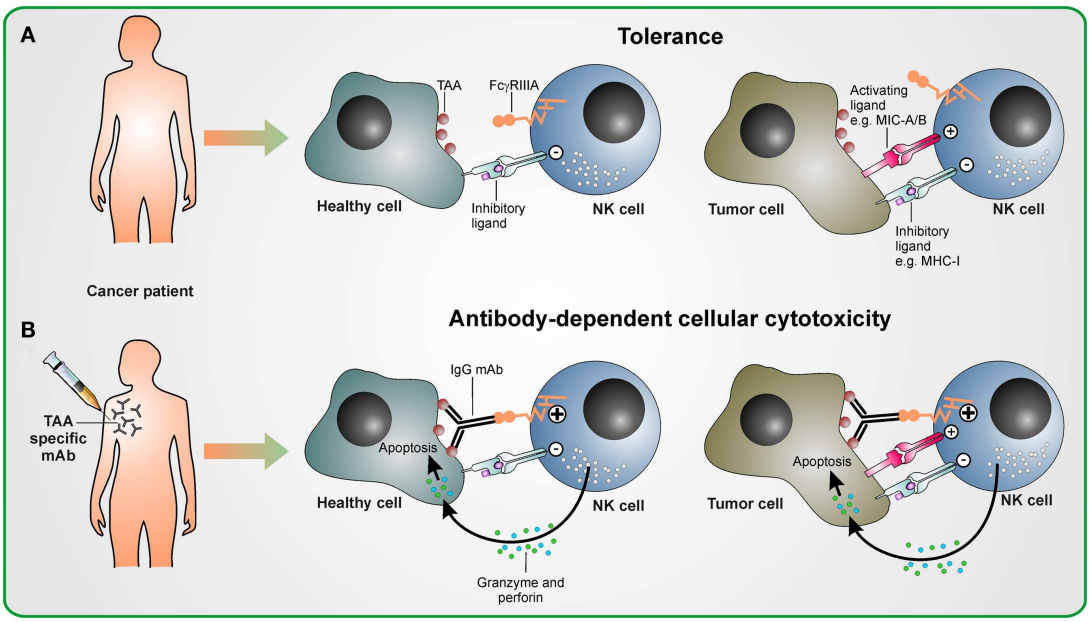

FIGURE 1 | Antibody-dependent cellular cytotoxicity in therapeutic antibody treatment. (A) Without antibody therapy, NK cells are tolerant to healthy cells and tumor cells, if the strength of activating signal they receive upon encountering activating ligands on these malignant cells does not overcome the inhibitory signaling delivered by inhibitory ligands as, e.g., MHC class I molecules. **(B)** Upon treatment with tumor antigen-associated (TAA) specifc antibody, the activating stimulus from FcγRIIIa induced by antibodies cross-linking NK cells with TAA-expressing healthy and malignant cells overcomes inhibitory signals. This leads to the activation of NK cells and ADCC is mediated by releasing cytotoxic granules containing perforin and granzyme.

NK cells (Rosental et al., 2011). However, several of those receptors need to be triggered by target cells for activating downstream signals to prevail over inhibitory signals and NK cells to be activated and to mediate target cell lysis (Moretta et al., 2001; Bryceson et al., 2006).

ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY

Most hematopoietic cells, except most T cell subsets, express Fcγ receptors (FcγRs) (Nimmerjahn and Ravetch, 2008). There are three types of FcγRs which recognize the Fc part of IgG antibody subclasses with different affinities. The activating FcγRI (CD64) binds to human IgG1 and IgG3 with high affinity, is expressed on macrophages and neutrophils, and mediates phagocytosis of target cells. The FcγRII (CD32) class comprises activating low affinity FcγRIIa (which binds human IgG1, IgG2, and IgG3) and inhibitory FcγRIIb (which recognizes human IgG1 and IgG3 with low affinity) and may attenuate signaling from activating receptors as FcγRI if engaged in phagocytes. FcγRIIIb is a protein expressed by neutrophils and may play a role in neutrophil activation. Activating low affinity FcγRIIIa (type III receptor for IgG; CD16) mediates antibody-dependent cellular cytotoxicity (ADCC) and is highly expressed on the cytotoxic $CD56^{dim}$ $CD16^{+}$ NK cell subset as well as on other hematopoietic cells. Nevertheless, NK cells are thought to be the key mediators of ADCC, since only NK cells do not co-express the inhibitory FcγRIIb. In contrast, all other FcγR-expressing cells are tightly controlled by the balance between activating and inhibitory FcγRs. Efficient FcγRIIIa signaling depends on high avidity for specific binding to antibodies which ensures that NK cells are activated when antibodies have bound to a multimeric or multivalent cognate antigen only (Banks et al., 2002). These antibodies of the subclasses IgG1 and IgG3 binding to FcγRIIIa induce a potent activating signal which overcomes inhibitory signals and results in both cytotoxicity and a cytokine response (Chan et al., 2012) (**Figure 1**). Co-engagement of other activating receptors has a synergistic effect and may enhance NK cell activation (Bryceson et al., 2006).

Several mouse model studies postulated the impact of activating FcγRs on anti-tumor effects in antibody therapy indicating that ADCC has a substantial effect on tumor rejection. A study using a xenograft breast carcinoma model has shown that an antibody engineered to prevent Fc binding to FcγRIIIa and specific to Her2/neu [Human Epidermal Growth Factor Receptor (EGFR) 2, CD340] which is overexpressed in certain aggressive types of breast cancer, was, in contrary to its murine parental antibody targeting Her2/neu, unable to arrest tumor growth *in vivo* (Clynes et al., 2000). Additionally, this study in mice showed in a murine lymphoma model that treatment with CD20-specific antibody rituximab was capable of preventing tumor growth in FcR wild-type mice whereas FcR knock-out mice failed to mediate antibody-dependent tumor protection. Another study demonstrated ADCC in mice *in vivo* by showing formation of ADCC synapses in immunocompetent mice bearing a murine breast tumor treated with an antibody specific to Tn, a glycopeptidic antigen which is expressed by breast cancer and a variety of other epithelial tumors in mice and humans (Hubert et al., 2011). Additionally, tumor rejection was abolished in mice deficient for FcγRs – emphasizing the potential role of ADCC (Hubert et al., 2011).

Another study investigated the relative contributions of complement-dependent cytotoxicity (CDC) and ADCC in a murine GD2-expressing metastatic lymphoma model treated with an antibody specific to the disialoganglioside GD2 utilizing wild-type, complement-deficient, complement-receptor-deficient, and FcγRI/III-deficient mice. Outcome after treatment with the ADCC and CDC mediating GD2 antibody was unaffected in mice incapable for CDC but was almost completely abrogated in FcγRI/III-deficient mice that were disqualified for ADCC (Imai et al., 2005). These results further highlight the key role of ADCC in anti-tumor effects in mice *in vivo*. In men it has been shown, that breast cancer patients who responded with partial or complete remission to Her2/neu-specific antibody trastuzumab have a higher capacity to mediate ADCC *in vitro* than patients which failed to respond to antibody therapy (Musolino et al., 2008).

These studies indicate that, at least in murine models, ADCC is a considerable component of the *in vivo* activity of therapeutic antibodies against tumors. However, studies with therapeutic antibodies in humans which demonstrate the direct influence of ADCC, as, e.g., ADCC as the single effector mechanism, remain to be conducted. Therefore, the direct *in vivo* effect of ADCC in tumor reactivity in humans remains to be shown.

INFLUENCE OF FcγRIIIa POLYMORPHISM AND KILLER-CELL IMMUNOGLOBULINE-LIKE RECEPTOR (KIR) RECEPTOR LIGAND INCOMPATIBILITY ON ADCC IN PATIENTS

In the past 15 years, 12 therapeutic antibodies have reached FDA approval for hematological malignancies as well as solid tumors (Scott et al., 2012). Their mechanisms of action include direct anti-tumor effects as induction of apoptosis, blocking receptor signaling or acting as an agonist, delivery of a cytotoxic agent, immune-mediated effects as CDC and ADCC as well as effects on the tumor microenvironment. At least five of these antibodies mediate efficient ADCC and a large number of new constructs are currently under investigation in early and late phase clinical trials (**Table 1**). In this review we will use anti-CD20-specific monoclonal antibody (mAb) rituximab, CD52-specific mAb alemtuzumab, Her2/neu-specific mAb trastuzumab, EGFR-specific mAb cetuximab, and anti-GD2 antibodies to discuss the influence of ADCC in treatment with therapeutic antibodies.

The CD16 encoding gene FCGR3A bears a single nucleotide polymorphism (SNP) at nucleotide 526 [thymidine (T) → guanine (G)] resulting in an amino acid (aa) exchange at aa position 158 of phenylalanine (F) to valine (V). It has been demonstrated that human IgG1 binds more efficiently to NK cells expressing the FcγRIIIa-158V allotype than to the FcγRIIIa-158F allotype (Koene et al., 1997; Wu et al., 1997). *In vitro* studies have shown that the increased binding is caused by a significantly higher affinity of IgG antibodies to FcγRIIIa-158V receptors, whereas expression levels of FcγRIIIa on NK cells are not influenced by the FcγRIIIa polymorphism (Dall'Ozzo et al., 2004; Congy-Jolivet et al., 2008). Furthermore, the adequate rituximab concentration exciting 50% lysis of a $CD20^+$ tumor cell line has been shown to be significantly lower in FcγRIIIa-158V/V donors compared to FcγRIIIa-158F/F donors (Dall'Ozzo et al., 2004).

Several clinical studies investigating antibodies which utilize an ADCC-mediating therapeutic antibody suggest that patients with the FcγRIIIa-158V allotype have a better clinical outcome. However, conflicting data have been published. First, we will discuss the chimeric IgG1 CD20-specific antibody rituximab which is approved for treatment of $CD20^+$ B-cell non-Hodgkin's lymphoma (NHL), $CD20^+$ follicular NHL, and chronic lymphocytic leukemia (CLL). Rituximab is the most extensively studied antibody mediating ADCC as its key effector mechanism (Cheson and Leonard, 2008; Alduaij and Illidge, 2011). The first study by Cartron et al. (2002) suggested that the homozygous FcγRIIIa-158V/V genotype is beneficial in patients with follicular NHL regarding clinical and molecular responses to single agent rituximab. These results were confirmed in a larger follicular lymphoma patient cohort treated with rituximab as monotherapy and an association of the FcγRIIIa-158V/V genotype with progression-free survival could be shown (Weng and Levy, 2003). Another study analyzing the effect of the FcγRIIIa genotype on the outcome of patients to single agent rituximab promoted some of the above mentioned reports by suggesting a significant improvement in event-free survival in FcγRIIIa-158V/V patients (Ghielmini et al., 2005). Furthermore, it has been postulated that NK cells from lymphoma patients with the FcγRIIIa-158V allotype but not from patients that are homozygous for FcγRIIIa-158F/F were activated after rituximab application (Veeramani et al., 2011).

On the contrary, no influence of the FcγRIIIa-F158V polymorphism on the outcome of follicular lymphoma patients treated with risk-adapted chemotherapy and rituximab has been shown (Prochazka et al., 2011). Similarly, in relapsed or refractory CLL

Table 1 | ADCC-mediating therapeutic antibodies currently FDA approved for cancer therapy.

Antibody	Antigen	Cancer indication	Mechanisms of action
Rituximab	CD20	$CD20^+$ B cell NHL, $CD20^+$ follicular NHL, CLL	ADCC, CDC, direct induction of apoptosis
Ofatumumab	CD20	CLL	ADCC, CDC
Trastuzumab	Her2/neu	Breast cancer	ADCC, abrogation of tumor cell signaling
Cetuximab	EGFR	colorectal cancer, SCCHN	ADCC, abrogation of tumor cell signaling
Alemtuzumab*	CD52	CLL	ADCC, CDC, direct induction of apoptosis

NHL, non-Hodgkin's lymphoma; CLL, chronic lymphocytic leukemia; ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; SCCHN, squamous cell carcinoma of the head and neck.

**Withdrawn from the market in August, 2012.*

patients treated with chemotherapy and rituximab, no significant influence of the FcγRIIIa-F158V polymorphism was demonstrated (Dornan et al., 2010). Another study evaluated the impact of the FcγRIIIa-F158V polymorphism on the response to rituximab in combination with a chemotherapy regimen (rituximab with cyclophosphamide, hydroxydaunorubicin, oncovin, and predniso(lo)ne, R-CHOP) in diffuse large B cell lymphoma (DLBCL) patients. The FcγRIIIa-158V allotype was shown to be significantly correlated with a higher complete response rate compared with the FcγRIIIa-158F allotype (Kim et al., 2006). However, overall survival did not correlate with the FcγRIIIa-158V/V genotype and several other studies investigating the impact of the FcγRIIIa-F158V polymorphism in DLBCL found no association with the response to R-CHOP (Carlotti et al., 2007; Mitrovic et al., 2007; Varoczy et al., 2012). Interestingly, all studies conducted with rituximab as monotherapy show an impact of the FcγRIIIa-F158V polymorphism and therefore indicate an important influence of ADCC on clinical outcome. However, the picture is less clear when rituximab is combined with chemotherapy. Since combined chemotherapy may hamper ADCC due to impaired NK cell function the combination of rituximab with chemotherapy might mask the influence of the FcγRIIIa genotype and ADCC. Nevertheless, a combination of rituximab with chemotherapy generally improves clinical results.

The humanized IgG1 mAb alemtuzumab was approved for treatment of B-CLL and is directed against the glycoprotein CD52 (Hillmen et al., 2007). The therapeutic antibody was withdrawn from the market in 2012 in order to optimize the pending launch of the agent as a treatment for multiple sclerosis. The predominant mechanism of action of alemtuzumab *in vivo* has not been clearly defined but involvement of ADCC mediated by NK cells and neutrophils has been suggested by studies employing a human CD52 transgenic mouse model and a xenograft model (Hu et al., 2009; Siders et al., 2010). In addition, CDC and direct induction of apoptosis have been identified as other potential mechanisms of action in *in vitro* studies (**Table 1**) (Crowe et al., 1992; Mone et al., 2006). A study exploiting the predictive value of FcγRIIIa polymorphism in a small CLL patient cohort ($n = 33$) treated with alemtuzumab found no association of responsiveness to the mAb with a FcγRIIIa-F158V allotype (Lin et al., 2005). However, further studies with larger patient cohorts are required in order to determine the potential influence of FcγRIIIa-F158V polymorphism and ADCC in treatment with alemtuzumab.

The humanized IgG1 mAb trastuzumab is applicable in HER2/neu-positive breast cancer patients and mediates abrogation of tumor cell signaling and ADCC (**Table 1**) (Hudis, 2007). A study investigating breast cancer patients treated with trastuzumab and a chemotherapeutic agent postulated a higher response rate and longer progression-free survival in homozygous FcγRIIIa-158V/V patients compared to patients with the FcγRIIIa-158F allotype (Musolino et al., 2008). In contrast, another study suggested a correlation of the FcγRIIa-131H/H genotype with objective response and progression-free survival in HER2/neu-positive non-metastatic and metastatic breast cancer patients treated with chemotherapy and trastuzumab but no significant correlation was observed for the FcγRIIIa-F158V/V genotype (Tamura et al., 2011). Furthermore, another recent study postulated a lack of correlation between FcγRIIIa genotypes and trastuzumab efficacy in HER2/neu-positive non-metastatic breast cancer patients treated with a combined trastuzumab-chemotherapy (Hurvitz et al., 2012). Since no studies with trastuzumab as single agent have been published and besides ADCC abrogation of tumor cell signaling being a major effector mechanism of trastuzumab the influence of FcγRIIIa-F158V polymorphism and ADCC on clinical outcome is hard to substantiate.

The chimeric IgG1 therapeutic antibody cetuximab targets EGFR in colorectal cancer (CRC) and has like trastuzumab ADCC and abrogation of tumor cell signaling as the modes of action (**Table 1**) (Van Cutsem et al., 2009). A study suggested a significantly higher clinical benefit after treatment with cetuximab and chemotherapy in homozygous FcγRIIIa-158V/V and or FcγRIIa-131H/H patients. These results were independent from the GTPase KRas (KRAS) mutation status in metastatic CRC (mCRC) (Bibeau et al., 2009). Furthermore, a higher disease control rate in KRAS-mutated mCRC patients harboring the FcγRIIa-131H/H genotype and treated with combined cetuximab-chemotherapy has been shown (Rodriguez et al., 2012). It has been suggested that the predictive value of FcγRIIa-H131R polymorphism may result from linkage disequilibrium between FcγRIIIa-V158F and FcγRIIa-H131R and therefore nevertheless indicates an effect of ADCC (Lejeune et al., 2008). Similarly, the association of the FcγRIIIa-F158V polymorphism with progression-free survival has been suggested for single agent cetuximab treated mCRC patients (Zhang et al., 2007). However, in this study and in another study from Dahan et al. investigating combined cetuximab-chemotherapy treatment, the FcγRIIIa-158F allele rather than the FcγRIIIa-158V allele had a favorable influence on overall and progression-free survival (Zhang et al., 2007; Dahan et al., 2011). Beyond that, a recent study found no association of clinical outcome in a patient cohort of 107 mCRC patients treated with cetuximab and chemotherapy and FcγRIIIa-F158V and FcγRIIa-H131R polymorphisms (Park et al., 2012). These discrepancies related to the predictive value of the FcγRIIIa-F158V polymorphism on cetuximab treatment are difficult to explain and indicate a demand for further elaborated investigations in larger patient cohorts. Interestingly, mutated KRAS induces a ligand-independent activation of EGFR downstream signaling pathways. Hence, the key effector mechanism of cetuximab besides ADCC, namely abrogation of tumor signaling, is invalidated by KRAS mutations. Clinical benefit of cetuximab yet seen in these patients and the predictive value of FcγRIIIa-V158F genotype emphasizes the impact of ADCC.

Disialoganglioside GD2 is a sialic acid containing glycosphingolipid which is uniformly expressed in neuroblastoma and most melanomas but also to a variable degree in some other tumors (Navid et al., 2010). Due to its tumor-associated expression, several anti-GD2 antibodies such as the chimeric IgG1 antibody ch14.18 and its humanized counterpart hu14.18 as well as 3F8 have been developed and deployed in the clinic. The mechanisms of actions of these antibodies are ADCC and CDC. Several years ago, a phase I study with pediatric neuroblastoma patients treated with anti-GD2 antibody ch14.18 was conducted at our institute and elicited some complete and partial tumor responses in neuroblastoma patients (Handgretinger et al., 1995). Currently, we are conducting an ongoing phase I/II-trial for relapsed metastatic neuroblastoma

with subsequent immunotherapy with ch14.18/Chinese hamster ovary (CHO) after HLA mismatched, haploidentical stem cell transplantation (SCT) at our institution. Preliminary results show effective ADCC and complement-mediated anti-tumor effects against neuroblastoma cells with donor-derived NK cells *in vitro* as well as significant anti-tumor effects *in vivo* (unpublished data). Due to yet small patient cohorts, the role of FcγRIIIa-F158V polymorphism in treatment with GD2 antibodies has not been studied up to now. However, there are a variety of further studies ongoing which investigate the *in vivo* efficacy of GD2 antibodies (Navid et al., 2010; Shusterman et al., 2010; Yu et al., 2010; Alderson and Sondel, 2011; Simon et al., 2011). Yu et al. demonstrated in a randomized trial a significantly better event-free survival for patients who received a combination of ch14.18, GM-CSF, and interleukin 2 than patients without ch14.18. Additionally, the third-generation antibody hu14.18K322A, which bears an aa substitution in the CH2 part, has been suggested to induce increased dose-dependent ADCC compared to ch14.18 and hu14.18, whereas dose-limiting CDC is intercepted by the Fc modification (Navid et al., 2010). In fact, results from studies conducted with this antibody *in vivo* will be interesting regarding anti-tumor effects caused by ADCC as single mode of action without involvement of any other effector mechanisms.

In adult acute myeloid leukemia (AML) and pediatric acute lymphoblastic leukemia (ALL) donor versus recipient NK cell alloreactivity is a key mechanism after HLA mismatched, haploidentical SCT, and has been reviewed elsewhere (Velardi et al., 2012). These "unlicensed" NK cells are characterized in the autologous setting by lacking self-KIRs and are thought to be beneficial in patients with neuroblastoma as well (Venstrom et al., 2009; Delgado et al., 2010). Interestingly, a recent study investigating the differential potential for ADCC of "licensed" and "unlicensed" NK cells in neuroblastoma, showed that "unlicensed" NK cells mediate ADCC most effectively against neuroblastoma cell lines under inflammatory conditions (Tarek et al., 2012). Based on these studies and our ongoing trial with HLA mismatched, haploidentical stem cell transplanted neuroblastoma patients treated with ch14.18/CHO we suggest that in the allogeneic, HLA mismatched SCT setting, KIR receptor ligand incompatibility may have synergistic effects with subsequent antibody therapy and propose further exploration of this hypothesis in larger clinical trials.

ENHANCED ADCC BY THIRD-GENERATION MONOCLONAL ANTIBODIES

Presuming that ADCC is a key mediator of anti-tumor effects *in vivo*, enhancing ADCC by engineering mAbs is expected to markedly improve clinical efficacy of therapeutic antibodies. Two main approaches of optimizing FcγRIIIa binding by enhancing the affinity of mAbs have been suggested and shown to induce 5- to 100-fold increased ADCC *in vitro* in recent years: molecular modifications in the Fc portion leading to aa substitutions (Shields et al., 2001; Lazar et al., 2006; Stavenhagen et al., 2007) and modifying Fc-linked glycosylation (Umana et al., 1999; Davies et al., 2001; Shinkawa et al., 2003).

The approach of optimizing the Fc portion of a therapeutic antibody via aa substitutions predominantly intends to enhance ADCC by increasing the affinity to activating FcγRIIIa and reducing the affinity to inhibitory FcγRIIb. A considerable number of these third-generation monoclonal antibodies are currently under early clinical investigation. These therapeutic antibodies target among others CD19, CD20, CD30, CD40, and FLT3 in hematological malignancies and all of them aim at achieving highly augmented ADCC *in vivo* (Awan et al., 2010; Foyil and Bartlett, 2010; Horton et al., 2010; Le Garff-Tavernier et al., 2011; Hofmann et al., 2012; Kellner et al., 2013). Furthermore, a CD19-specific Fc-optimized therapeutic antibody is currently evaluated in pediatric patients with refractory acute B-lineage leukemia after allogeneic SCT within the scope of a compassionate use program at our institution. In four out of six patients at very high risk of relapse, minimal residual disease could be significantly reduced or completely eradicated with a longest follow up of 15 months (unpublished data). Other attempts aim at reduction of CDC related toxicity as applied in GD2 targeting hu14.18K322A in neuroblastoma (Lazar et al., 2006; Sorkin et al., 2010) or enhancing affinity to low affinity allele FcγRIIIa-158F (Bowles et al., 2006; Nordstrom et al., 2011). Recently, a phase I trial investigating a Fc-engineered antibody targeting CD20 in follicular lymphoma patients has suggested encouraging results even in patients with the less favorable FcγRIIIa-F allotype (Forero-Torres et al., 2012).

Glyco-engineered therapeutic antibodies either bear oligosaccharides modified by bisecting GlcNAc, a β1,4-GlcnAc residue attached to a core β-mannose residue, or lack the core fucose of the Fc oligosaccharides and have been found to exhibit significantly higher ADCC than their unmodified counterparts (Lifely et al., 1995; Shinkawa et al., 2003). These modifications of oligosaccharides are mainly obtained by either transfecting CHO cell line with *N*-acetylglucosaminyltransferase III (GnTIII) or eliminating its intrinsic α-1,6-fucosyltransferase (FUT8) activity. Alternative expression systems such as Sf21 insect cells, resulting in fucosylated paucimannosidic *N*-linked glycosylation, have been suggested to elicit an enhanced ADCC and have been investigated with a chimeric CD19 antibody at our institution (Lang et al., 2004; Barbin et al., 2006). Several other glyco-engineered antibodies targeting a variety of different tumor associated antigens like CD19, CD20, EGFR, and GD2 are currently investigated in early clinical studies (Robak, 2009; Navid et al., 2010; Paz-Ares et al., 2011; Ward et al., 2011). All glyco-engineered antibodies in clinical trials have been reviewed in more detail elsewhere (Beck and Reichert, 2012). Recently, the C–C chemokine receptor 4 (CCR4) targeting glyco-engineered antibody mogamulizumab has been approved in Japan for use in patients with relapsed and refractory CCR4-positive adult T cell leukemia/lymphoma (ATL) (Beck and Reichert, 2012). Clinical superiority of these third-generation mAbs over clinically established therapeutic antibodies remains to be shown and may further portend the key role of ADCC for clinical use.

CONCLUSION

The studies discussed here clearly demonstrate a substantial role of ADCC *in vitro* and in mouse tumor models. However, the direct *in vivo* effect of ADCC in tumor reactivity in humans remains to be shown since no therapeutic antibody with ADCC as single mechanism of action has been investigated in clinical trials to date. Conflicting results have been obtained about the role of FcγRIIIa-V158F polymorphism in mAb treatment. Several studies have shown a predictive value, which underlines

the influence of ADCC *in vivo* and suggests that ADCC is one of the key mechanisms for clinical efficacy of therapeutic antibodies. On the other hand, some studies could not confirm these results. Further studies investigating the clinical relevance of FcγRIIIa-V158F polymorphism may be required for each therapeutic antibody and its indications. Although additional chemotherapy can mask the influence of the FcγRIIIa genotype and may hamper ADCC due to impaired NK cell function, a combination of both is able to improve clinical results. In particular, the use of therapeutic antibodies after hematopoietic SCT is an interesting treatment option, as the new donor-derived immune system usually faces minimal tumor burden. Further exploration of the role of the FcγRIIIa-V158F polymorphism and the influence of KIR-receptor-ligand incompatibility on ADCC in allogeneic hematopoietic SCT may contribute to future transplantation strategies in this setting. Furthermore, clinical superiority of ADCC improved third-generation mAbs over clinically established second-generation antibodies remains to be shown and may further portend the key role of ADCC in cancer therapy with monoclonal antibodies.

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Cellular and molecular basis of haploidentical hematopoietic stem cell transplantation in the successful treatment of high-risk leukemias: role of alloreactive NK cells

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Natural killer (NK) cells are involved in innate immune responses and play a major role in tumor surveillance and in defense against viruses. Human NK cells recognize human leukocyte antigen (HLA) class I molecules via surface receptors [killer immunoglobulin-like receptor (KIR) and NKG2A] delivering signals that inhibit NK cell function and kill HLA class I-deficient target cells, a frequent event in tumors or virus-infected cells. NK cell triggering is mediated by activating receptors that recognize ligands expressed primarily on tumors or virus-infected cells. NK cells play also a key role in the cure of high-risk leukemias. Thus, donor-derived "alloreactive" NK cells are fundamental effectors in adult acute myeloid leukemia and in pediatric acute lymphoblastic leukemia patients undergoing haploidentical hematopoietic stem cell transplantation (HSCT). Alloreactive NK cells mediate killing of leukemia cells and patient's dendritic cell, thus preventing respectively leukemic relapses and graft-vs-host responses. Cytofluorimetric analysis of KIRs expressed by NK cells allows to define the size of the alloreactive NK subset and the selection of the best potential donor. Recently, it has been shown that also the expression of activating KIRs, in particular the (C2-specific) KIR2DS1, may contribute to donor NK alloreactivity. It has also been established a correlation between the size of the alloreactive NK cell population and the clinical outcome. Notably, the alloreactive NK cells derived from donor's hematopoietic stem cells are generated and persist in patients over time. The high survival rates of patients undergoing haploidentical HSCT highlight an important new reality in the setting of allograft performed to cure otherwise fatal leukemias. Novel approaches are in progress to further improve the clinical outcome based on the infusion of donor alloreactive NK cells either as a component of the transplanted cell population or as *in vitro* expanded NK cells.

Keywords: natural killer cells, killer Ig-like receptors, NK alloreactivity, acute myeloid leukemia, acute lymphoblastic leukemia, haploidentical hemopoietic stem cell transplantation, graft-vs-host disease

HAPLOIDENTICAL HEMOPOIETIC STEM CELL TRANSPLANTATION

For over 40 years, allogeneic hematopoietic stem cell transplantation (allo-HSCT) from an human leukocyte antigen (HLA)-matched donor, either related or unrelated, has been increasingly used to treat patients affected by several malignant or non-malignant disorders. Thanks to this procedure, thousands of subjects have been cured of their original disease (Copelan, 2006). However, only 25% of patients who need an allograft have an HLA-identical sibling available and for <60% of the remaining patients a suitable, HLA-compatible, unrelated volunteer can be found (Rocha and Locatelli, 2008). In the absence of an HLA-matched donor, alternative donors/sources of hematopoietic stem cells (HSC), such as unrelated umbilical cord blood (UCB) and HLA-haploidentical relatives, are being increasingly used (Gluckman, 2006; Rocha and Locatelli, 2008; Locatelli et al., 2009). In particular, the majority of patients have a family member, identical for one HLA-haplotype and fully mismatched for the other (i.e., haploidentical), who can immediately serve as HSC donor (Martelli et al., 2002; Locatelli et al., 2009). Thus, HSCT from an HLA-haploidentical relative (haplo-HSCT) offers an immediate transplant treatment *virtually* to *any* patients lacking a matched donor or a suitable UCB unit.

A major breakthrough in the history of successful haplo-HSCT was the demonstration that an efficient T cell-depletion of the graft prevented both acute and chronic graft-vs-host disease (GvHD), even when the donor was a relative differing for an entire HLA-haplotype from the recipient (Reisner et al., 1983). The importance

of T cell-depleted haplo-HSCT was first shown in children with severe combined immunodeficiency (SCID; Reisner et al., 1983) and it can now be estimated that hundreds of SCID patients have been transplanted worldwide using an HLA-haploidentical related donor, with a high rate of long-term, either partial or complete, immune reconstitution (Antoine et al., 2003). However, while the infusion of bone marrow (BM) cells obtained from an HLA-haploidentical relative was associated with a high engraftment rate in children with SCID, it was associated with an unacceptably high incidence of graft failure in patients with acute leukemia (Reisner and Martelli, 1999). In these cases, due to the extensive T cell-depletion of the graft, the balance between competing host and donor T cells shifts in favor of the unopposed host-vs-graft rejection (Reisner and Martelli, 1999). As a possible solution to this obstacle, the use of "megadoses" of granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood-derived HSC was shown, in animal models, to overcome the barrier of HLA incompatibility and to elude the residual anti-donor T lymphocyte reactivity of the recipient (Bachar-Lustig et al., 1995). An effective translation of this approach into the clinical setting was first reported in a pilot study performed in adults with acute leukemia (Aversa et al., 1994). In this study, Aversa et al. (1994) transplanted "megadoses" of T cell-depleted HSC from BM or G-CSF-mobilized peripheral blood without any subsequent pharmacological GvHD prophylaxis. The reported engraftment rate was above 90% with a cumulative incidence of both grade II–IV acute and chronic GvHD below 10%. Clinical trials performed using purified $CD34^+$ cells have confirmed that sustained engraftment of donor hematopoiesis, without the occurrence of GvHD, can be obtained in the majority of adult patients and that a substantial proportion of them, especially when affected by acute myeloid leukemia (AML) or myelodysplastic syndromes, become long-term survivors (Aversa et al., 1998; Ruggeri et al., 2002).

In view of the role played by donor T cells in mediating the graft-vs-leukemia (GvL) effect, it could be expected that a relevant proportion of patients given this type of allograft would experience leukemia relapses. This expectation was only partly confirmed by clinical results, since among adult patients affected by AML, a subgroup of patients given T cell-depleted HSCT from an HLA-disparate relative had a particularly low risk of leukemia relapse (Aversa et al., 1998; Ruggeri et al., 2002). These patients were transplanted from a donor having natural killer (NK) cells that were "alloreactive" toward recipient targets. NK cell alloreactivity was originally described by Moretta et al. (1990a) over 20 years ago when killing of allogeneic lymphoblasts was observed *in vitro* and associated with defined NK cell subsets (Moretta et al., 1990a) identified by the expression or lack thereof of novel surface molecules (Moretta et al., 1990b), subsequently identified as HLA class I-specific receptors (Ciccone et al., 1992b, 1994; Moretta et al., 1993, 1996; Wagtmann et al., 1995). The emergence of the concept of the efficacy of NK cell alloreactivity in this transplantation setting has represented a sort of revolution in the field of haplo-HSCT, underlining for the first time that not only adaptive immunity, but also innate immunity is a crucial element for guaranteeing a successful clinical outcome (Moretta et al., 2008; Locatelli et al., 2009). Indeed, it became evident that the therapeutic effect of haplo-HSCT is largely dependent on the GvL effect exerted by NK cells which originate from donor HSC (Ruggeri et al., 2002; Moretta et al., 2008; Locatelli et al., 2009) and largely contribute to eradicate leukemia cells surviving the preparative regimen.

Thus, while for many years the absence of the T cell-mediated GvL effect was considered to render the recipients of a T cell-depleted allograft more susceptible to leukemia relapse (Horowitz et al., 1990), it is now evident that, in haplo-HSCT, an efficient GvL effect can be mediated by donor-derived alloreactive NK cells which compensate for the lack of T cell intervention.

NK CELL RECEPTORS AND FUNCTION

Natural killer cells are important players of the innate immunity. They are regulated by a number of receptors that finely tune potent effector functions, including cytolytic activity against different target cells and release of cytokines that play a major role in inflammation and immunoregulation (Trinchieri, 1989; Moretta et al., 1994; Janeway and Medzhitov, 2002; Moretta and Moretta, 2004).

A group of inhibitory receptors interact specifically with major histocompatibility (MHC) class I molecules (Ciccone et al., 1992b; Moretta et al., 1993, 1996; Long, 1999). These receptors prevent NK cell-mediated attack against normal (i.e., MHC class I^+) autologous cells. Cells in which MHC class I expression is compromised/downregulated (e.g., by tumor transformation or viral infection) become susceptible to NK-mediated killing. In humans, the inhibitory receptors for HLA class I molecules, namely: (1) killer immunoglobulin (Ig)-like receptors (KIR2DL/3DL) that belong to the Ig superfamily and are specific for determinants shared by groups of HLA-A, -B, or -C allotypes (referred to as KIR-ligands; reviewed in Moretta et al., 1996; Lanier, 1998; Long, 1999; **Table 1**), (2) CD94/NKG2A, a heterodimer related to C-type lectins that recognizes HLA-E, an HLA class Ib molecule (Lanier, 1998; Lopez-Botet et al., 2000), and (3) LILRB1 (ILT2, LIR-1, CD85j) that displays broad HLA class I specificity and interacts with UL18 human cytomegalovirus (HCMV) glycoprotein (Colonna et al., 1997; Cosman et al., 1997). Notably, activating forms of KIRs (KIR2DS/3DS; Moretta et al., 1995, 1996; Lanier, 1998), and CD94/NKG2C also exist. Activating KIRs may be relevant for recognition and killing of leukemia cells and dendritic cells (DCs; see below), while CD94/NKG2C appears to be involved in the control of HCMV infections (Gumà et al., 2004; Della Chiesa et al., 2012; Foley et al., 2012). In addition, NK cells are equipped with several triggering receptors responsible for NK cell activation in the process of natural cytotoxicity. An important role in tumor cell killing is exerted by NKp46 (Sivori et al., 1997; Pessino et al., 1998), NKp30 (Pende et al., 1999), and NKp44 (Vitale et al., 1998; Cantoni et al., 1999), a group of activating receptors that are mostly restricted to NK cells and that are collectively named "natural cytotoxicity receptors" (NCRs). In particular, NKp46 expressed both in human and in mouse NK cells represents the most reliable marker for NK cell identification (Sivori et al., 1997; Walzer et al., 2007). The cellular ligands recognized by these receptors are still elusive, with the exception of B7-H6, a ligand for NKp30 (Brandt et al., 2009). Another receptor that plays a major role in NK cell-mediated recognition and killing of some tumors is NKG2D, a type II membrane protein characterized by a lectin-like

Table 1 | KIRs and KIR-ligands.

KIR	Domain composition	KIR-ligand	Function	Reference
2DL1	D1 + D2	HLA-C^{Lys80} (C2)	Inhibitory	Ciccone et al. (1992a), Biassoni et al. (1995)
2DL2/2DL3	D1 + D2	HLA-C^{Asn80} (C1), HLA-B*46:01, HLA-B*73:01 Low affinity: HLA-C^{Lys80} (C2)	Inhibitory	Ciccone et al. (1992a), Biassoni et al. (1995), Moesta et al. (2008)
2DL4	D0 + D2	HLA-G	Inhibitory and activating*	Rajagopalan et al. (2001)
2DL5	D0 + D2	Unknown	Inhibitory	
3DL1	D0 + D1 + D2	HLA-B^{Bw4} and some HLA-A^{Bw4}	Inhibitory	Gumperz et al. (1997), Stern et al. (2008)
3DL2	D0 + D1 + D2	HLA-A*03 and HLA-A*11	Inhibitory	Döhring et al. (1996), Pende et al. (1996)
2DS1	D1 + D2	HLA-C^{Lys80} (C2)	Activating	Stewart et al. (2005), Chewning et al. (2007)
2DS2	D1 + D2	Unknown	Activating	
2DS3	D1 + D2	Unknown	Activating	
2DS4	D1 + D2	HLA-A*11 and some HLA-C alleles	Activating	Graef et al. (2009)
2DS5	D1 + D2	Unknown	Activating	
3DS1	D0 + D1 + D2	HLA-B^{Bw4} (?)	Activating	Martin et al. (2002)

**KIR2DL4 may function as an inhibitory receptor in cytotoxicity while it triggers IFN-γ production.*

domain (Wu et al., 1999). NKG2D recognizes the stress-inducible MHC class I-related chain A/B (MICA/B) or UL16-binding proteins (ULBP; Raulet, 2003). Other activating receptors include 2B4 (Moretta et al., 1992; Valiante and Trinchieri, 1993) specific for CD48, NK, T, and B cell antigen (NTB-A; Bottino et al., 2001) mediating homotypic interactions, NKp80 (Vitale et al., 2001) specific for AICL1 (Welte et al., 2006), DNAM-1 (Shibuya et al., 1996) specific for poliovirus receptor (PVR, CD155), and Nectin-2 (CD112; Bottino et al., 2003) also involved in cell-to-cell adhesion and in leukocyte extravasation (Reymond et al., 2004). Notably, PVR and Nectin-2 are frequently over-expressed on tumor cells and leukemia blasts (Bottino et al., 2003). Recognition of self-ligands that are induced by viral infection, tumor transformation, and in general cell stress may represent an important mechanism by which NK cells can identify and remove abnormal cells.

KIR REPERTOIRE AND SPECIFICITY FOR HLA CLASS I ALLELES

The ability of NK cells to sense allelic differences on hematopoietic target cells was first suggested by the hybrid resistance phenomenon in which NK cells can reject parental BM grafts in F1 hybrid mice (Bennet, 1987). Studies in both humans and mice clarified the general mechanisms underlying NK cell function and their capability of selectively killing tumor cells. In humans, two surface molecules expressed by subsets of NK cells that were capable of modulating NK cell function were identified (Moretta et al., 1990a,b, 1993; Wagtmann et al., 1995). They were shown to function as inhibitory receptors specific for distinct HLA-C alleles (Moretta et al., 1993). Molecular cloning revealed novel members of the Ig superfamily characterized by two extracellular Ig-like domains (KIR2D) and by a cytoplasmic tail containing two immunoreceptor tyrosine-based inhibition motif (ITIM; Moretta et al., 1990a,b, 1993; Wagtmann et al., 1995). Three Ig-like domain KIRs (KIR3D) were also identified (Colonna and Samaridis, 1995). They recognize either a group of HLA-B alleles sharing the HLA-Bw4 supertypic specificity or certain HLA-A alleles.

Among the activating forms of KIRs, the specificity for HLA class I molecules has been unequivocally documented only for KIR2DS1 and KIR2DS4 (**Table 1**; Moretta et al., 1995; Stewart et al., 2005; Chewning et al., 2007; Graef et al., 2009). KIRs are clonally distributed on NK cells and individual cells express different sets of inhibitory or activating KIRs. Notably, most (but not all) NK cells express at least one self-reacting inhibitory receptor, either a KIR or CD94/NKG2A (Moretta et al., 1996).

While in an autologous setting NK cells can kill only cells that do not express sufficient HLA class I molecules (Ciccone et al., 1994), in a non-self environment NK cells may kill allogeneic cells. It became evident that such "alloreactive" NK cells could kill allogeneic cells, both *in vitro* and *in vivo*, when they expressed inhibitory KIRs that did not recognize HLA class I alleles on target cells (Ciccone et al., 1992b, 1994; Moretta et al., 1993; Pende et al., 2005). In addition, these alloreactive NK cells should not express CD94/NKG2A^{+} (Pende et al., 2005) because HLA-E molecules are present in all HLA class I^{+} cells.

Notably, other factors may greatly contribute to NK alloreactivity. In particular, killing of target cells may also depend on the surface density of certain activating receptors (such as NCRs) on NK cells and on the expression of their ligands on target cells (Costello et al., 2002; Pende et al., 2005). More importantly, activating KIRs (in particular KIR2DS1) were shown to play a substantial role in mediating alloreactivity (Chewning et al., 2007; Pende et al., 2009). KIR2DS1 activating receptor recognizes the C2 specificity (Chewning et al., 2007). It is worthy to note that, in NK cells derived from C1/C2 or C1/C1 donors, activation via KIR2DS1 may overcome also the KIR2DL2/3-mediated inhibition, resulting in an efficient lysis of C2/C2 leukemic cells (Pende et al., 2009).

In addition, KIR2DS1 can overcome the CD94/NKG2A-mediated inhibition, again resulting in killing of C2/C2 leukemias. Thus, the expression of KIR2DS1 may reveal NK cells endowed with potent alloreactivity and allow a more precise definition of the size of the alloreactive NK cell subset (Pende et al., 2009).

IDENTIFICATION OF ALLOREACTIVE NK CELLS

Phenotypic identification of the alloreactive NK cell subset and assessment of the NK cytolytic activity against leukemic cells represent important criteria in donor selection. Multi-color flow-cytometric analysis using appropriate combinations of monoclonal antibodies (mAb) allows the identification and definition of the size of the alloreactive NK cell population (Chewning et al., 2007; Pende et al., 2009). Substantial progress has been made recently after the identification of mAbs discriminating between inhibitory and activating KIRs. Thanks to these mAbs, it is now possible to distinguish KIR3DL1 from KIR3DS1, KIR2DL1 from KIR2DS1, and KIR2DL3 (but not KIR2DL2) from KIR2DS2 (Pende et al., 2009). This is most important because the expression of activating KIRs, in particular KIR2DS1, recognizing alleles belonging to the C2 specificity may exert a positive effect and greatly contribute to NK alloreactivity, provided that patient's cells express C2 alleles. Notably, the beneficial effect is more evident in leukemia blasts of pediatric acute lymphoblastic leukemia (ALL) that express higher levels of HLA class I molecules than AML blasts. In addition, the presence of activating KIRs can also be assessed by analyzing the KIR genotype and using appropriate redirected killing assays (Chewning et al., 2007). Cytolytic activity of donor NK cells against patient's leukemic blasts or, alternatively, against appropriate EBV-induced B cell lines should be evaluated to select the HSCT donor with the best alloreactive capacity. In general, the degree of cytolytic activity correlates with the size of phenotypically defined alloreactive NK cell subsets (Chewning et al., 2007; Pende et al., 2009).

The fact that alloreactive NK cells are generated in the recipient after the allograft was documented in the early studies by Ruggeri et al. (2002). More recent studies by our group have confirmed and extended these findings. Donor's alloreactive NK cell populations have been identified on the basis of both phenotypic and functional (i.e., cytolytic activity) criteria in a large cohort of pediatric patients with high-risk leukemias even over 5 years after transplantation (Moretta et al., 2008, 2011). In these studies, a great variability in the size of the alloreactive NK cell population was detected in different donors and in post-transplantation patients. Importantly, most patients characterized by high proportions of alloreactive NK cells were disease-free after long time intervals (Pende et al., 2009). In addition, a correlation between the size of the alloreactive NK subset and the clinical outcome was found. After transplantation of positively selected $CD34^+$ cells, KIR^+ alloreactive NK cells were detectable at 6–7 weeks after transplantation and, in most instances, the pattern of expressed KIRs was similar to that originally found in the donor (Moretta et al., 2008, 2011; Pende et al., 2009).

A major and fascinating question is why alloreactive NK cells do not mediate GvHD. Early experimental evidence suggested that NK cells predominantly attack the hematopoietic cells of the host, while sparing tissues that are common targets of T cell-mediated GvHD. For example, in the hybrid resistance phenomenon in the mouse, NK cells rejected BM graft, but did not attack other tissues (Bennet, 1987). More recent studies in mice showed that allogeneic cells can mediate GvL effect in the absence of GvHD (Asai et al., 1998). Ruggeri et al. (2002) obtained direct evidence that murine alloreactive NK cells did not cause GvHD, whereas infusion of allogeneic T cells killed all the mice. In the same murine model, alloreactive NK cells were also shown to kill host antigen-presenting cells. This effect can contribute to reduce the risk of GvHD. The molecular basis of the resistance of recipient normal tissues other than the hematopoietic ones is the lack of ligands for activating NK receptors. These ligands become expressed or up-regulated by cells of different histotypes upon cell stress, viral infection, or tumor transformation (Moretta et al., 2006). Accordingly, NK cells cannot attack normal resting cells.

Notably, recent reports have proposed a novel approach for optimal donor selection based on the KIR genotype analysis. These studies provide evidence that the selection of donors with KIR B haplotypes was associated with significant improvements in both overall and relapse free survival, suggesting that activating KIRs, particularly those located in the centromeric portion, play a positive role in GvL in adult AML patients (Cooley et al., 2010; Symons et al., 2010).

It should be mentioned that some studies failed to establish an association between the presence of donor NK alloreactivity and a favorable clinical outcome of transplanted patients (Leung et al., 2004; Nguyen et al., 2005; Vago et al., 2008). This can be explained taking into account (1) the type of grafted cells (manipulated vs un-manipulated), (2) the type of conditioning regimen, (3) the source (PBSC vs BM) and, importantly, the number of stem cells used ("megadoses" in haplo-HSCT), (4) the type of GvHD prophylaxis, and (5) the clinical status of the patient at the time of the allograft (early vs advanced disease).

RECENT ADVANCES AND FUTURE PERSPECTIVES

There is no doubt that studies on NK cell receptor specificity and function allowed a rapid exploitation of these results in the treatment of high risk leukemias. Nonetheless, further relevant progresses are expected from the use of donor alloreactive NK cells as a tool for improving the clinical outcome of severe malignancies and for preventing GvHD.

The capability of alloreactive NK cells to kill host DCs, which are known to initiate T cell-mediated GvHD through presentation of host alloantigens to donor T cells, suggested a novel and interesting experimental approach in mice (Asai et al., 1998; Shlomchik et al., 1999). Infusion of mature, donor-vs-recipient alloreactive NK cells prevented GvHD to such an extent that mice that were given these cells could receive mismatched BM grafts containing up to 30 times the lethal dose of allogeneic T cells in the absence of clinical or histological evidence of GvHD (Asai et al., 1998). Transfer of such an approach to humans is particularly promising to prevent or treat GvHD, in view of the role of the lytic activity of donor-derived NK cells toward recipient T lymphocytes in the control/prevention of graft rejection.

As mentioned above, in the haplo-HSCT setting, after the infusion of pure $CD34^+$ cells, the first appearance of KIR^+ alloreactive

NK cells from HSC precursors may require 6–8 weeks and thus their anti-leukemia effect is relatively delayed. In case of high residual tumor burden and/or of rapidly proliferating leukemia blasts, this may result in leukemic relapses. To minimize this risk, mature alloreactive NK cells isolated from the haploidentical donor may be infused at short time intervals after HSCT. These mature donor NK cells could be properly activated *ex vivo* with interleukin-15 for further improving the clinical results of haplo-HSCT. Another promising and even less cumbersome approach is represented by the use of a recently developed method of graft manipulation based on the negative selection of T lymphocytes carrying the α/β chains of the T cell receptor (TCR) coupled with a B cell-depletion through an anti-CD19 mAb. T lymphocytes carrying the α/β chains of TCR are the lymphocyte subset responsible for the occurrence of GvHD, and thus their elimination allows to prevent the occurrence of this life-threatening complication of an allograft. This novel approach permits to transfer to the recipient not only high numbers of $CD34^+$ cells, but also of mature donor NK cells and $TCR\gamma/\delta^+$ T cells which can display their protective effect against leukemia re-growth and life-threatening infections (Chaleff et al., 2007; Handgretinger, 2012). Alloreactive NK cells are immediately available and may promptly exert their anti-leukemic and GvHD-preventing effect (**Figure 1**). A formal clinical trial using this approach is ongoing in our department and the preliminary results are extremely encouraging (Locatelli et al., unpublished). Likewise, preliminary experimental data indicate that, already 1 month after the allograft, pediatric patients receiving this novel type of HSCT from an HLA-haploidentical donor have peripheral mature NK cells that fully express KIRs and are endowed with a good lytic capacity against leukemia cells.

Regarding other possible settings in which alloreactive NK cells can be of relevant clinical interest, recent studies reported on the infusion of third-party purified NK cells in patients with either relapsed or first CR AML, who had not received allogeneic HSCT (Miller et al., 2005; Rubnitz et al., 2010). These patients were given immunosuppressive chemotherapy (combining fludarabine and cyclophosphamide) and interleukin-2,

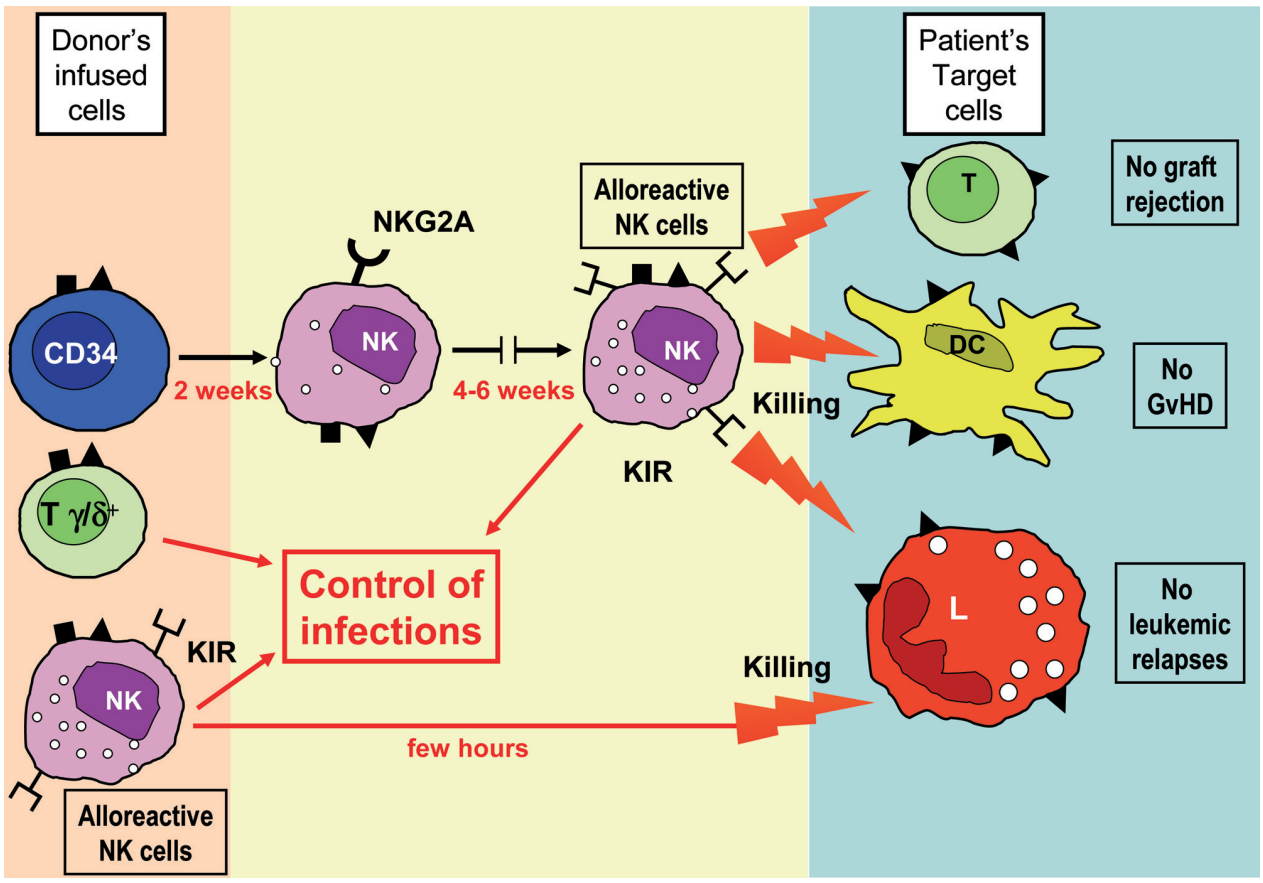

FIGURE 1 | A novel strategy for HSC transplantation from haploidentical donors. In this protocol, HSC-enriched cell populations are obtained by negative selection upon removal of TCR α/β^+ T cells and $CD19^+$ B cells. Notably, in addition to $CD34^+$ cells, these cell suspensions contain mature NK cells and TCR γ/δ^+ T cells. Using this strategy, two sources of alloreactive NK cells will come into play: (1) those generated from $CD34^+$ cells after 6–8 weeks from transplantation and (2) those present in the fresh cell suspension infused into patients. It is evident that the prompt availability of alloreactive effector cells may greatly improve the anti-leukemia effect and the removal of residual patient's DCs and T lymphocytes, thus ensuring a more efficient prevention of leukemic relapses, GvHD and graft-rejection. In addition, transplanted NK and γ/δ T cells may provide a first line of defense against different infectious agents.

respectively, before and after NK cell infusion in order to prevent rejection and favor NK cell function. NK cells transiently engrafted and expanded *in vivo*. The clinical results were particularly encouraging. This appears as a promising novel therapy for reducing the risk of relapse in patients with AML treated with conventional chemotherapy. Another promising approach to control leukemia progression resides in the NK cell manipulation using anti-KIR mAb (Romagné et al., 2009). This mAb, currently tested in phase II clinical trials on patients with AML or multiple myeloma, confers specific, stable blockade of KIR and induces NK-mediated killing of HLA-matched tumor cells *in vitro* and *in vivo*.

Altogether these data indicate that the discovery of NK receptors and NK cell alloreactivity has represented a true revolution in the field of allo-HSCT, underlining that not only adaptive immunity, but also innate immunity may be crucial for guaranteeing a successful clinical outcome.

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Improving engraftment and immune reconstitution in umbilical cord blood transplantation

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Umbilical cord blood (UCB) is an important source of hematopoietic stem cells (HSC) for allogeneic transplantation when HLA-matched sibling and unrelated donors (MUD) are unavailable. Although the overall survival results for UCB transplantation are comparable to the results with MUD, UCB transplants are associated with slow engraftment, delayed immune reconstitution, and increased opportunistic infections. While this may be a consequence of the lower cell dose in UCB grafts, it also reflects the relative immaturity of cord blood. Furthermore, limited cell numbers and the non-availability of donor lymphocyte infusions currently prevent the use of post-transplant cellular immunotherapy to boost donor-derived immunity to treat infections, mixed chimerism, and disease relapse. To further develop UCB transplantation, many strategies to enhance engraftment and immune reconstitution are currently under investigation. This review summarizes our current understanding of engraftment and immune recovery following UCB transplantation and why this differs from allogeneic transplants using other sources of HSC. It also provides a comprehensive overview of promising techniques being used to improve myeloid and lymphoid recovery, including expansion, homing, and delivery of UCB HSC; combined use of UCB with third-party donors; isolation and expansion of natural killer cells, pathogen-specific T cells, and regulatory T cells; methods to protect and/or improve thymopoiesis. As many of these strategies are now in clinical trials, it is anticipated that UCB transplantation will continue to advance, further expanding our understanding of UCB biology and HSC transplantation.

Keywords: umbilical cord blood, transplantation, hematopoietic stem cells, engraftment, immune reconstitution

INTRODUCTION

Over the last 25 years, umbilical cord blood (UCB) has become an established alternative source of hematopoietic stem cells (HSC) for use in allogeneic HSC transplantation (1, 2). Due to the lower immunogenicity of UCB grafts and the low rates of graft-versus-host disease (GvHD) compared to bone marrow (BM) and peripheral blood stem cell (PBSC) transplants, less stringent HLA-matching has traditionally been required (3–5). At present, only HLA typing at HLA-A and -B (serological) and HLA-DRB1 (allelic) are commonly used, with mismatches at one or two loci usually being tolerated if sufficient cell doses are transplanted (6). Consequently, UCB transplantation is a potential treatment option for many patients who lack a suitable HLA-matched sibling or unrelated donor. With over 600,000 frozen cord blood units (CBU) stored in cord blood banks worldwide, UCB also has the advantage of being immediately available, avoiding long delays to transplantation, and without any associated risks to the donors (2).

While UCB has increased the applicability of HSC transplantation, UCB transplantation can be associated with delayed engraftment, poor immune reconstitution, and higher rates of infection compared to conventional sources of HSC (4, 5, 7–9). This is due to the quantitative and qualitative differences in the composition of UCB grafts (10). While UCB contains a higher concentration of HSC than adult peripheral blood (PB), each unit contains a one to two log lower total cell dose compared to BM and PBSC harvests (PBSCH). Furthermore, the vast majority of T cells within UCB are antigen-inexperienced, i.e., naïve ($CD45RA^+$), being less responsive to allogeneic stimulation, having reduced expression of transcription factors associated with T-cell activation (e.g., nuclear factor of activated T cells, NFAT), and producing lower levels of effector cytokines compared to activated T cells from adult PB (11–13). UCB also contains more immunoregulatory cells, including regulatory T cells (Tregs), with greater potential for expansion and increased suppressive function compared to adult Tregs (13–15). The immaturity of UCB dendritic cells is also associated with lower antigen presenting activity, reduced expression of co-stimulatory molecules (CD80, CD86), reduced cytokine production (TNFα, IL-12), and an inherent ability to induce immune tolerance through peripheral expansion of Tregs (13, 16, 17).

Within this review, the pattern and factors affecting engraftment and immune reconstitution following UCB transplantation will be summarized. We will then provide an overview of current strategies being used to improve engraftment and immune reconstitution following UCB transplantation and potential areas for future research and development (**Table 1**).

Table 1 | Methods to improve engraftment and immune reconstitution in UCB transplantation.

1. INCREASING CELL DOSE
Improved collection and processing of cord blood
Infusion of two cord blood units (double cord blood transplantation)
Ex vivo expansion of cord blood HSC/HPC
Infusion of cord blood with third-party donor cells (haploidentical graft)
2. IMPROVING DELIVERY AND HOMING OF HSC
Direct intrabone infusion of cord blood
Increased stromal-derived factor-1 (SDF-1) (CXCL12)/CXCR4 interaction (e.g., inhibition of CD26 peptidase)
Ex vivo fucosylation of HSC/HPC
3. IMPROVING SELECTION OF CORD BLOOD UNITS
Enhanced HLA-matching
Detection of donor specific anti-HLA antibodies
4. MODIFYING UCB TRANSPLANT REGIMENS
Using reduced-intensity conditioning
Using T-replete protocols
5. EXPANDING SPECIFIC CELL POPULATIONS (*EX VIVO* OR *IN VIVO*)
Natural killer (NK) cells
T cells/pathogen-specific T cells (CMV, EBV, adenovirus)
Regulatory T cells (Tregs)
6. CO-INFUSING CORD BLOOD WITH ACCESSORY CELLS
Mesenchymal stem cells (MSC)
7. IMPROVING THYMOPOIESIS
Interleukin-7 (IL-7), interleukin-2 (IL-2), and interleukin-15 (IL-15)
Reducing sex steroid hormones (androgen, estrogen)
Growth hormone (GH), insulin-like growth factor 1 (IGF-1)
Keratinocyte growth factor (KGF)
Tyrosine kinase inhibition (sunitinib)
FMS-like tyrosine kinase receptor III ligand (FLT-3-L)
Stem cell factor (SCF)
Inhibition of p53 [pifithrin-β (PFT-β)]

HSC, hematopoietic stem cells; HPC, hematopoietic progenitor cells; UCB, umbilical cord blood; HLA, human leukocyte antigen.

PATTERN AND KINETICS OF ENGRAFTMENT AND IMMUNE RECONSTITUTION FOLLOWING UCB TRANSPLANTATION

NEUTROPHIL AND PLATELET ENGRAFTMENT

Following conditioning [chemotherapy and/or total body irradiation (TBI)] and infusion of the UCB graft, there is an initial period of aplasia during which time the donor UCB HSC and early hematopoietic progenitors engraft, differentiate, and proliferate within the BM environment. The total nucleated cell (TNC) dose and/or $CD34^+$ cell dose within the UCB unit(s) (per recipient body weight) are important contributing factors to the probability and rate of neutrophil and platelet engraftment (6, 18). UCB grafts contain a lower TNC dose compared to BM and PBSCH and, therefore, the time to neutrophil engraftment (defined as the first of three consecutive days with a neutrophil count $>0.5 \times 10^9$/l) is prolonged, with a median time of approximately 30 days for UCB, 21 days for BM harvests, and 14 days for PBSCH (19). Similarly, platelet recovery in UCB transplantation is also prolonged with a median time to engraftment (defined as the first of three consecutive days with an unsupported platelet count $>20 \times 10^9$/l) ranging from 50 to 100 days. Other important factors influencing engraftment are the degree of HLA-matching, the intensity of the conditioning regimen, and the type of immunosuppression used for GvHD prophylaxis (20).

CORD BLOOD NK CELLS: PROPERTIES AND RECOVERY AFTER UCB TRANSPLANTATION

In keeping with allogeneic HSCT using BM or PBSCH, lymphocyte reconstitution following UCB transplantation typically begins with rapid recovery of natural killer (NK) cells (21) (**Figure 1**). UCB contains both main NK-cell populations found in PB, i.e., $CD16^+CD56^{dim}$ and $CD16^-CD56^{bright}$, although differences in their phenotype, maturity, and function have been reported (22, 23). UCB also contains precursor NK-cell populations, including $CD16^+CD56^-$ cells (24). UCB NK cells have lower cytotoxicity, although their function can be enhanced following interleukin-2 (IL-2), interleukin-7 (IL-7), interleukin-12 (IL-12) or interleukin-15 (IL-15) stimulation (22). They also have lower expression of critical adhesion molecules, e.g., CD2, CD54, and L-selectin, and higher expression of inhibitory receptors including killer-cell immunoglobulin-like receptor (KIR) and NKG2A/CD94 (23). Post-UCB transplant, NK-cell recovery initially occurs through expansion of $CD16^-CD56^{bright}$ cells with total NK-cell numbers returning to normal values within the first 3 months (21, 25, 26). Beyond this, UCB transplantation is then associated with an increase in both the absolute number and proportion of $CD16^+CD56^{dim}$ cells compared to BM transplantation (27). Total NK-cell numbers may temporarily exceed the normal range seen in the healthy population, possibly through a compensatory expansion that occurs during periods of profound T-cell lymphopenia (28). This pattern has been reported in both children and adults, following single and double UCB transplants [NK cells are derived from the predominant unit in the case of double UCB transplantation] and can last up to 9 months post-transplantation (21, 25, 26).

CORD BLOOD T CELLS: PROPERTIES AND RECOVERY AFTER UCB TRANSPLANTATION

In allogeneic HSCT, T-cell reconstitution typically occurs in two phases (**Figure 1**). The first involves early allo-antigen driven homeostatic proliferation of memory T cells, contained either within the graft or, in the setting of T-cell depleted grafts, from residual host T cells escaping pre-transplant conditioning therapy ("thymic-independent"). This, however, produces a restricted T-cell population with limited T-cell receptor (TCR) repertoire against infection. Homeostatic proliferation also occurs faster in $CD8^+$ T cells compared to $CD4^+$ T cells, producing a reversal of the normal CD4:CD8 T-cell ratio (9, 19). In contrast to BM and PBSCH, UCB mainly contains antigen-inexperienced naïve T cells. Early T-cell reconstitution can therefore only occur via the more stringent *in vivo* priming, activation, and proliferation of the limited naïve T-cell repertoire contained within the graft. The immaturity of UCB T cells is also associated with reduced effector cytokine expression (IFNγ, TNFα) and reduced expression transcription factors involved in T-cell activation (NFAT, STAT4, and T-bet) (11). Consequently, longitudinal studies of immune reconstitution in UCB transplantation have consistently

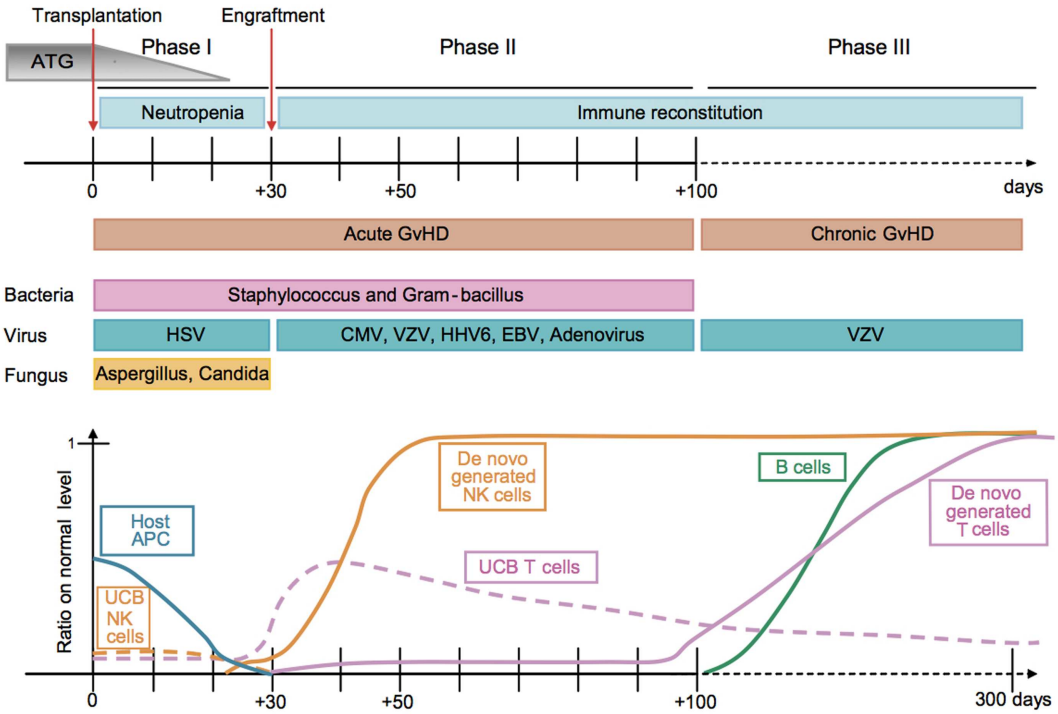

FIGURE 1 | Kinetics of immune reconstitution and transplant-related complications in children following UCB transplantation. The post-transplantation period can be divided in three phases. The pre-engraftment period (Phase I, days 0–30) is characterized by general immunosuppression (i.e., depletion of lymphocytes in the recipient by ATG), neutropenia, low platelet counts, and high susceptibility to fungal infections. Host residual APCs progressively disappear during Phase I. Massive proliferation leads NK cells to reach normal levels within 1 month post-transplantation. They may represent 80% of the recipient's PBLs during the post-engraftment period (Phase II, days 31–100). During Phase II, patients experience high susceptibility to bacterial infections, viral infections (i.e., CMV), and acute GvHD. Some UCB T cells are believed to persist in the recipient and proliferate through HE. This period culminates with the *de novo* generation of T cells through thymopoiesis and the end of the 100-day high-risk window. The late phase (Phase III) is characterized by a higher incidence of VZV infection/reactivation and a progressive reconstitution of B cell and T-cell subsets, which can reach normal levels at 6–9 months post-transplant [figure and legend originally published by Merindol and colleagues (226); used with the permission of H. Soudeyns and the Journal of Leukocyte Biology (Copyright FASEB Office of Publications, Bethesda, MD, USA)].

demonstrated profound early T-cell lymphopenia with impaired functional immunity and limited responses to viral infections, in keeping with a primary immune response (9, 28–30).

For long-term effective immune reconstitution with a broad T-cell repertoire, a second T-cell expansion phase is necessary involving thymic production of new naïve T cells ("thymic-dependent"). Hematopoietic progenitors, produced from the engrafted HSC within the BM, enter the thymus to form early T-cell progenitors (ETPs). During T-cell development in the thymus, double positive thymocytes ($CD4^+CD8^+$) are exposed to self-MHC on the thymic cortical epithelial cells. Only those thymocytes that bind to self-MHC with appropriate affinity will be "positively" selected to continue their development into single positive T cells; $CD4^+$ T cells interact with MHC Class II molecules, $CD8^+$ T cells interact with MHC Class I molecules. Double positive thymocytes that bind too strongly or too weakly to self-MHC undergo apoptosis. As the thymocytes pass through the thymic medulla they are then exposed to self-antigens presented in association with self-MHC molecules. Thymocytes that bind to self-antigens are removed by "negative" selection, thus preventing the production of autoreactive T cells (31). The presence of naïve T cells with markers of recent thymic emigration, i.e., T-cell receptor rearrangement excision DNA circles (TRECs), usually begins around 3–6 months post-UCB transplant (32, 33). However, the timing and effectiveness of thymopoiesis can be impaired by age-related thymic atrophy and/or thymic damage from conditioning therapy and GvHD. Escalon and Komanduri reported a longer delay in the recovery of thymopoiesis, as measured by TREC, in UCB transplantation compared to other HSC sources, possibly due to the limited dose of lymphoid progenitors within the UCB grafts (30). As a consequence, T-cell reconstitution was delayed with a median time to recovery of approximately 9 months for $CD8^+$ cytotoxic T cells and 12 months for $CD4^+$ helper T cells (25). Similarly, in a retrospective Eurocord analysis of 63 children transplanted with related and unrelated UCB grafts, the median time to T-cell reconstitution was 8 months for $CD8^+$ T cells and 12 months for $CD4^+$ and total T cells (21). Factors favoring T-cell recovery were HLA-matched UCB, higher nucleated cell dose, and positive recipient cytomegalovirus (CMV) serology prior to transplantation. Conversely, the presence of acute GVHD delayed T-cell recovery. Interestingly, in a recent Eurocord study of children with severe combined immunodeficiency (SCID) transplanted with either

UCB ($n = 74$) or haploidentical grafts ($n = 175$), there were no significant difference in T-cell recovery (total T cells and $CD4^+$ T cells) between the groups at any time, although the UCB transplant recipients had significantly faster recovery of total lymphocyte counts (34).

Over the last few years, the use of double cord blood transplants has significantly increased, mainly in adults following reduced-intensity conditioning (RIC) regimens. In this setting, although two CBUs are initially transplanted, only one provides prolonged engraftment, i.e., the "predominant" unit. Few studies have reported data on T-cell recovery after double CBT. Ruggeri and colleagues reported outcomes, infection rates, and immune reconstitution after 35 double UCB transplants in recipients with high-risk hematological diseases (25). Lymphocyte subset analyses were performed at 3, 6, 9, and 12 months post-transplant and demonstrated reduced T and B cell counts until 9 months. Recovery of thymopoiesis, as measured by TRECs, was also impaired until 9 months post-transplant. Somers and colleagues also analyzed engraftment kinetics in leukocyte subsets following non-myeloablative double UCB transplants (26). $CD4^+$ T cells, $CD8^+$ T cells, and NK cells all showed early engraftment predominance by day 11, followed by predominance in myeloid cells by day 18. Based upon these findings, it is proposed that T cells and/or NK cells from the predominant unit may elicit an early immune response against the second unit.

CORD BLOOD B CELLS: PROPERTIES AND RECOVERY AFTER UCB TRANSPLANTATION

B cell reconstitution occurs over the first 6 months post-UCB transplantation, although full recovery of immunoglobulins takes longer (**Figure 1**). In 63 children given related or unrelated UCB transplants, the median time to B cell recovery was 6 months (21). PB $CD19^+CD28^{high}CD38^{high}$ transitional B cells are first detectable at 1–2 months post-transplant. Over the next 3–6 months, total B cell numbers gradually rise with an associated increase in the proportion of mature $CD19^+CD28^{int}CD38^{int}$ mature B cells to approximately 90% by 9 months (35). As reported with NK cells, total B cell numbers may also expand above the normal range during the period of T-cell lymphopenia before returning to normal values when T-cell recovery occurs (28). At the same time, levels of immunoglobulins to commonly encountered antigens typically rise to normal levels by the end of the first year. Recovery of B cell function, as measured by the time to discontinuation of intravenous immunoglobulin replacement therapy, was compared following UCB ($n = 74$) and haploidentical transplants ($n = 175$) in children with SCID (34). At 3 years after transplantation, 45% of the UCB transplant recipients had discontinued immunoglobulins, compared with 31% of the haploidentical recipients ($P = 0.02$). Other factors associated with improved B cell function were the absence of pre-transplantation infections and use of myeloablative conditioning (MAC).

IMPLICATIONS OF DELAYED IMMUNE RECONSTITUTION FOLLOWING UCB TRANSPLANTATION

As a direct consequence of delayed immune reconstitution, UCB transplantation is associated with a significant risk of opportunistic infections, particularly viral infections including CMV, varicella zoster virus (VZV), Epstein–Barr virus (EBV), adenovirus (ADV), human herpesvirus-6 (HHV-6), and BK virus (**Figure 1**). In a recent retrospective review of 332 UCB transplants performed at the University of Minnesota between 1994 and 2007, 51% of CMV-seropositive patients ($n = 92/180$) experienced CMV reactivation, with a median time to reactivation of 40 days (range, 9–95 days) (36). CMV infection was infrequent in CMV-seronegative recipients (1.3%, $n = 2/152$). Neither pre-transplant CMV-seropositive status nor CMV reactivation was associated with increased transplant-related mortality (TRM). However, in the 14% ($n = 25/180$) of CMV seropositive patients that developed CMV disease (16 respiratory; 6 gastrointestinal; 3 multi-organ), TRM was significantly higher and overall survival reduced. It is hypothesized that the high incidence of CMV reactivation/infection post-UCB transplantation is related to impaired CMV-specific T-cell responses. In a study of T-cell reconstitution in 28 pediatric UCB transplants using MAC, $CD4^+$ and $CD8^+$ T cells were persistently low for the first 3 months post-transplant (37). By 3 months, only two patients had developed CMV-specific T cells and only one patient had VZV-specific T cells, as measured by antigen-specific IFNγ ELISPOTS. CMV and VZV responses developed over the next 3 years in 30% and 40% of the recipients, respectively. However, development of CMV or VZV disease was not necessary to acquire these responses and no subject developed infections once satisfactory responses had been attained (>150 spot forming units/10^6 PBMC). More recently, McGoldrick and colleagues were able to demonstrate CMV-specific polyclonal $CD4^+$ and $CD8^+$ T-cell responses from the donor UCB graft by day 42 but at insufficient numbers to be able to control viral reactivation (38). The lack of sufficient CMV-specific $CD8^+$ T cells to control CMV reactivation appears to be due to insufficient *in vivo* expansion, either due to immunosuppressive therapy and/or early deficiency of $CD4^+$ T cells. Later control of CMV reactivation was due to improved function of the T cells primed early after transplant rather than *de novo* responses from the thymic-dependent pathway.

In a retrospective study of EBV reactivation, 4.5% ($n = 15/335$) patients receiving an UCB transplant between 1994 and 2005 developed EBV related complications (4 viremia; 11 PTLD) (39). More recently, in a retrospective Eurocord study of 175 UCB transplants in which EBV viral load was monitored by RT-PCR during the first 3 months post-transplant, 24 patients had EBV reactivation with a median time of 86 days (range, 14 days to 2.7 years) (40). The cumulative incidence of EBV reactivation by 100 days was 8% ($n = 15$) and four patients developed EBV-PTLD (cumulative incidence 2%) at a median time of 73 days (range, 63–80 days) (median of 28 days after the first positive EBV RT-PCR result). Other viral infections also have a higher prevalence in UCB transplants compared to BM and PBSCH. VZV reactivation is more frequent in UCB transplantation than BM transplants [RR 2.27 (95% confidence interval (CI), 1.18–4.34), $P = 0.013$], as is VZV dissemination (41). HHV-6 is a double stranded DNA virus that can reactivate following HSCT and may cause encephalitis and/or pneumonitis. In a recent meta-analysis, the prevalence of HHV-6 reactivation and HHV-6 encephalitis was higher in patients receiving UCB than other stem cell sources (72 vs. 37%, $P < 0.0001$; 8 vs. 0.5%, $P < 0.0001$, respectively) (42). BK virus is another double

stranded DNA virus, belonging to the polyomavirus family, and has been associated with hemorrhagic cystitis post-HSCT. A retrospective analysis of 209 HSCT demonstrated that both BK PCR positivity pre-transplant and receiving an UCB or haploidentical graft with MAC was associated with a significantly higher risk of developing hemorrhagic cystitis (43).

Opportunistic infections are associated with significant mortality following UCB transplantation and are a major contributing factor in >40% of deaths following UCB transplants, particularly in the first 3 months (5, 7). In a review of 330 single unit UCB transplants at Duke University, 110 patients died within the first 6 months, of which 58% were due to opportunistic infection (viral, fungal, or protozoal) (9). Twenty-two patients died from ADV and 12 from CMV infection. In a Grupo Español de Trasplante Hematopoyético (GETH) study of 192 consecutive adult unrelated allogeneic HSCT, the 100-day and 3-year infection-related mortality (IRM) for UCB transplants ($n = 48$) was 30% (95% CI, 10–40%) and 40% (95% CI, 12–58%), respectively (44). However, although IRM post-UCB transplantation remains a concern, it is unclear whether UCB transplantation has a higher proportion of deaths due to infection compared to other forms of HSCT. In an IBMTR comparison of unrelated donor transplants for leukemia, Laughlin and colleagues reported the proportion of deaths due to infection within 100 days as 45, 21, and 24% ($P = 0.01$) for UCB ($n = 150$), HLA-matched marrow ($n = 367$), and HLA-mismatched marrow ($n = 83$), respectively (8). However, Rocha and colleagues observed that a similar proportion of transplant deaths were due to infection when comparing cord blood (42%, 18/62) to unrelated BM (41%, 41/320) (5). In the Spanish GETH study, although UCB transplants had a higher risk of severe infection compared to BM/PBSC transplants (85 vs. 69%, $P < 0.01$), the 100-day IRM (30 vs. 28 vs. 22%; $P = 0.2$) and 3-year IRM (40 vs. 42 vs. 38%, $P = 0.5$) were not significantly different (44). Likewise, in a study of 136 pediatric unrelated donor transplants, the proportion of patients in which infection was causal or contributing toward death was not significantly different (BM 36%, T-cell depleted BM 33%, UCB 30%) (45).

IMPROVING ENGRAFTMENT AND IMMUNE RECONSTITUTION FOLLOWING UCB TRANSPLANTATION

USE OF DOUBLE CORD BLOOD TRANSPLANTATION

In UCB transplantation, the cell dose (TNC dose and/or $CD34^+$ dose) and HLA-matching of the graft are important factors for successful engraftment in both pediatric and adult patients (3, 5, 7). Therefore, in 2009, Eurocord published recommendations for selection of CBUs for transplantation (6). In summary, when a single CBU (6/6 or 5/6 HLA-matched) does not contain sufficient number of cells (TNC $>2.5 \times 10^7$/kg upon freezing; $>2.0 \times 10^7$/kg on thawing), double cord blood transplantation should be considered, aiming for a combined TNC dose $>3.0 \times 10^7$/kg. Even higher doses are recommended if the single CBU is only 4/6 HLA-matched (TNC $>3.5 \times 10^7$/kg upon freezing; $>2.5 \times 10^7$/kg on thawing for malignant disorders; TNC $>4.0 \times 10^7$/kg upon freezing; $>3.5 \times 10^7$/kg on thawing for non-malignant disorders). UCB transplants using two units from different donors were first reported in 2001 by the Minneapolis group in an attempt to increase cell dose infused in adults and older children (46). Both units contribute to early engraftment, although eventually, one unit predominates (47). In an analysis of 23 double UCB transplants following MAC, hematopoiesis was observed from a single donor in 76% patients at day 21 and 100% patients by day 100 (48). Likewise, on review of 81 patients with sustained chimerism after receiving a double UCB transplant using a non-myeloablative regimen, single donor chimerism was detectable in 57, 81, and 100% patients at day 21, 100, and 365, respectively (47). Double UCB transplants show high rates of engraftment (85–100%) with the median time to neutrophil engraftment ranging from 9 to 33 days depending on the conditioning regimen and/or the use of granulocyte colony stimulating factor (GCSF) (26, 47, 49). Interestingly though, a significant difference in the rate of engraftment has not been demonstrated between patients receiving one or two CBUs (47, 50). However, a lower relapse risk was found in patients receiving two CBUs for acute leukemia (CR1/CR2), possibly through an enhanced graft-versus-leukemia (GvL) effect (50). Recently, Ruggeri and colleagues reported the outcomes of 35 double cord blood transplants in recipients with high-risk hematological diseases (25). The cumulative incidence of neutrophil recovery was 86%, acute GvHD 47%, and first viral infection 92%. Immune recovery was delayed with reduced T and B cell counts and compensatory expansion of NK-cells observed until 9 months post-transplant, followed by the appearance of new thymic precursors.

CORD BLOOD EXPANSION

An alternative approach being used to increase cell dose in UCB transplantation is *ex vivo* expansion of cord blood. As well as increasing the total number of HSC cells for long-term engraftment, this may also increase the number of committed progenitors to reduce the initial period of neutropenia. *Ex vivo* expanded CB can then be given alone or in combination with an unmanipulated unit. In this setting, although the expanded unit improves early hematopoietic recovery, it is the unmanipulated unit that usually provides long-term engraftment (51). UCB expansion has been achieved using several different methods. The first is liquid culture in which isolated $CD34^+$ or $CD133^+$ HSC are expanded in the presence of selected cytokines and growth factors, including stem cell factor (SCF), thrombopoietin (TPO), GCSF, and/or FMS-like tyrosine kinase 3 ligand (FLT-3-L) (52, 53). The optimal milieu of cytokines and growth factors remains uncertain but several groups have shown improved expansion by the addition of IL-3 and/or IL-6 (54). Shpall and colleagues performed a feasibility study in which $CD34^+$ cells were isolated from a fraction (40–60%) of the CBU and expanded in liquid culture with SCF, GCSF, TPO, and megakaryocyte growth and differentiation factor (52). The remainder of the unit was then infused with the expanded cells following MAC. The median TNC dose infused was 0.99×10^7/kg and the median time to engraftment was 28 days (range, 15–49 days) for neutrophils and 106 days (range, 38–345 days) for platelets. Using a modification to this approach, a Phase I/II trial was performed in which $CD133^+$ cells were isolated from a portion of the CBU and expanded in liquid cultures with SCF, FLT-3-L, IL-6, TPO, and the copper chelator TEPA (55). The median TNC fold expansion was 219 (range, 2–260). Both expanded and unexpanded cells were infused with a median TNC

of 1.8×10^7/kg. Nine of the 10 patients engrafted with a median time to neutrophil and platelet engraftment of 30 days (range, 16–46 days) and 48 days (range, 35–105 days), respectively. Delaney and colleagues reported results from a Phase I trial using an immobilized Notch ligand Delta-1 in addition to SCF, FLT-3-L, TPO, IL-3, and IL-6 (56). Ten patients with high-risk leukemia were treated with a myeloablative double UCB transplant in which one unit was expanded using this protocol. The average fold expansion was 562 (range, 146–1496) for TNC and 164 (range, 41–471) for $CD34^+$ cells. Nine of the 10 patients engrafted with a median time to neutrophil engraftment of 16 days (range, 7–34 days). However, in contrast to other reported studies, there was a predominance for donor $CD33^+$ and $CD14^+$ cell engraftment from the expanded unit.

The second expansion method uses co-culture with a supporting network of mesenchymal stromal cells to provide a hematopoietic microenvironment that supports HSC proliferation (57). de Lima and colleagues reported the results of 31 patients receiving 2 CBUs, 1 of which was expanded *ex vivo* with mesenchymal stem cells (MSC). This *ex vivo* culture system expanded TNC and $CD34^+$ cells by a median factor of 12.2 and 30.1, respectively, and the median TNC dose infused was 8.34×10^7/kg (51). Of the 24 patients who received *ex vivo* expanded cells, 23 achieved neutrophil engraftment, at a median time of 15 days (range, 9–42 days), and 18 had sustained platelet engraftment, at a median time of 42 days (range, 15–62 days). Both compared favorably to 80 CIBMTR historical controls that received unmanipulated double UCB transplants only [neutrophil engraftment 24 days (range, 12–52 days), $P < 0.001$; platelet engraftment 49 days (range, 18–264 days), $P = 0.03$]. In addition, while the expanded CBU improved early hematopoietic recovery, in all cases, the unmanipulated unit provided long-term engraftment.

HSC expansion has also been achieved using a continuous perfusion culture system in which cells are supplied with fresh culture media and gaseous exchange (58, 59). In a Phase I study, Jaroscak and colleagues expanded a portion of a CBU using a continuous perfusion culture device and infused these expanded cells 12 days after the remainder of the original unit (58). The median fold increase in TNC was 2.4 (range, 1.0–8.5). Twenty-one of the 26 patients attained neutrophil engraftment with a median time of 22 days (range, 13–40 days). The median time for platelet engraftment was 71 days (range, 39–139 days; $n = 16$). Ongoing early phase clinical trials of all three methods are in progress.

COMBINED USE OF UCB WITH THIRD-PARTY DONOR

Another strategy being used to improve engraftment following UCB transplantation is the combined use of cord blood and haploidentical transplants (60, 61). While the haploidentical graft provides early engraftment, it is the UCB graft that usually provides long-term engraftment. Sebrango and colleagues reported the results of 55 combined UCB/haploidentical transplants for high-risk myeloproliferative and lymphoproliferative disorders (60). The maximum cumulative incidence of neutrophil and platelet engraftment was 96 and 78% with a median time to recovery of 10 and 32 days, respectively. Full UCB chimerism was achieved in 50 patients [cumulative incidence 91% (95% CI, 84–99%)] with a median time of 57 days (range, 11–186 days). Immune reconstitution analysis showed NK-cell recovery occurred within 3 months, at a time when patients had dual UCB/haploidentical chimerism. However, B and T cells recovered by 6 months and 1 year, respectively when full UCB chimerism had been attained. Liu and colleagues transplanted 45 patients using a RIC regimen with an unrelated UCB graft and $CD34^+$ selected cells from a haploidentical donor (61). The cumulative incidence of neutrophil engraftment was 95% at day 50 with a median time to recovery of 11 days. The cumulative incidence of platelet engraftment was 83% at day 100 with a median time to recovery of 19 days. The median percentage of PB cells of UCB origin was 10, 78, and 95% at day 30, 100, and 180, respectively. Conversely, the median percentage of PB cells from the haploidentical graft was 86, 22, and 2% at the corresponding times. The cumulative incidence of acute and chronic GvHD was 25 and 5%, respectively, with non-relapse mortality (NRM) at 1 year 38%, relapse 30%, and overall survival 55%.

IMPROVING DELIVERY AND HOMING OF UCB HSC

To improve HSC engraftment following UCB transplantation, direct intrabone infusion of cord blood cells is currently being investigated. In a Phase I/II study, 32 consecutive patients with acute leukemia received UCB transplants with intrabone infusion between 2006 and 2008 (62). No complications occurred during administration. The median time to neutrophil and platelet engraftment was 23 days (range, 14–44 days; $n = 28$) and 36 days (range, 16–64 days; $n = 27$), respectively, and all engrafted patients showed full donor chimerism from day 60 onward. Sixteen patients were alive and in remission with a median follow-up of 13 months. Okada and colleagues demonstrated in a Phase I study that intrabone infusion of unwashed cord blood following a RIC regimen was also well tolerated (63). In 10 patients, there were no injection related complications and the median time to neutrophil recovery was 17 days. Saglio and colleagues showed that intrabone injection was also well tolerated in children (64). In a recent Eurocord retrospective comparison of single unit intrabone UCB transplants ($n = 87$) with double unit intravenous UCB transplants ($n = 149$), intrabone infusion was associated with improved neutrophil engraftment by day 30 (76 vs. 62%, $P = 0.014$) and improved platelet engraftment by day 180 (74 vs. 64%, $P = 0.003$). Intrabone infusion was also associated with a lower incidence of acute GvHD and had a trend toward improved disease-free survival (DFS) (65). These results are encouraging and further clinical trials are currently ongoing to evaluate engraftment kinetics and immune reconstitution following intrabone infusion (66).

Factors that promote homing of UCB HSC to the BM niche may also improve engraftment. Stromal-derived factor-1 (SDF-1) (CXCL12) is produced by BM stromal cells and binds to its receptor, CXCR4, on the surface of HSC, pre-B lymphocytes, and T cells. SDF-1 levels are increased following HSCT conditioning and the infused HSC follow the SDF-1 gradient toward the BM niche. Once engrafted, SDF-1 may also promote HSC proliferation and survival (67, 68). Many factors increase the sensitivity of CXCR4 on HSC to SDF-1, including complement (C3a), hyaluronic acid, VCAM-1, fibrinogen, and thrombin. Therefore, *ex vivo* priming of UCB HSC with these molecules may promote homing and engraftment of the HSC (69, 70). Inhibition

of the membrane bound extracellular peptidase (CD26), which cleaves SDF-1, also enhances long-term engraftment in UCB $CD34^+$ cells in NOD/SCID/beta 2 microglobulin null mice (71, 72). Fucosylation of ligands expressed on HSC is also required for their interaction with selectins expressed in the BM microvasculature. In NOD-SCID interleukin-2Rγ (null) mice, Robinson and colleagues demonstrated that only fucosylated UCB $CD34^+$ were responsible for engraftment and that *ex vivo* fucosylation improved UCB engraftment rates (73). All these pre-clinical studies show encouraging results and, as such, further investigation is warranted to determine whether these techniques can improve HSC engraftment in clinical UCB transplantation.

ENHANCED HLA-MATCHING AND DETECTION OF HLA ANTIBODIES

Until recently, CBUs for transplantation were selected using cell dose (TNC and/or $CD34^+$) and HLA-matching at HLA-A and -B (antigen) and HLA-DRB1 (allele). However, the importance of enhanced HLA-matching has now been recognized. In 2011, Eapen and colleagues retrospectively reviewed the results from 803 single UCB transplants (leukemia/MDS), analyzing the impact of HLA typing at HLA-A, -B, and -C (intermediate resolution) and HLA-DRB1 (allelic) (74). Neutrophil recovery (day 28) was inferior in UCB transplants mismatched at three or more HLA-loci (70, 64, 64, 54, and 44% with zero, one, two, three, and four mismatches, respectively). In addition, TRM was higher when CBU were mismatched at two or more HLA-loci [HR 3.27 ($P = 0.006$); HR 3.34 ($P = 0.005$); HR 3.51 ($P = 0.006$) for two, three, and four mismatches]. Compared to fully matched transplants (8/8), CBU mismatched at HLA-C had higher TRM [HR 3.97 ($P = 0.018$)]. TRM was also higher in UCB transplants with a single HLA-mismatch at HLA-A, -B, or -DRBI and mismatched at HLA-C compared to transplants with a single HLA-mismatch at HLA-A, -B, or -DRBI but matched for HLA-C [HR 1.70 ($P = 0.03$)]. Additional matching for HLA-C was therefore recommended. More recently, a joint CIBMTR and Eurocord study analyzed the effect of high resolution (allele) typing at HLA-A, -B, -C, and -DRB1 on the outcomes of 1658 MAC single UCB transplants (75). Neutrophil recovery (day 28) was inferior in transplants mismatched at three or more alleles compared to fully matched CB [odds ratio (OR) 0.56 (95% CI, 0.36–0.88) $P = 0.01$; OR 0.55 (95% CI, 0.34–0.88) $P = 0.01$; OR 0.45 (95% CI, 0.25–0.82), $P = 0.009$ for three, four, and five allelic mismatches]. NRM was also associated with the degree of HLA-mismatching. Single HLA-allele mismatches at HLA-A, -C, or -DRB1 had higher NRM [HR 3.05 (1.52–6.14), $P = 0.02$; HR 3.04 (95% CI, 1.28–7.20), $P = 0.01$; HR 2.93 (95% CI, 1.38–6.25), $P = 0.005$, respectively]. Importantly, CBUs with TNC $<3.0 \times 10^7$/kg were associated with significantly higher NRM, independent of HLA-matching. Therefore, Eapen and colleagues proposed that single UCB transplants should have a minimum TNC dose of 3.0×10^7/kg. The best HLA-allele matched CBU should then be selected. However, units with three or more HLA-allele mismatches should only be used with caution due to increased graft failure and higher NRM.

In UCB transplantation, screening for donor specific anti-HLA antibodies (DSA) should also be considered. In a retrospective analysis of 386 MAC single UCB transplants performed for hematological malignancies, 89 patients had anti-HLA antibodies, 20 with specificity against the CBU (76). In multivariate analysis, neutrophil and platelet recovery were significantly worse in these 20 patients compared to the antibody negative group [RR 0.23 (0.09–0.56), $P = 0.001$; RR 0.31 (95% CI, 0.12–0.81), $P = 0.02$, respectively]. Similarly, in 73 double UCB transplants, the presence of DSA was associated with increased graft failure (5.5 vs. 18.2 vs. 57.1% for none, single, or dual DSA positivity; $P = 0.0001$) and a longer median time to neutrophil recovery [29 days (any DSA) vs. 21 days (no DSA), $P = 0.04$] (77). More recently, a retrospective Eurocord analysis on the impact of DSA in 294 RIC UCB transplants was performed. 21% recipients had anti-HLA antibodies of which 14 (5%) had donor specificity. Day 60 neutrophil engraftment (44 vs. 81%, $P = 0.006$) and 1 year TRM (46 vs. 32%, $P = 0.06$) were inferior in the presence of DSA (78). In 70 children receiving single UCB transplants, the presence of antibodies to major-histocompatibility-complex Class I-related chain A antigen (MICA) was also associated with delayed platelet engraftment [HR 4.2 (95% CI, 1.02–17.08), $P = 0.04$] (79). While not all studies have found this association between DSA and engraftment, possibly due to use of lower thresholds for DSA detection, it is recommended that potential recipients be screened for anti-HLA antibodies before UCB transplantation (79, 80). CBUs for which the recipient has high levels of DSA should then be avoided. The full implication of DSA at lower levels remains less clear and further evaluation is required.

REDUCED-INTENSITY CONDITIONING AND T-REPLETE TRANSPLANTS

The use of RIC regimens in all forms of allogeneic HSCT has increased over the last decade. RIC regimens are less myeloablative but provide sufficient immunosuppression to allow donor engraftment. Disease eradication is then dependent upon the donor-derived T cells recognizing residual tumor as "non-self," producing an immune mediated graft-versus-tumor (GvT) response (81). RIC regimens have less toxicity and lower TRM, therefore allowing transplantation to be performed in older patients and/or in those with other significant co-morbidities that would otherwise prevent HSCT. RIC regimens are now commonly used in both pediatric and adult UCB transplants (47, 82). However, immune reconstitution following RIC UCB transplantation has not been extensively reported. Geyer and colleagues monitored immune subset recovery in 88 consecutive UCB transplants of which 49 had MAC and 39 had RIC (83). In this series, no significant difference was observed in T, B, or NK-cell recovery or immunoglobulin reconstitution, although the two groups were not evenly matched for other potential confounding factors, e.g., disease type and status at transplant.

To improve engraftment and limit GvHD, many UCB transplant regimens also use *in vivo* T-cell depletion with anti-thymocyte globulin (ATG). ATG is a polyclonal antibody, prepared in rabbits or horses, raised against thymocyte antigens (84). Although infused into the recipient pre-transplant, the half-life of ATG is such that it reduces T cells in both the recipient and infused grafts, producing profound T-cell depletion and delaying immune reconstitution. Chiesa and colleagues recently published a study measuring early immune reconstitution in 30 pediatric UCB transplants without *in vivo* T-cell depletion (85). In keeping with T-depleted transplants, NK-cell recovery was rapid with a median

time to recovery of 1 month (1–3 months). However, in contrast to ATG-based protocols, T-cell recovery was faster with a rapid "thymic-independent" expansion, broader T-cell repertoire with virus-specific responses, and rapid conversion from naïve to central memory phenotype. In particular, $CD4^+$ T-cell recovery was faster with a median count of 0.6×10^9/l at 2 months post-UCB transplant. Although $CD8^+$ T-cell recovery was delayed, it was still faster than previously reported for T-depleted UCB transplants. Similarly, B cell reconstitution was also faster. Twenty-nine of the 30 patients engrafted with the median time to neutrophil engraftment of 22 days (range, 13–38 days) and platelet engraftment of 42 days (range, 17–123 days). The cumulative incidence of grade II–IV acute GvHD was 50% but chronic GvHD was relatively low at 14%. In a similar review in adult patients, T-cell reconstitution was measured in 72 double UCB transplants (52 myeloablative and 20 non-myeloablative) without ATG (86). In this group, the median $CD4^+$ T-cell count was 0.27×10^9/l at 4 months. Again, $CD4^+$ T-cell recovery was faster than that seen in similar series of single and/or double UCB transplants using ATG. Four patients had graft failure and the median time to neutrophil engraftment was 23 days (range, 11–43 days) in the myeloablative group and 11 days (range, 7–36 days) in the non-myeloablative group. The cumulative incidence of acute GvHD at day 100 was 43% (95% CI, 33–56%) and survival at 1 year was 68% (95% CI, 57–79%). Therefore, both studies show promising results with potentially improved immune reconstitution compared to T-depleted UCB transplants although this has to be balanced against the risk of GvHD. In addition, as recognized by the authors, the limitations of comparing results from different retrospective series must be considered and further studies will be necessary.

INCREASING SPECIFIC CELL POPULATIONS

NK cells

Natural killer cells have an important role in early immunity against infection and the GvL response following HSCT. NK-cells expressing the activation receptor, NKG2C, and producing IFNγ rapidly expand following acute CMV reactivation, reflecting a primary NK-cell response (87). These cells persist long after viral clearance and develop a mature phenotype being $CD56^{dim}$ with high KIR expression and reduced expression of NKG2A. In HLA-haploidentical transplants, donor-versus-recipient NK-cell alloreactivity also protects against rejection, GvHD, and acute myeloid leukemia (AML) relapse (88, 89). NK-cell alloreactivity arises from a mismatch between the inhibitory receptors for self-MHC Class I molecules on NK cells and MHC Class I antigens on recipient cells (90). Furthermore, KIR ligand incompatibility in the GvH direction is an independent predictor of overall survival in AML (89). In light of these observations, NK-cell immunotherapy has been used as consolidation treatment in high-risk AML patients and following leukemic relapse after haploidentical HSCT (91, 92).

In relation to UCB transplantation, isolation and infusion of NK cells to improve immune recovery and/or treat relapse has proven difficult due to the low number of cells available in CBUs. To resolve this issue, several groups have developed *ex vivo* NK-cell expansion protocols using UCB (93–96). Beck used Notch receptor ligand Delta-4 to differentiate and expand UCB $CD34^+$ cells into NK cells (93). Most were $CD16^-CD56^{bright}$ and did not express inhibitory receptors that bind Class I MHC (NKG2A, KIR) but did express activating NK receptors (NKG2D) required for cytotoxicity against leukemia cells. Tanaka and colleagues expanded NK cells from unmanipulated UCB cells (1×10^6) using Tacrolimus and low molecular weight heparin without feeder cells (95). This good manufacturing practice (GMP) compliant method produced 40×10^6 NK cells with high levels of stimulatory NK-cell receptors (NKG2C, NKG2D, NKp44). Recently, the same group also reported that the tyrosine kinase inhibitor, Dasatinib, can enhance expansion of NK cells from unseparated UCB and, therefore, could be potentially used to expand NK cells both *ex vivo* and *in vivo* (97). Use of NK cells in UCB transplantation is currently being investigated in clinical trials (98).

T cells

In view of the prolonged T-cell lymphopenia and high rates of viral infection observed post-HSCT, there has been much interest in using adoptively transferred viral-specific mature T cells to improve immune reconstitution and prevent/treat infection. Pathogen-specific T cells can be expanded *ex vivo* from small quantities (<2.5%) of GCSF-mobilized PBSCH and have been used in Phase I/II trials or in selected cases of refractory CMV or EBV infection (99–102). *In vitro* techniques are also being developed to detect, enrich, and produce multiple virus-specific T cells allowing simultaneous adoptive transfer of T cells directed against CMV, EBV, BKV, and ADV (103, 104). For UCB transplantation, however, this strategy has proved much more difficult due to the small number of cells available and because the majority of UCB T cells are antigen naive. Initial strategies employed *ex vivo* polyclonal expansion of all UCB T cells from an aliquot of the CBU using anti-CD3/CD28 coated Dynabeads and IL-2 (105, 106). Although expansion and maturation of the T cells was possible, significant apoptosis of $CD4^+$ T cells occurred and there was a reversal of the normal CD4:CD8 ratio. Addition of IL-7 to the cultures reduced apoptosis, increased proliferation to an average fold expansion of 165, and promoted functional maturation (107). Importantly, the expanded cells lacked alloreactivity against allogeneic cells but could be primed against leukemia cells to generate tumor-specific cytotoxic T cells. Clinical trials of these *ex vivo* expanded T-cell products will be necessary. In relation to pathogen-specific T cells, Park and colleagues first demonstrated that CMV-specific T cells could be produced from UCB by isolation of T cells, priming with IL-7 and IL-12 and stimulation with monocytes and dendritic cells containing CMV antigen (108). More recently, Hanley and colleagues further developed these techniques to produce GMP-compliant cytotoxic T cells against CMV, EBV, and ADV (109). In the first expansion phase, UCB derived T cells are stimulated with UCB dendritic cells transduced with adenoviral vector containing the CMV antigen, pp65, in presence of IL-7, IL-12, and IL-15. A second stimulation is then performed using EBV-transformed B cells. This method only requires 20% of the CBU and can produce 150×10^8 viral specific cells that lyse antigen-pulsed targets and release effector cytokines in response to antigen stimulation. Phase I/II trials using the pathogen-specific expanded UCB T cells are currently in recruitment (110).

Regulatory T cells

The fundamental principle of the human immune system is to protect the body from harmful pathogens ("non-self") while being unresponsive to self-antigens ("self-tolerance"). In addition to the passive central mechanisms of tolerance ("positive" and "negative" selection in the thymus), tolerance is also maintained by peripheral immune mechanisms. Several cells ("suppressors") can suppress autoreactive clones through dominant mechanisms. Of these, Tregs are arguably the most understood. In 1995, Sakaguchi and colleagues described a population of $CD4^+$ T cells expressing the IL-2 receptor alpha chain (CD25) (111). When $CD4^+CD25^-$ cells, from BALB/c nu/$^+$ mice were transferred into BALB/b nu/nu mice they induced a widespread autoimmune disease that could be prevented by co-transfer of donor $CD4^+CD25^+$ cells. The $CD4^+CD25^+$ T cells subsequently became known as Tregs and, in 2001, human $CD4^+CD25^+$ Tregs were described (112, 113). In 2003, the transcription factor forkhead box P3 (Foxp3) was found to be specifically expressed in Tregs and is thought to be the master regulator of Treg differentiation and function (114, 115). Scurfy mice have functional mutations in *Foxp3* producing a deficiency of Tregs and a severe multi-systemic autoimmune disorder (114, 116, 117). Conversely, ectopic expression of Foxp3 in murine naïve $CD4^+$ T cells produces suppressive function and expression of other Treg phenotypic markers, e.g., cytotoxic T lymphocyte antigen 4 (CTLA-4) (114, 115). FOXP3 is also found in human Tregs and mutations in *FOXP3* cause the IPEX syndrome (Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked) (118, 119).

Tregs induce peripheral tolerance by inhibiting the proliferation and cytokine secretion of T, B, NK, NKT, and antigen presenting cells. Many functional mechanisms have been proposed although the contribution of each one *in vivo* remains unclear (120). Cell-contact independent mechanisms include sequestration of IL-2 and the production of inhibitory cytokines such as IL-10 and IL-35. As Tregs express high levels of CD25, Tregs may preferentially absorb IL-2, causing Bim-mediated apoptosis in effector cells due to relative IL-2 deficiency (121). IL-10 [human cytokine synthesis inhibitory factor (CSIF)] is an anti-inflammatory cytokine that is important for maximal Treg function (122). Treg-produced IL-10 suppresses Th17 cells and inhibits IFNγ production by $CD4^+$ T cells in inflamed tissues (123, 124). Cell-contact dependent mechanisms include CTLA-4, cell surface TGFβ, and granzyme mediated cell apoptosis (125). CTLA-4 on Tregs down-regulates CD80 and CD86 expression on APC, preventing activation of effector T cells through TCR–MHC–APC interactions (126). Activated Tregs also express GARP-latent TGFβ complex on their cell surface, which may induce FOXP3 expression in activated T cells in areas of inflammation, i.e., infectious tolerance (127).

There has been particular interest in Tregs in the setting of allogeneic HSCT. In murine GvHD models, depletion of $CD25^+$ Tregs from BM grafts given to lethally irradiated MHC-mismatched mice significantly increased the severity and mortality from GvHD (128, 129). Conversely, co-transfer of $CD4^+CD25^+$ Tregs with $CD4^+CD25^-$ effector T cells (1:1) from C57BL/6 mice into MHC-mismatched BALB/c mice prevented the lethal GvHD seen with the transfer of $CD4^+CD25^-$ T cells alone (130). More importantly, co-transfer of isolated $CD4^+CD25^+$ Tregs with $CD4^+CD25^-$ conventional T cells into an MHC-mismatched mouse with leukemia were able to prevent GvHD but did not prevent the GvT response (131). Co-transfer of Tregs specific for recipient alloantigens were also able to improve immune reconstitution with faster recovery of total lymphocytes, T, and B cells (132). In human allogeneic HSCT, reduced numbers of $CD4^+CD25^{high}$, $CD4^+FOXP3^+$, or $CD4^+CD25^{high}FOXP3^+$ cells and reduced FOXP3 mRNA have been observed in the PB of patients with GvHD (133–138). Furthermore, Magenau and colleagues demonstrated that Treg frequency at the start of the GvHD reduced linearly with increasing GvHD severity, correlated with the maximum grade of GvHD, and predicted response to treatment (138). In skin and intestinal biopsies, patients with GvHD also have fewer $FOXP3^+$ cells per $CD8^+$ lymphocyte compared to patients without GvHD or with non-GvHD inflammation (136, 139). Several studies have also demonstrated that the number of $CD4^+FOXP3^+$ Tregs in PBSCH is an independent predictor of acute GvHD in MAC transplants (140, 141). A low graft CD3/Treg ratio in MAC T-replete transplants was also found to be an independent predictor of acute GvHD, NRM, and overall survival (142, 143). Of note, UCB grafts contain $CD4^+CD25^+$ Tregs with the proportion of cells inversely correlating with gestational age up to the levels found in adults (2–5% $CD4^+$ T cells) (144). In contrast to adults, the majority of UCB Tregs express naïve markers (CD45RA/CD38), possess a more undifferentiated gene expression profile, and do not show initial suppressor activity upon TCR stimulation (15, 145). However, following antigenic stimulation, these cells upregulate CD25, CTLA-4, and FOXP3, proliferate with a high capacity, and possess potent suppressive activity with high IL-10 production (15, 145). These properties may, therefore, partly explain why UCB transplantation is associated with a lower incidence of GvHD (14).

Using Tregs in allogeneic HSCT may promote immune reconstitution and prevent/treat GvHD while maintaining a GvT response. In recent years, focus has been on the isolation and transfer of Tregs around the time of transplantation. Unfortunately, isolation of human Tregs has been hampered by the lack of Treg-specific surface markers, low Treg frequency, and the limitations of GMP. Most strategies use GMP-grade magnetic bead selection of $CD25^{high}$ cells with the depletion of $CD8^+$, $CD19^+$, or $CD127^{high}$ cells. These Treg-rich products contain 40–60% $CD4^+CD25^{++}FOXP3^+$ T cells with *in vitro* suppressive function (146–148). Using this approach, two clinical trials of adoptive transfer of Tregs have been performed in humans. The first was a Phase I study in which patients with a high-risk of relapse were pre-emptively given up to 5×10^6/kg Tregs prior to donor lymphocyte infusions (DLI) (149). In nine patients, there were no adverse events related to the Tregs. In the second study, 28 HLA-haploidentical transplants were given 2–4 $\times 10^6$/kg isolated Tregs (50% $FOXP3^+$) 4 days prior to receiving $CD34^+$ cells and 0.5–2.0 $\times 10^6$/kg conventional T cells (150). The administration of Tregs into the lymphopenic environment was to allow pre-activation and homeostatic expansion of Tregs *in vivo* (151). Despite the absence of other immunosuppression, only two patients developed grade II–IV acute GvHD. In the context of UCB transplantation, Treg isolation strategies have been problematic due to the lower number of Tregs per CBU. Therefore, *ex vivo*

Treg expansion has been necessary. In pre-clinical murine studies, *ex vivo* expanded human UCB $CD4^+CD25^+$ Tregs can prevent allogeneic GvHD, reduce Il-17 production, and tip the Treg/Th17 balance in favor of Tregs (152). Initial expansion protocols of adult Tregs used *in vitro* CD3/CD28 stimulation of Treg-rich isolations in the presence of high dose IL-2 (146, 153). However, small numbers of contaminating cells rapidly expanded in culture, reducing the purity of the final product. In addition, repetitive *in vitro* stimulation was associated with down-regulation of FOXP3 (154). Therefore, several refinements to these protocols have been proposed. First, is the combined use of CD25 and CD45RA to isolate naïve Tregs only and improve Treg purity by depleting contaminating effector cells. Naïve Tregs ($CD4^+CD25^+CD45RA^+$) are highly proliferative *in vitro* and have stable phenotype and function following culture (146). Second, is the addition of Rapamycin as mTOR inhibition prevents expansion of conventional T cells whilst allowing expansion of Tregs (155–157). Using a combination of Rapamycin, IL-15, and TGFβ, Asanuma and colleagues obtained more than 500-fold expansion of $CD4^+CD25^+$ T cells from UCB and the expanded cells could suppress allogeneic cell cultures by more than 50% (158). Rapamycin may also convert conventional T cells into $FOXP3^+$ Tregs. Although the stability of these cells remains contentious, reports suggest that *ex vivo* expansion of Tregs in the presence of Rapamycin and all-trans retinoic acid (ATRA) may be superior to expansion with Rapamycin alone (159). Only Brunstein and colleagues have reported results using expanded human Tregs to prevent GvHD (160). Twenty-three double UCB transplant patients were given expanded Tregs at a dose of $1–30 \times 10^5$/kg on day 1, with 13 of these patients receiving an additional dose of 30×10^5/kg on day 15. Tregs were obtained by CD25 bead isolation from third-party UCB and expanded with CD3/CD28 beads and IL-2 for 18 days. There were no reported adverse events from Treg infusion and grade II–IV acute GvHD was reduced compared to 108 historical controls. These studies therefore provide encouraging results and are the first tentative steps toward Treg cellular therapies in UCB transplantation.

While adoptive transfer of human Tregs remains promising, the ultimate goal is to expand Tregs *in vivo* and modification of current GvHD prophylaxis regimens may enable this. Ciclosporin reduces FOXP3 expression in Tregs, significantly reducing *in vitro* and *in vivo* function of allo-stimulated Tregs and Treg expansion (161–163). Conversely, Rapamycin suppresses conventional T cells but does not reduce FOXP3 expression or *in vivo* Treg function (155–157, 164). *In vivo* murine studies have shown that the combination of IL-2 and Rapamycin can selectively expand Tregs and reduce lethal acute GvHD (165). In Phase II clinical studies, the combination of Rapamycin and Tacrolimus has also shown effective prevention of GvHD and lower NRM compared to historical controls (166, 167). However, there are concerns about the increased risk of thrombotic microangiopathies. Novel therapies to increase Tregs *in vivo* are also being investigated. In mice, *in vitro* inhibition of DNA methylation using the hypomethylating agents, Azacitidine or Decitabine, or by knocking out DNA methyltransferase 1 (DNMT-1) induced Foxp3 expression in proliferating naïve $CD4^+CD25^-$ T cells (168–171). Furthermore, Choi and colleagues demonstrated that Decitabine treated naive $CD4^+CD25^-$ T cells from B6 mice (60% $Foxp3^+$) were able to protect lethally irradiated BALB/c mice from GvHD when given T-cell depleted BM and conventional T cells from the same B6 mice (171). Using this model, *in vivo* Azacitidine post-transplant (days 15–21) prevented GvHD without inhibiting the GvL response, increased $FOXP3^+$ Tregs in the PB, and improved overall survival (171). Azacitidine has also been shown to increase FOXP3 expression in human naive T cells although it has been reported that these cells produce pro-inflammatory cytokines, including IFNγ and TNFα (169, 172). Therefore, it remains unclear whether demethylation agents will increase functional human Tregs post-HSCT and whether this will improve immune reconstitution and/or prevent GvHD. It also remains to be determined whether these agents can be used following UCB transplantation without effecting engraftment. Early phase clinical trials are in process.

USE OF MESENCHYMAL STEM CELLS

Mesenchymal stem cells are multipotent undifferentiated stromal cells with capacity to self renew and/or differentiate into mesenchymal cells including chondrocytes, osteocytes, adipocytes, cardiomyocytes, and neurons. They are present in PB, BM, UCB, and non-hematopoietic tissues including fat, muscle, and UC connective tissue, e.g., Wharton's jelly, although their exact function *in vivo* remains unclear. MSC are a heterogeneous population that lack hematopoietic markers (CD45/CD34/CD14) but express the antigens SH-3/SH-4 (CD73), Thy-1 (CD90), and Endoglin (CD105) (173). However, there is considerable phenotypic variation between MSC obtained from different sources and there is no universal marker allowing specific isolation of these cells.

In relation to UCB transplantation, MSC have low immunogenicity and potent immunosuppressive function that may be useful for improving engraftment and preventing GvHD. MSC do not express Class II MHC molecules or co-stimulatory molecules and, thus, do not elicit allo-antigenic responses. They can also suppress T and NK-cell proliferation, cytokine secretion, and B cell function (174–176). Possible mechanisms include cell-contact dependent and independent responses including IL-10, TGFβ, nitric oxide, and PGE2 and induction of Tregs (174, 177–179). Pre-clinical murine studies showed that co-transplantation of MSC with UCB $CD34^+$ cells in NOD/SCID mice improved engraftment (180–182). In addition, UC MSC supported *ex vivo* expansion of UCB HSC in long-term cultures (183). In 2009, MacMillan and colleagues performed a Phase I/II study of *ex vivo* expanded haploidentical BM-derived MSC in pediatric unrelated UCB transplants (184). Eight patients received MSC [median dose 2.1×10^6/kg (range, 0.9–5.0)] in addition to UCB [median TNC 3.1×10^7/kg (range, 2.0–12.4)], with three patients receiving an additional infusion of MSC on day 21. There were no harmful side effects related to infusion of the MSC. All patients achieved neutrophil engraftment at a median time 19 days (range, 9–28 days). Six patients achieved platelet engraftment at a median of 53 days (range, 36–98 days). Rates of engraftment, GvHD, and survival were comparable to equivalent historical group demonstrating the safety and feasibility of this approach. In a similar pilot study, nine patients received MAC followed by UCB transplants with co-infusion of BM-derived MSC and T-depleted HSC from a third-party donor (185). All patients achieved neutrophil engraftment at a median of 12 days (range, 10–31 days) with full

CB chimerism at a median of 51 days (range, 20–186 days). The maximum cumulative incidence of platelet engraftment was 88% (95% CI, 70–100%) at a median of 32 days (range, 13–97 days). However, there was no difference in engraftment rates compared to a control group of 46 transplants from the same center not receiving MSC. In addition, they reported no difference in recovery of lymphocyte populations although their data were not published. Bernardo and colleagues reported similar findings in 13 pediatric UCB transplants using paternal MSC with no difference in engraftment or rates of rejection compared to 39 matched historical controls (186). Recently, a Phase I/II study of UCB transplants with UC-derived MSC has been performed (187). Five patients received *ex vivo* expanded MSC obtained from Wharton's jelly without any adverse events. Neutrophil engraftment [median 11 days (range, 7–13 days)] and platelet engraftment [median 32 days (range, 22–41 days)] were significantly faster than in nine control patients not receiving MSC. There was no significant difference in total lymphocyte recovery. However, other studies have demonstrated that co-infusion of MSC at the time of UCB transplantation has a negative effect on thymopoiesis and TREC reconstitution, and was associated with reduced survival (188). Therefore, the full implications of co-infusion of MSC with UCB transplantation on engraftment and immune reconstitution require further clarification.

In murine BMT models, MSC have also reduced GvHD, although the timing, dose, and frequency appeared critical as greatest effect was seen when the cells were given into a pro-inflammatory environment with high levels of IFNγ. MSC were first administered to a HSCT patient in 2004 when a 9-year-old boy with refractory GvHD, following a haploidentical transplant, was given BM-derived MSC with complete resolution of symptoms. In 2006, Ringden and colleagues reported a Phase I study in which eight patients with steroid-refractory grade III–IV acute GvHD were given MSC at a median dose of 1×10^6/kg (range, 0.7–9.0) (189). There were no acute effects related to the infusions and six out of eight patients had complete resolution of all symptoms. Overall survival was reported to be better than in a similar group of 16 patients not receiving MSC. In a multi-center EBMT Phase II study, 55 HSCT patients received *ex vivo* expanded BM-derived MSC for steroid-refractory severe acute GvHD (190). Thirty had a complete response and nine had a partial response with median time from infusion to CR of 18 days (range, 3–63 days). Both TRM and overall survival were better in the complete responders. In a multi-center Phase III study, 244 patients with steroid-refractory grade II–IV acute GvHD were randomized to receive third-party MSC (eight infusions of 2×10^6/kg). For liver and gastrointestinal GvHD, the overall response rate at 28 days was higher in the MSC group. However, no significant difference was found in the rate of durable responses (>28 days). MSC have also been used in first-line treatment of GvHD. In a Phase II study, 31 patients with *de novo* grade II–IV acute GvHD were randomized to receive third-party MSC at 2 or 8×10^6/kg in addition to standard corticosteroid therapy. Complete responses were seen in 77% patients and 16% showed a partial response although there was no difference between the low and high dose cohorts. To date, few published studies have examined the use of MSC to treat GvHD following UCB transplantation. In the study by Gonzalo-Daganzo and colleagues using third-party MSC and UCB HSC at the time of transplantation, no difference was observed in acute GvHD (185). However, in this study, two patients who developed steroid-refractory GvHD were subsequently treated with therapeutic infusions of MSC and both had complete resolution of symptoms. In Bernardo and colleagues study, using co-transplantation of UCB and parental MSC, those patients receiving MSC had less grade III–IV acute GvHD (0 vs. 26%, $P = 0.05$) although there was no significant difference for grades II–IV (186). Therefore, the use of MSC to prevent a/or treat GvHD following UCB transplantation may have a role but further investigation is required.

USE OF CYTOKINES, GROWTH FACTORS, AND TYROSINE KINASES

Many cytokines and growth hormones involved in normal thymic function are currently under investigation in pre-clinical studies to determine whether they improve thymopoiesis and immune reconstitution post-HSCT. However, to date, few of these have been specifically tested in the setting of UCB transplantation.

Interleukin-7 is a 25-kDa glycoprotein growth factor produced by the BM stroma and thymic epithelial cells (TEC) and is important for T-cell development. IL-7 binds to its cognate receptor (IL-7R) on immature thymocytes, promoting their differentiation and proliferation into immature T lymphocytes. Mutations of the IL-7 gene or IL-7R produce severe immunodeficiency syndromes (191). In murine allogeneic BMT models, IL-7 increased thymopoiesis and peripheral expansion of recent thymic emigrants and mature T cells, as well as increasing B cells, NK cells, NKT cells, monocytes, and macrophages (192–194). Importantly, in these studies, GvHD was not exacerbated although GvL was maintained (192). However, other similar models have shown increased GvHD using IL-7 (195). In non-BMT human studies, administration of rhIL-7 increases $CD4^+$ and $CD8^+$ T cells with a preferential expansion of naïve T cells and an increased TCR repertoire (196, 197). More recently, Perales and colleagues reported results from a Phase I trial of rhIL-7 (CYT107) after T-cell depleted allogeneic HSCT (198). Twelve patients were treated with escalating doses of rhIL-7 (10–30 μg/kg). IL-7 produced an increase in effector memory T cells with an associated increase in viral specific T cells and enhanced TCR diversity.

Interleukin-2 is a 15-kDa soluble cytokine produced by activated T cells and is critical for their differentiation and proliferation into effector cells. It also promotes proliferation and expansion of B and NK cells and increases cytotoxic activity and production of effector cytokines. IL-2 binds via its cell surface receptor containing the IL-2Rα subunit (CD25), IL-2Rβ subunit (CD122), and the common gamma chain (CD132). As with IL-7, deficiency in IL-2 or its receptor leads to profound immune dysregulation with chronic infection and severe autoimmunity (199). In HSCT, several groups have shown the IL-2 administered after chemotherapy or DLI may reduce relapse and increase responses in refractory disease, possibly through an enhanced GvL response (200, 201). However, low dose IL-2 produces enhanced Treg proliferation with increased thymic Treg output while having little effect on conventional T cells (202). Therefore, such strategies may improve T-cell homeostasis post-HSCT, preventing GvHD and/or thymic damage. Clinical trials using IL-2 in T-cell

depleted double UCB transplants for refractory AML are currently in recruitment (203).

Interleukin-15 is a 14- to 15-kDa glycoprotein belonging to the same family as IL-2 and IL-7. It is expressed by monocytes, macrophages, and dendritic cells and binds to the IL15Rα subunit, IL-2/15Rβ subunit (CD122), and the common gamma chain (CD132). Functionally, it causes proliferation of T cells, B cells, and NK cells and is the primary survival growth factor for NK cells. In murine BMT models, post-transplant IL-15 administration increased donor-derived $CD8^+$ T cells, NK cells, and NKT cells with enhanced NK and T-cell function (204). IL-15 was able to enhance the GvL effect but also increased GvHD in T-replete BMT.

Sex steroids have important effects on myeloid and lymphoid recovery following HSCT. The sex steroid hormones (androgen, estrogen) impair thymic function and cause apoptosis in thymic stromal cells and developing thymocytes following puberty. Conversely, reducing sex steroid levels, either via castration or by modifying the hypothalamic–pituitary–gonadal axis, increases thymopoiesis by reducing the rate of apoptosis and increasing the proliferation of TECs. When a luteinizing hormone releasing hormone agonist (LHRHa) is administered in a continuous fashion at high doses, LHRH receptors become desensitized and there is a subsequent reduction in the production of follicle stimulating hormone (FSH) and luteinizing hormone (LH). In turn, this leads to a reduction in the production of sex steroids (205, 206). Using this approach in a murine BMT model produced a significant increase in myeloid and lymphoid progenitors and enhanced thymic reconstitution with increased peripheral T cells (205). GvHD was not increased but GvL was maintained. Furthermore, combined use of keratinocyte growth factor (KGF) and LHRHa increased thymopoiesis with enhanced reconstitution on naïve $CD4^+$ and $CD8^+$ T cells, reduced homeostatic expansion, and produced a broader T-cell repertoire (207).

Growth hormone (GH) and its mediator insulin-like factor-1 (IGF-1) may also improve thymopoiesis. GH is a 22-kDa protein produced by the pituitary gland, under the control of the hypothalamus via the production of growth hormone releasing hormone (GHRH) and growth hormone inhibiting hormone (GHIH). It is also produced by thymocytes and TEC and acts locally with its corresponding receptor (GHR) in an autocrine fashion. GH induces proliferation and cell growth within the thymus and increases thymic size, cellularity, and TCR repertoire when administered to GH-deficient mice (208). It also accelerates T-cell and immune recovery when given in murine T-depleted HSCT (209). Furthermore, GH may also protect against the effects of radiation, increasing production of HSC and promoting recovery of leukocytes, T, B, and NK cells in murine models (210). IGF-1 is a 7-kDa protein, which is mainly produced by the liver in response to GH but is also produced by other cell types in an autocrine fashion. IGF-1R is expressed on thymocytes, T cells, and TECs and binding of IGF-1 to its receptor increases the proliferation of thymocytes and peripheral T cells (211). Administration of IGF-1 in murine HSCT models also increased *in vivo* thymic precursor populations, donor-derived T cells as well as pro-, pre-, and mature B cells, and myeloid cells (212).

Keratinocyte growth factor is a 28-kDa protein produced by mesenchymal stromal cells and mature thymocytes. Several groups have shown that KGF protects mucosal, cutaneous, and epithelial cells from cytotoxic and irradiation induced injury (213, 214). In murine BMT models, pre-treatment with KGF protected the TECs and increased donor-derived thymocytes, peripheral naïve T cells, and production of IL-7 (215). KGF induces p53 and NF-kappa pathways in immature TECs, promoting proliferation and differentiation into mature TECs (216). In a murine UCB transplant model, KGF pre-treatment increased day 35 thymic outputs with higher T-cell and NKT-cell numbers within the spleen and increased the proportion of TREC (217). However, in human clinical trials, although KGF (Palifermin) reduced mucositis, it has not been shown to have any significant impact on lymphocyte reconstitution, GvHD, infectious complications, or overall survival (218–221).

Finally, other cellular pathways currently being investigated to improve thymopoiesis post-HSCT are the tyrosine kinases, Flt-3 and c-Kit, and the tumor suppressor gene, p53. FLT-3-L, produced in the thymus and expressed on the surface of perivascular fibroblasts, is upregulated following irradiation and increases proliferation of Flt-3 positive thymic precursors (222). Flt-3-L also promotes thymocyte maturation and homeostatic expansion of peripheral T cells following HSCT (223). Stem cells factor (SCF) also promotes early thymocyte development through its receptor tyrosine kinase, c-Kit. Pre-clinical studies in mice using the tyrosine kinase inhibitor, Sunitinib, suggest that pre-treatment of the recipient may promote improved donor-derived thymopoiesis by blocking c-Kit in the host's early thymic progenitors and, thus, improving accessibility to the thymic niche (224). p53 is a tumor suppressor gene that activates DNA repair, arrests cell growth, and induces apoptosis in response to cell damage. Given the degree of epithelial damage following conditioning chemotherapy and/or irradiation, it has been proposed that temporary inhibition of p53 may reduce thymic apoptosis and promote T-cell recovery following HSCT. In murine HSCT models, Kelly and colleagues demonstrated that temporary inhibition of p53 using the small molecule pifithrin-β (PFT-β) prevented damage in TECs (225). Moreover, when combined with KGF, thymic function was improved post-HSCT with higher numbers of donor-derived naïve $CD4^+$ and $CD8^+$ T cells and enhanced responses against *Listeria monocytogenes*.

CONCLUSION

The scientific disciplines of immunology, cellular biology, and immune regulation following UCB transplantation continue to rapidly advance and over recent years, our understanding of how cord blood and neonatal immune cells differ from those found in adults has become clearer. This information is essential to improve our understanding of immune reconstitution, and perhaps, the means to accelerate recovery after UCB transplantation. Despite our extensive knowledge on the unique biology of CB graft lymphocytes, many of the characteristics of these cells and their relevance to immune cell recovery have still not been adequately evaluated after UCB transplantation. Therefore, more in depth pre-clinical and clinical studies in these areas are warranted, both in terms of recovery of normal immune cell function and their effectiveness in antitumor cell activity.

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