# MOVING TOWARDS ALLOGENEIC CELLULAR THERAPIES: OPPORTUNITIES AND CHALLENGES

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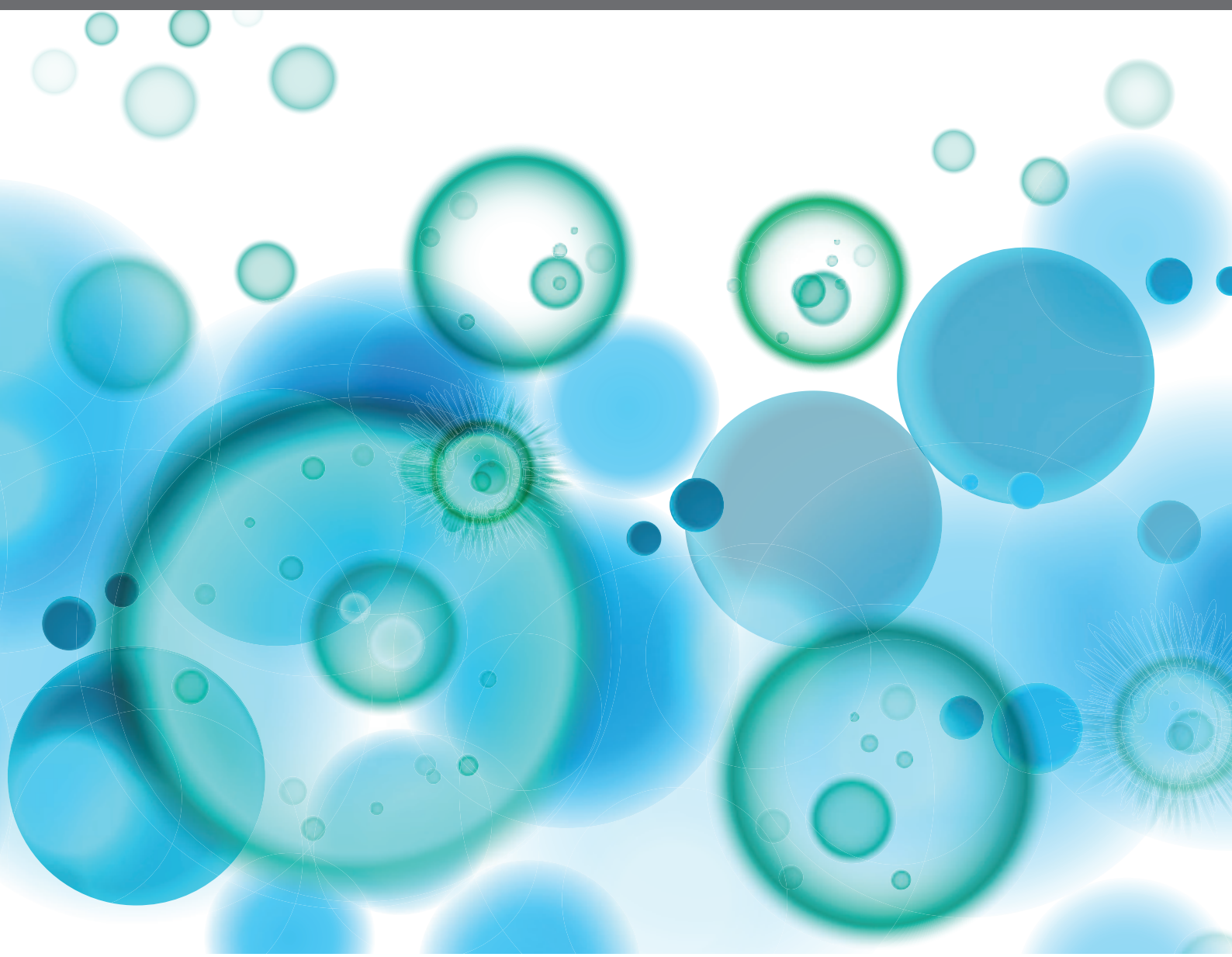

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# MOVING TOWARDS ALLOGENEIC CELLULAR THERAPIES: OPPORTUNITIES AND CHALLENGES

Topic Editors:
**Federico Simonetta,** Geneva University Hospitals (HUG), Switzerland
**Alice Bertaina,** Stanford University, United States
**Peter Bader,** University Hospital Frankfurt, Germany

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REVIEW
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# The Immunomodulatory Effects of Mesenchymal Stem Cells on Regulatory B Cells

*Jialing Liu*[1], *Qiuli Liu*[1]* *and Xiaoyong Chen*[1,2,3]*

[1] The Biotherapy Center, The Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, China, [2] Center for Stem Cell Biology and Tissue Engineering, Key Laboratory for Stem Cells and Tissue Engineering, Ministry of Education, Sun Yat-sen University, Guangzhou, China, [3] Department of Pathophysiology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, China

The therapeutic potential of mesenchymal stem cells (MSCs) has been investigated in many preclinical and clinical studies. This potential is dominantly based on the immunosuppressive properties of MSCs. Although the therapeutic profiles of MSC transplantation are still not fully characterized, accumulating evidence has revealed that B cells change after MSC infusion, in particular inducing regulatory B cells (Bregs). The immunosuppressive effects of Bregs have been demonstrated, and these cells are being evaluated as new targets for the treatment of inflammatory diseases. MSCs are capable of educating B cells and inducing regulatory B cell production via cell-to-cell contact, soluble factors, and extracellular vesicles (EVs). These cells thus have the potential to complement each other's immunomodulatory functions, and a combined approach may enable synergistic effects for the treatment of immunological diseases. However, compared with investigations regarding other immune cells, investigations into how MSCs specifically regulate Bregs have been superficial and insufficient. In this review, we discuss the current findings related to the immunomodulatory effects of MSCs on regulatory B cells and provide optimal strategies for applications in immune-related disease treatments.

**Keywords: mesenchymal stem cells (MSCs), regulatory B cells (Bregs), cell-to-cell contact, soluble factors, extracellular vesicles (EVs)**

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***Edited by:***
*Alice Bertaina,*
*Stanford University, United States*

***Reviewed by:***
*Honglin Wang,*
*Shanghai Jiao Tong University, China*
*Marcella Franquesa,*
*Germans Trias i Pujol Health Science Research Institute (IGTP), Spain*

***Correspondence:**
*Qiuli Liu*
*liuqli3@mail.sysu.edu.cn*
*Xiaoyong Chen*
*cxy-1216@163.com*

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## INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent stromal cells existing in many human tissues that can be rapidly expanded *in vitro* to meet the needs of clinical and basic research. The term MSCs was coined by Caplan in 1991 (1). Since Friedenstein and coworkers demonstrated the osteogenic potential of a minor subpopulation of BM cells that rapid adherence to tissue culture vessels and have a fibroblast-like appearance of their progeny in culture (2), MSCs have been derived from lots of tissues in different species (3, 4). However, MSCs still lack specific markers for identification. The International Society for Cell Therapy (ISCT) established three basic criteria for the identification of MSCs in 2006: (1) demonstration of plastic-adherent growth; (2) exhibition of the following phenotypic characteristics: expression of CD105, CD73, and CD90 in more than 95% of cells; a lack of expression of CD45, CD34, CD14, CD11b, CD79a, and CD19 in the majority of cells; and a lack of expression of HLA-DR; and (3) demonstration of the ability to differentiate into osteoblasts, adipocytes, chondroblasts *in vitro* (5). MSCs can exhibit important roles in tissue regeneration and repair (6), maintenance of bone marrow hematopoietic microenvironment homeostasis (7), and immunomodulation of inflammation (8).

Given the current considerable safety and efficacy in preclinical and clinical studies, the roles of MSCs in regenerative medicine have attracted widespread attention, especially their immunomodulatory effects on autoimmune diseases and transplantions, such as Crohn's disease (CD) (9), rheumatoid arthritis (RA) (10), and systemic lupus erythematosus (SLE) (11), as well as graft-versus-host disease (GvHD) (12), kidney transplantation (KTx) (13, 14), liver transplantation (LTx) (15, 16), chronic lung allograft dysfunction (CLAD) (17) and small bowel transplantation (SBTx) (18), and even their roles in immune-mediated cell therapies (19). MSCs exhibit functional characteristics related to immune regulation and have consistently been shown to play roles in regulating innate and adaptive immune responses via a variety of pathways, such as cell-to-cell contact (20), soluble factors (21), and exosomes derived from MSCs (22). For instance, MSCs possess the ability to secrete regulatory molecules and cytokines that can modulate PBMC maturation, proliferation, differentiation, migration, and functional activation (23–25).

B cells are essential immune effector cells that are pivotal in adaptive immune responses and play roles in autoimmunity through antigen presentation, antibody secretion, and complement activation. Previous studies have shown that MSCs are capable of regulating B cell proliferation and differentiation, inhibiting B cell apoptosis, etc., and they can also suppress the adaptive immune response by indirectly regulating dendritic cell (DC)-mediated antigens. Another mechanism by which MSCs may exert effects on autoimmune diseases in the short and long term is their induction of regulatory B cells (Bregs), especially types that promote the secretion of interleukin (IL)-10, which promote B cells to exhibit immunosuppressive functions and modulate the immune environment homeostasis of patients with autoimmune diseases or solid organ transplantation such kidney transplantation and liver transplantation.

A relatively large number of studies have been published to confirm the clinical phenomenon and mechanisms regarding MSCs regulating regulatory B cells. In addition, previous studies have shown the regulatory effects in animal disease models and the safety, feasibility and potential effectiveness of allogeneic transplantation of MSCs in clinical trials to treat immune-related diseases. It seems necessary to better understand how the underlying mechanisms of MSC-mediated Breg or combined MSC/Breg cell therapies can be successfully applied in clinical fields. In this review, we discuss MSC functions related to Bregs and the possible mechanisms by which MSCs induce Bregs *in vivo* and *in vitro*, especially with regard to IL-10-producing Bregs.

## CURRENT DEFINITION AND UNDERSTANDING OF REGULATORY B CELLS

B cells, an important cells for the adaptive immune response, have the ability to present antigens, secrete antibodies, and activate the immune system (26), which have been observed in autoimmune diseases, infections and cancers. Several subsets of B cells exert regulatory functions similar to those of regulatory T cells (Tregs) and are collectively termed regulatory B cells (Bregs). Previous studies have shown that Bregs could inhibit Th1 and Th17 responses and induce $FoxP_3^+$ Treg pools to play a key role in maintaining peripheral tolerance (27). Regulatory B cells have been found in various B cell subpopulations, including B1 B cells, B2 B cells, and plasma cells (28). Breg-mediated immunosuppression is an important manner for the maintenance of peripheral tolerance (29). However, there is still no clear consensus on the definition and classification of Bregs. As their heterogeneity, Bregs may express one or more of regulatory factors [including IL-10, IL-35, transforming growth factor (TGF)-β, and programmed cell death 1 ligand 1 (PD-L1)] and exert suppressive effects on cognate T cells (27, 30–32). Since three inhibitory cytokines, IL-10, TGF-β, and IL-35, having been identified as key inhibitory inflammatory factors for Bregs, Bregs can be divided into three categories: IL-$10^+$, TGF-$\beta^+$, and IL-$35^+$ Bregs. Among these, the IL-$10^+$ Bregs, also called B10 cells, are the major cell type in mediating immunosuppression. IL-$10^+$ Bregs have been widely regarded as important immunoregulatory cells in various inflammatory diseases, such as RA (33), chronic intestinal inflammatory conditions (34), SLE (35), CD (36), Collagen Induced Arthritis (CIA) (37), and GVHD (38). Besides, Bregs also play an important role in transplantation, including KTx (39, 40), cardiac allografts (41), liver transplantation (42) and so on. The various subpopulation phenotypes among IL-$10^+$ Bregs are shown in **Table 1**.

The term "regulatory B cells" were firstly introduce by Bhan and Mizoguchi. Using T-cell receptor (TCR)-$\alpha^{-/-}$mice, μMT mice, and TCR-$\alpha^{-/-}$μMT mice, they found that colitis pathogenesis does not require B cells, but B cells are presumably involved in the elimination of apoptotic cells, which contributed to suppressing colitis (60). Similarly, Michael Hahne et al. reported that LPS-activated B cells expressing FAS ligands (FasL) can clear activated T cells such as FAS-expressing T cells, and transfer of LPS-activated B cells could ameliorate the development of diabetes in NOD mice (61). Subsequently, Atsushi Mizoguchi et al. found that under conditions of chronic enteritis, B cell subsets, characterized by upregulation of CD1d expression, can produce IL-10 and attenuate IL-1 upregulation and signal transducer and activator of transcription (STAT)3 activation, which indicates that B cells producing IL-10 could serve as regulatory cells in immunologically mediated inflammatory responses (34). Later, Claudia Mauri et al. used agonistic anti-CD40 and collagen to stimulate arthritic B cells, increasing the secretion of IL-10 in B cell subsets to control the proinflammatory Th1 type response while reducing secretion of interferon (IFN)-γ; the findings proved that these B cells play important roles in immune regulation in arthritis models (33). Niamh E. Mangan et al. have also reported that the induction of IL-10-producing B cells can modulate allergic responses in worm-infected mice (62, 63). Besides, studies of Bregs in transplantation have also been conducted. Lal Girdhari et al. proved that CD40 costimulatory blockade induces IL-10 producing Marginal Zone Precursor (MZP) Bregs, especially IL-$21R^+$ MZP Bregs, performing a key function in restoring graft survival (50).

**TABLE 1 |** Phenotypes of IL-10$^+$ Bregs.

| Species | Phenotype | Function |
|---|---|---|
| Mouse | CD138$^{high}$ (43) | Anti-Salmonella immunity |
| | CD19$^+$CD5$^+$CD1d$^{high}$ (44, 45) | Treg induction; inhibition of Th17 response |
| | CD1d$^{high}$CD23$^{high}$CD21$^{int}$ (46) | Protective role in the mucosa |
| | CD19$^+$CD43$^+$CD80$^+$CD86$^+$CD40$^+$ (47) | Inhibition of Th1 response |
| | CD19$^+$CD43$^+$CD5$^+$ (48) | Amelioration of cGVHD |
| | CD1d$^{high}$ (49) | Treg induction |
| | CD5$^+$CD1d$^{hi}$ (41) | Inhibition of Th1 cells activation; induction of islet allograft tolerance |
| | CD19$^+$CD24$^{high}$CD38$^{high}$ (30) | Suppression of Th1 cell differentiation |
| | IL-21R$^+$ MZP (50) | Induction transplantation tolerance |
| Human | CD5$^+$IL-10$^+$ (51) | Inhibition of Th1 response |
| | CD19$^+$CD25$^{high}$CD27$^{high}$ CD86$^{high}$CD1d$^{high}$IL-10$^{high}$TGF-β$^{high}$ (52) | Suppression of CD4+ T cell proliferation |
| | CD19$^+$CD38$^+$CD1d$^+$IgM$^+$CD147$^+$ CD25$^+$ (53) | Suppression of antitumor immune responses |
| | CD24$^{high}$CD27$^+$ (42, 54) | Negatively regulate monocyte cytokine production; predicted the occurrence of acute allograft rejection in liver transplantation |
| | CD154$^+$ (55) | A character of SLE patients |
| | CD25$^+$CD71$^+$CD73$^{low}$PD-L1$^+$ (56) | Suppress antigen-specific immune responses |
| | CD27$^{int}$CD38$^+$ (57) | Production of IL-10 |
| | CD5$^{high}$CD38$^{low}$PD-1$^{high}$ (58) | Inhibition of Th1 and Th17 differentiation |
| | CD23$^+$CD43$^+$(59) | Inhibition of T cell response |

## MSCS PLAY ANTI-INFLAMMATORY ROLES IN IMMUNE DISEASES BY INCREASING BREGS

There have been many discoveries shown that MSCs exert immunomodulatory functions to affect B cells. In 2006, Anna Corcione et al. first discovered that hMSCs can directly interact with B cells to prevent their proliferation and death while promoting arrest during the G0-G1 phase of the cell cycle. They found that the expression of CXCR4, CXCR5, and CCR7 in B cells was downregulated as a result of inhibition of human B cell proliferation, differentiation into antibody-secreting cells, and chemotaxis *in vitro* (64). In 2007, Patrizia Comoli et al. reported MSCs induced by allo-stimulation *in vitro* are capable of modulating B-cell allo-responses via inhibiting antibody production, suggesting that third-party MSCs are able to suppress allo-specific antibody production *in vitro*, and may therefore help overcome a positive cross-match in sensitized transplant recipients (65). In 2009, it was reported that MSCs inhibit B cell terminal differentiation by releasing cytokines to downregulate B cell Blimp-1 expression both *in vitro* and *in vivo* (66). Moreover, Elisabetta Traggiai et al., through polyclonal stimulation of B cells isolated from children with systemic lupus erythematosus and healthy donors, found that bone marrow MSCs can promote the proliferation of transitional cells and naive B cells and their differentiation into immunoglobulin-secreting cells, moreover, MSCs strongly promote the proliferation of memory B cells and their differentiation into plasma cells (67).

Many previous studies have focused on MSCs inducing the production of regulatory T cells to exert immunosuppressive functions. Similarly, the modulation of regulatory B cells by MSCs also plays important roles in the treatment of many diseases. For example, in experimental autoimmune encephalomyelitis (EAE), an experimental model of human multiple sclerosis (MS), CD1d$^{high}$CD5$^+$ regulatory B cells were upregulated after MSC administration and exert anti-inflammatory and immunosuppressive effects (68). Experiments have also found that human umbilical cord MSCs (hUC-MSCs) protect experimental mice by increasing the numbers of CD5$^+$ Bregs that produce IL-10 and correcting Treg/Th17/Th1 imbalance in a colitis model (69). Furthermore, Y Peng et al. have reported that the numbers of CD5$^+$IL-10$^+$ regulatory B cell subset are increased in patients with refractory chronic graft-versus-host disease after MSC treatment (70). Minglu Yan et al. reported that human synovial membrane-derived MSCs can inhibit the maturation and differentiation of B cells; induce CD21$^{high}$CD23$^{high}$ transitional 2 (T2) cells, CD23$^{low}$CD21$^{high}$ marginal zone (MZ) cells, and CD5$^+$CD1d$^+$IL-10 cells in the spleen; and increase the numbers of immature transitional B cells, such as IL-10$^+$ cells, thus reducing the severity of arthritis in mice (71). Kunal S. Gupte et al. reported that co-culture of adipose tissue-derived MSCs (AD-MSCs) from 15 potential kidney donors with peripheral blood PBMCs could induce IL-10-secreting B cells, demonstrating the promise of cell therapies for immune diseases after transplantation (72). Studies demonstrated that MSC infusions contributed to long-term stabilization of renal allograft function, likely via triggering an active peripheral immunomodulation to induce long term immunophenotyping of naïve and CD24$^{high}$CD38$^{high}$ transitional B-cell subsets in kidney allograft recipients (73). Along with this, another recent study held by Davide Piloni el at. proved that CD19$^+$CD24$^{high}$CD38$^{high}$ Breg cell subset also showed key functions in the long term acceptance of lung graft (74). These discoveries of B-cell subsets provide not only a potential marker of MSC-induced immunomodulation associated with transplantation tolerance, but also a prospective view in IL-10 producing B cells key functions among SOT applications. Recently, Di Lu et al. found that allogeneic MSC transplantation can promote the levels of IL-4 and IL-10 and the induction of Bregs in an aGVHD mouse model with complete mismatch of MHC and significantly inhibit the expression of CD69 and CD86 on B lymphocytes to prolong survival, thus demonstrating that B lymphocytes play an important role in the development of aGVHD and that B lymphocytes are targets of the immune regulatory cascade in MSCs (75). Studies based *in vitro* experiments or preclinical and clinical researches have

**TABLE 2 |** Summary of the studies on MSC-mediated effects to Bregs.

| Study | Disease or study type | Key findings |
|---|---|---|
| Chen et al. (76) | Clinical trial: BOS after allo-HSCT | Increased $CD5^+$B cells and IL-10-producing $CD19^+CD5^+$ Bregs |
| Chen et al. (59) | Colitis model | Induced the novel $CD23^+CD43^+$Bregs subset |
| Planella et al. (77) | *Invitro* study | The PF as well as the CM could increase induced $CD24^{high}CD38^{high}$ B cells |
| Lu et al. (75) | Acute GVHD model | Decreased IL-4 and increased IL-$10^+$Bregs |
| Li et al. (78) | EAE model | Increased $CD5^+$ IL-$10^+$ B cells |
| Mehdipour et al. (79) | *Invitro* study | Decreased TNF-$\alpha^+$/ IL-$10^+$ B cells ratio in B cell-ASCs co-culture |
| Luk et al. (80) | *Invitro* study | Under immunological quiescent conditions, MSC increased IL-$10^+CD38^{high}$ $CD24^{high}$ Bregs |
| Yan et al. (71) | CIA model | Increased $CD21^{high}CD23^{high}$ T2 cells, $CD23^{low}CD21^{high}$ MZ cells, and $CD5^+CD1d^+$IL-$10^+$Bregs |
| Gupte et al. (72) | *Invitro* study | Increased IL-10-secreting Bregs from baseline of patients |
| Cho et al. (81) | Animal *in-vivo* study | Induced IL-10-expressing Bregs in an EBI3-dependent manner |
| Zhang et al. (82) | Clinical trial: NS after allo-HSCT | Induced $CD19^+CD5^+$IL-$10^+$ Bregs |
| Hermankova et al. (83) | *Invitro* study | IFN-γ-treated MSCs inhibited IL-10 production by activated B cells via cell-contact and the Cox-2 pathway |
| Chao et al. (69) | Colitis model | Boosted the numbers of $CD5^+$ B cells and IL-10-producing $CD5^+$ Bregs |
| Peng et al. (70) | Clinical trial: refractory cGvHD | Increased IL-10-producing $CD5^+$ B cells |
| Franquesa et al. (84) | *Invitro* study | Reduced plasmablast formation and induce IL-10-producing $CD19^+CD24^{high}CD38^{high}$ Bregs |
| Park et al. (85) | SLE model | Increased IL-10-producing Bregs |
| Garimella et al. (86) | CIA model | Increased the $CD19^+CD1d^{high}CD5^+$ Bregs in the spleens of ASC-treated CIA mice |
| Wang et al. (87) | Cardiac allograft model | MSC-expressing B7-H1 neutralization reduced IL-$4^{high}$IL-$10^{high}CD83^{low}$ B cells |
| Guo et al. (68) | EAE model | Upregulated $CD1d^{high}CD5^+$Bregs |

*BOS, Bronchiolitis obliterans syndrome; HSCT, Hematopoietic Stem Cell Transplantation; GVHD, graft-versus-host disease; EAE, experimental autoimmune encephalomyelitis; CIA, collagen-induced arthritis; NS, Nephrotic syndrome; SLE, systemic lupus erythematosus.*

reported the induction of Bregs by MSCs as we summarized in **Table 2**.

## HOW MSCs REGULATE BREG GENERATION

Accumulating evidence has revealed the importance of Bregs and Tregs in the maintenance of immune tolerance, and MSC-mediate disease improvements are often associated with the induction of Bregs and Tregs. It's well-known that MSCs regulate Tregs proliferation, survival, and function mainly through several pathways. Firstly, cell-to-cell contact, through which interactions among different molecules expressed by MSCs and T lymphocytes (such as ICOSL and ICOS, Notch and Notch ligands), upregulates the production of IL-10 and the proliferation of Tregs. Followed, the secretion of soluble factors by MSCs, including TGFβ, CCL2, IL-6, IL-7, PGE2, IDO, HO-1, and HLA-G5, can regulate Treg generation (88–90). Moreover, antigen-presenting cell dependence; in this pathway, MSCs affect antigen-presenting cells (dendritic cells, monocytes, macrophages) to induce regulatory phenotypes and promote Treg activity through IL-10 and TGF-β1, although the detailed mechanism has not been fully elucidated. In addition, MSC-derived extracellular vesicles, containing specific RNA, proteins and other biological molecules, induce the polarization of $CD4^+$ T cell into Tregs (91).

Compared to MSCs inducing Tregs, the specific mechanisms by which MSCs regulate the generation of Breg are still not sufficiently clear. Several studies have focus on the mechanism that induces the generation of Bregs. H Li et al. found that T follicular regulatory (Tfr) cells could induce IL-$10^+$ Breg cells, as higher frequency of IL-$10^+$ Breg cells was observed when incubation with Tfr cells (92). Moreover, tolerogenic DC (tolDC), one type of DC with immuno-suppressive properties, were reported to induce the IL-10 producing Breg, as wells as the IL-10 producing type 1 regulatory T cells (Tr1) (93). Cynthia M. Fehres et al. described that a proliferation-inducing ligand (APRIL) induced IL-$10^+$B cells production in EAE and CHS models, as APRIL promoted the differentiation of naïve human B cells to IL-10-producing $IgA^+$ B cells (94). It has been postulated by some investigators that the conditions in the microenvironment are key factors for the induction of Bregs. Notably, Toll-like receptor (TLR), CD40, and BCR-induced signaling are vital for Breg function (95–97). In view of previous studies that have assessed multiple modulatory mechanisms of MSCs, we illustrate below the relationship between MSCs and Bregs from several perspectives, which also summarized in the **Figure 1**.

### Cell-to-Cell Contact

MSCs can regulate immune responses through direct cell-to-cell contact. Via interaction with surface molecules and/or receptors, MSCs might directly regulate their downstream pathways in B cells, thereby affecting B cell activation, proliferation,

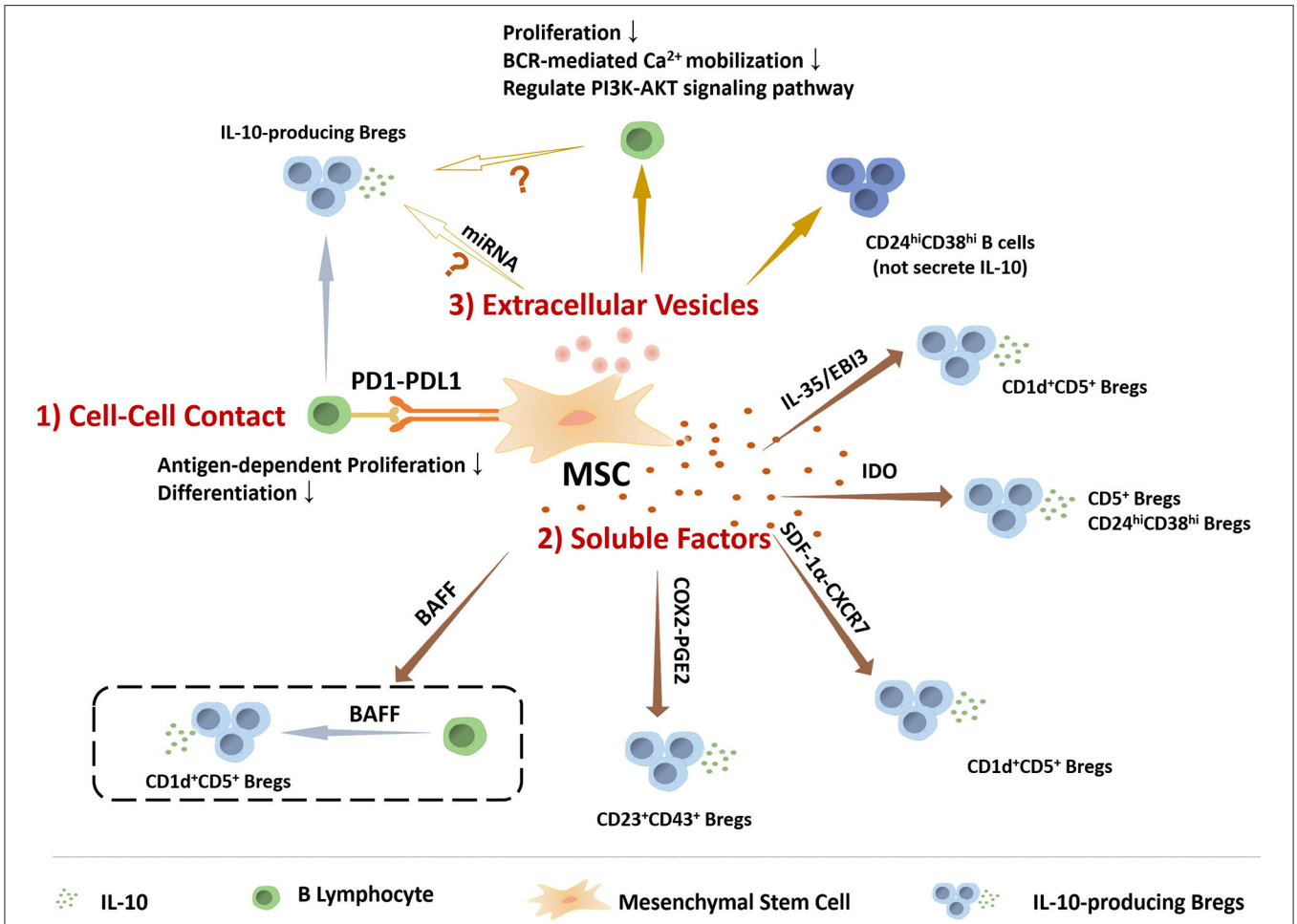


**FIGURE 1 |** The role of MSCs in regulating the IL-10 producing regulatory B cells. MSCs perform functions on modulating IL-10 producing regulatory B cells via many manners, including (1) Cell-to-cell contact: MSCs play roles in B cells via PD1-PDL1 pathway to inhibit antigen-dependent proliferation and differentiation, and induce Bregs. (2) Soluble factors: IL-10-producing Breg subsets, including $CD5^+$ Bregs, $CD24^{high}CD38^{high}$ Bregs, $CD1d^+CD5^+$ Bregs, and $CD23^+CD43^+$ Bregs, are mediated by MSCs-secreting soluble factors. (3) Extracellular Vesicles: MSCs-EVs could inhibit B cell proliferation and BCR-mediated $Ca^{2+}$ mobilization, regulate PI3K-AKT signaling pathway in B cells that is critical for Breg cell development, and induce $CD24^{high}CD38^{high}$ B cell subpopulation, a classic phenotype of Bregs, but without IL-10 production. Based on the current data, MSCs-EVs might be involved in MSCs regulating IL-10 producing B cells.

survival, differentiation, and Bregs induction. For instance, M. Franquesa et al. experimentally demonstrated that hASCs can act independently of T cells and directly on B cells to promote the production of $CD19^+CD24^{high}CD38^{high}$ and IL-10-production regulatory B cells (84). Although cell-to-cell contact manner have been confirmed to be involved in MSCs inducing Bregs by the transwell co-culture (59, 80), little is known about the particular molecules. One of the major molecules involved in this cell-to-cell interaction of MSCs is the costimulatory molecules is programmed death ligand-1 (PD-L1, also known as B7-H1). PD-L1 is well-known for its role in immune checkpoint regulation (98). Its receptor, programmed cell death protein 1 (PDCD1; also known as PD1), is an immunoglobulin-superfamily member that over-expressed upon programmed cell death as its primary function described to attenuate the immune response (99, 100). Francesca Schena et al. found that BM-MSCs inhibit antigen-dependent proliferation and differentiation of follicle and MZ B cells *in vitro* through the PD-1/PD-L1pathway, and ameliorate the inflammatory response in systemic lupus erythematosus mice (101). H Wang et al. reported that the expression of B7-H1 on MSCs was required for IL-10-producing Bregs development in recipients and MSC-mediated suppression of antibody production and B cell proliferation, which contribute to the induction of immune tolerance to allografts in mouse cardiac allograft model by the combination therapy of MSCs and rapamycin (RAPA) (87).

## Soluble Molecule Interactions

### Cyclooxygenase-2 (COX-2)/PGE2

Prostaglandins (PGs) are small molecule derivatives of arachidonic acid produced by cyclooxygenase (102). Prostaglandin E2 (PGE2), the main product of cyclooxygenase in myeloid cells and stromal cells, is a biologically active factor whose synthesis was regulated by COX-2 and shown to regulate

multiple aspects of inflammation in immune cells (103). Many studies have shown that MSCs exert their therapeutic ability mainly dependent on PGE2 secretion (104, 105). MSCs-derived PGE2 also contribute to their induction of Tregs (106). In B cells, Tae-Hoon Shin et al. show that COX-2 signals are necessary for MSCs to inhibit the proliferation and maturation of B lymphocytes, result of inhibiting the secretion of IgE by mature B cells in a mouse atopic dermatitis (AD) model (107). R Chen et al. shown that PGE2 could induce B10 cells via the MAPKs/AKT-AP1 axis or aryl hydrocarbon receptor (AhR) signaling (108). Recently, COX-2/PGE2 pathway is also found to involved in MSCs induce $CD23^{+}CD43^{+}$ Bregs, which significantly reducing the clinical and histopathological severity of induced colon inflammation and ameliorating gastrointestinal mucosal tissue damage in mice (59). However, IFN-γ-primed MSCs were reported to inhibit the production of IL-10 by LPS-activated B cells through the COX-2 pathway (83). PGE2 has shown to exert paradoxes function in regulating immune response (103), more experiments might need to uncover the key mechanisms and targets of PGE2-mediated effects on MSCs inducing Bregs. The immune status of MSCs may be another cause needed pay attention to, as the microenvironment is one of the major factors that affecting the immuno-regulatory ability of MSCs.

### Indolamine-2,3-dioxygenase (IDO)

IDO catalyzes the first and rate-limiting step of tryptophan catabolism in the kynurenine pathway, and its downstream metabolites include kynurenine (KYN) and 3-hydroxyanthranilic acid. It is worth noting that IDO has been shown to regulate the expression of inflammation-related genes, either by itself as a signaling factor or through the production of biologically active intermediates via the kynurenine pathway, such as 3-hydroxyanthranilic acid and kynurenic acid (KYNA). IDO could inhibited T cell proliferation and modulated regulatory T cell differentiation (109, 110). G Wang et al. demonstrated IDO is necessary to the therapeutic effects of human umbilical cord-derived MSC (hUC-MSC) for treating acute lung injury (ALI) (111). Based on previous studies, the IDO expression in MSCs require priming by IFN-γ and pro-inflammatory cytokines that enhance IDO levels via JAK/STAT signaling (112, 113). IFN-γ-pretreated MSCs inhibit the production of IgG and the proliferation of B cells, largely dependent on tryptophan catabolism by IDO (80). Human umbilical cord-derived MSCs (hUC-MSCs) can control EAE by increasing the proportion and promoting the function of $CD5^{+}IL\text{-}10^{+}$ B cells. After co-culture with MSCs, $CD5^{+}$ B cells show a stronger ability to inhibit T cell proliferation and proimmflamatory cytokines secretion, as well as to induce Tregs (78), and these enhanced immunomodulation of $CD5^{+}$ B cells by MSCs were reversed when blocking the IDO pathway. Moreover, MSCs increased the frequency of $CD5^{+}$ Breg cells by enhancing their proliferation and survival via the IDO pathway (70).

### IL-35

Interleukin-35 is a novel anti-inflammatory cytokine belonging to the IL-12 cytokine family that can be applied as a potential therapy for chronic inflammation and autoimmune diseases (114). Human IL-35, which functions as an important immunomodulator, seems to inhibit mature inflammation rather than prevent inflammation as IL-35 is not constitutively expressed in human tissue (27, 115, 116). IL-35 has reported to induce both Tregs and Bregs (117). IL-35 could induce the conversion of B cells into Bregs, including $IL\text{-}35^{+}$ Bregs and $IL\text{-}10^{+}$ Bregs. Mice deficient in p35 or EBI3, the two subunits of IL-35, exhibit an exacerbation in EAE and experimental autoimmune uveitis (EAU) with less Bregs (27, 43). Studies have revealed that overexpression of IL-35 in hMSCs can increase the proportion of Tregs among lamina propria lymphocytes (LPLs) and induce an immunosuppressive microenvironment via inhibition of the expression of TNF-α, IFN-γ, and IL-17 in the lamina propria (114). Similarly, IL-35 also takes part in MSCs inducing Bregs. Kyung-Ah Cho et al. have proven that MSCs are capable of ameliorating B-cell activation induced by hormonal stimulation, and directly inducing the population of immunosuppressive IL-10-secreting Breg cells in an IL-35-dependent manner without acting on T cells; both these MSCs-mediated effects require MSCs-derived EBI3, a critical subunit of IL-35 (81).

### SDF-1α-CXCR7

Stromal cell-derived factor-1α (SDF-1α, also known as CXCL12) is a crucial process involved in the chemotaxis of stem cells/progenitor cells (118). Previous studies have reported that the migration and survival of MSCs have been enhanced via up-regulation of SDF-1 receptors, CXCR4 and CXCR7, under hypoxic preconditioning stimulation, which likely contribute to improving the therapeutic effect in renal ischemia/reperfusion (I/R) injury in animal model (119). According to Marie-Luise Humpert et al. research, CXCR7, is an atypical chemokine receptor, binds CXCL12 and CXCL11 to regulate CXCR4/SDF-1-mediated the migration of plasmablasts during B-cell maturation (120). Moreover, Yan Qin et al. demonstrated that low concentration of SDF-1 promoted MSCs to induce IL-10-producing Bregs while high concentrated inhibited MSCs induction of $IL\text{-}10^{+}$Breg cells, but overexpressed CXCR7 of MSCs can reverse this inhibitory effect. The result supported that SDF-1α-CXCR7 axis play key roles in MSCs regulating IL-10-producing Bregs, especially $CD1d^{+}CD5^{+}IL\text{-}10^{+}$Bregs, by regulating paracrine actions (121). In addition, endometrial regenerative cells (ERCs), mesenchymal-like stromal cells, have been found to induce a donor-specific allograft tolerance in mouse cardiac allograft models, which is depended on SDF-1 mediated increasing levels of regulatory immune cells including IL-10 producing $CD1d^{high}$ $CD5^{high}$ $CD83^{low}$ Bregs (122).

### B Cell-Activating Factor (BAFF)

B cell-activating factor (BAFF) is a member of the tumor necrosis factor superfamily known to play a critical role in the survival and maturation of B cells by binding to the receptors BCMA (B cell maturation antigen) and TACI (transmembrane activator and CAML interactor) (123). BAFF is also critical for naive circulating B cell and MZ B cell homeostasis. BAFF is expressed in a wide variety of cell types, including macrophages, dendritic cells and neutrophils, and even functions in an autocrine manner (124).

Using BAFF-transgenic (Tg) mice, BAFF has been demonstrated to induce $CD4^+Foxp3^+$ Treg cells to suppress T-cell responses (125), suggesting a regulatory role of BAFF *in vivo*. Followed, low dosages of BAFF was found to possess the ability to induce IL-10 producing Bregs with the phenotype of $CD1d^{hi}CD5^+$, moreover, the number of IL-10-producing B cells in the marginal zone regions were increased when treated with BAFF *in vivo* (126). Interestingly, MSCs were reported to express BAFF both in mRNA and protein (127), indicating that MSCs might have the ability to induce Bregs via secreting BAFF. In clinical studies, MSCs are shown to decrease the plasma levels of BAFF in patients with cGVHD or refractory rheumatoid arthritis (RA), accompanied with regulating the activity of B cells and alteration in B cell subpopulation (128, 129). However, more experiments still need to confirm the BAFF-mediated effects on MSCs inducing Bregs, and reveal the underling mechanisms.

## MSC-EVs

An increasing number of studies have shown that MSCs perform many paracrine functions by releasing extracellular vesicles (EVs). In particular, small EVs (50–200 nm in diameter) (130) can be obtained from cell culture supernatants of MSCs cultured under different culture conditions and have been reported to possess therapeutic effects in different preclinical models. MSC-derived exosomes function through horizontal transfer of proteins, mRNA, and regulatory microRNAs (131). MSC-EVs have become promising therapeutic agents (132). Drirh Khare et al. identified 39 upregulated genes by sequencing exosomes derived from MSCs cocultured with B cells, including SerpinB2, PTGS2, CXCL8 (IL8), and MZB1 (marginal zone B and B1 cell specific protein) (133–136). These genes are involved in a variety of classic immunosuppressive effects, including inhibition of T cell activation, B cell proliferation, and BCR-mediated $Ca^{2+}$ mobilization, proving that mesenchymal stromal cell exosomes affect the expression and function of B lymphocytes (137). Recently, L Guo et al. reported that MSC-EVs prevent fibrosis of skin in sclerodermatous cGVHD mouse model via blocking the TFH/GC B cells interaction and reduce the ratio of BAFF to B cells *in vivo* (138). MSC-derived soluble protein-enriched fractions (MSC-PFs) have effects comparable to those of MSCs and can promote B cells to produce IL-10. MSC-EVs induce $CD24^{high}CD38^{high}$ B cells to the same extent as MSCs while the resulting cells do not produce IL-10 (77). MiR-155, a microRNA that significant increase in MSCs prime with IFN-γ and TNF-α (139), promotes IL-10 production in $CD24^{hi}CD27^+$ Bregs directly by inhibiting the expression of Jarid2, resulting in reduction of H3K27me3 binding to the IL10 promoter (139). In addition, MSCs-EVs are found to regulate the PI3K-AKT signaling pathway in B cells (140), combined with PI3K-Akt pathway in B cells is critical for Breg cell development, it is conceivable that MSCs might regulate the Bregs via their EVs to modulate the PI3K-AKT signaling pathway in B cells. Of course, there are still many unknowns in this field, and more research is needed to uncover the role of MSCs-EV in regulating Bregs.

Nevertheless, the potential for MSC-EVs immunomodulation remains promising, although the mechanisms of MSC-EVs in Breg induction is not yet well-understood. Moreover, MSC-EVs are traditionally derived from highly heterogeneous MSC cells. Due to the diversity of MSCs, the complexity of MSCs preparation, the lack of standardized quality assurance procedures for various methods of production and isolation of EVs, and the limited reproducibility of *in vitro* and *in vivo* functional assays. Four associations (SOCRATES, ISEV, ISCT, and ISBT) have proposed specific harmonized standards for MSC-EV preparation, which will help promote the development of clinical applications in this field (141).

# CONCLUSIONS

Investigations in the past few years have provided new insights into the functions of MSCs in immune system modulation and the potential of MSC-based cell therapies, which have been extensively assessed in clinical studies for their efficacy in degenerative, autoimmune, or inflammatory diseases. The mechanisms by which MSCs perform their therapeutic functions are multifaceted, but in general, these cells are thought to be able to balance the inflammatory and regenerative microenvironment of damaged tissue in the presence of severe inflammation. Studies on the interactions between immune systems and MSCs have shown that enhancement of the immunoregulatory activity of MSCs is essential during tissue regeneration. Over past decades, numerous studies have been conducted to clarify the immunomodulatory effects of MSCs on immune cells. Completed and ongoing clinical trials and *in vivo* studies on the therapeutic effects of MSCs against immune-mediated diseases have proven that MSCs can increase the generation of Bregs. It has been suggested that MSCs can increase the secretion of IL-10 by Bregs to treat inflammatory diseases, but research on specific mechanisms is still relatively scarce. Undeniably, the effectiveness of related B cell-based treatments greatly depends on the functions of Bregs, especially IL-10-secreting Bregs. Numerous studies on Bregs have revealed that B10 cells have powerful potential to ameliorate inflammatory disorders, exhibiting promise for use in the treatment of autoimmune diseases. On the one hand, regulatory B cells have not been clearly defined, and there is a lack of identified markers. At present, Bregs are still defined on the basis of their functions, which make breakthroughs in related research difficult. We have reviewed previous studies on effective MSC-mediated promotion of the production of $IL\text{-}10^+$ Bregs. To a certain extent, MSCs have multiplicative potential; they are able to induce Bregs and/or increase Breg production through a wide range of verified direct and indirect mechanisms. In the future, further studies are needed to discover reliable markers for defining different subpopulations of Bregs, clarify the heterogeneity among different subpopulations of Bregs used in specific treatments and clarify the potential mechanisms by which MSCs regulate Bregs. In clinical applications of MSCs combined with Bregs for the treatment of immune diseases, the stability and flexibility of the treatments should be closely considered and optimized to achieve appropriate modulation of inflammatory responses at different stages of disease progression.

## AUTHOR CONTRIBUTIONS

JL and XC searched the literature and wrote the manuscript. XC and QL critically revised the manuscript and final approval of the work. All authors contributed to the article and approved the submitted version.

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# Helper Innate Lymphoid Cells in Allogenic Hematopoietic Stem Cell Transplantation and Graft Versus Host Disease

*Linda Quatrini*[1], *Nicola Tumino*[1], *Francesca Moretta*[2], *Francesca Besi*[1], *Paola Vacca*[1] *and Lorenzo Moretta*[1]*

[1] Department of Immunology, IRCCS Bambino Gesù Children's Hospital, Rome, Italy, [2] Department of Laboratory Medicine, IRCCS Sacro Cuore Don Calabria Hospital, Negrar, Italy

Helper Innate Lymphoid Cells (hILCs), including ILC1s, ILC2s, and ILC3s, are mainly localized at the mucosal barriers where they play an important role in tissue regeneration and homeostasis through the secretion of specific sets of cytokines. The recent identification of a circulating ILC precursor able to generate all ILC mature subsets in physiological conditions, suggests that "ILC-poiesis" may be important in the context of hematopoietic stem cell transplantation (HSCT). Indeed, in HSCT the conditioning regimen (chemotherapy and radiotherapy) and Graft *vs* Host Disease (GvHD) may cause severe damages to mucosal tissues. Therefore, it is conceivable that rapid reconstitution of the hILC compartment may be beneficial in HSCT, by promoting mucosal tissue repair/regeneration and providing protection from opportunistic infections. In this review, we will summarize the evidence for a role of hILCs in allogenic HSCT for the treatment of hematological malignancies in all its steps, from the preparative regimen to the immune reconstitution in the recipient. The protective properties of hILCs at the mucosal barrier interfaces make them an attractive target to exploit in future cellular therapies aimed at improving allogenic HSCT outcome.

**Keywords: innate lymphoid cells, hematopoietic stem cell transplantation, graft *vs* host disease, hematological malignancies, innate lymphoid cell development**

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*Yongxia Wu,*
*Medical University of South Carolina, United States*

****Correspondence:***
*Lorenzo Moretta*
*lorenzo.moretta@opbg.net*

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## HELPER INNATE LYMPHOID CELLS

Innate lymphoid cells (ILCs) comprise five subsets—Natural Killer (NK) cells, ILC1s, ILC2s, ILC3s, and lymphoid tissue inducer (LTi) cells—that represent the innate counterparts of T lymphocytes, as they lack the expression of rearranged antigen-specific receptors (1). In particular, NK cells mirror the functions of $CD8^+$ cytotoxic T cells, and the other subsets (ILC1s, ILC2s, and ILC3s, collectively referred to as "helper ILCs") mirror $CD4^+$ T helper (Th)1, Th2, and Th17 cells, respectively, in terms of function (1). While NK cells are mainly circulating in the peripheral blood (PB), helper ILCs (hILCs) are mainly resident at mucosal barrier interfaces, where they play a pivotal homeostatic and protective role. They are activated by inflammatory cytokines and, because they are localized in the lungs, skin, and intestine, their function has mainly been studied in the

context of bacteria, parasite, and virus infections at these mucosal sites. ILC1s have several features in common with NK cells: they both produce IFN-γ as their principal cytokine output and require the transcription factor T-bet for this function. In addition, NK cells require Eomes, whereas ILC1s can develop in the absence of this transcription factor, which is, therefore, often used as a marker to distinguish ILC1s from NK cells. In addition, ILC1s are identified by the expression of the surface marker CD127 (shared by all hILC subsets) and the lack of expression of CD117 and CRTH2 (**Figure 1**). ILC1s are generally non-cytotoxic and act as a first line of defense against viruses like murine cytomegalovirus (MCMV) (2), enteric bacteria such as *C. difficile* (3), and parasites like *T. gondii* (4). ILC2s are defined by the expression of higher amounts of the transcription factor GATA3 compared to the other subsets, by the surface expression of CD127 and CRTH2, and by their capacity to produce the type 2 cytokines IL-4, IL-5, and IL-13 in response to IL-25, TSLP, and IL-33 (5, 6) (**Figure 1**). ILC2s are mainly involved in the innate immune response to parasites in the lung and intestine. After resolving the infection, ILC2s contribute to tissue repair by producing amphiregulin (7, 8). ILC3s are abundant at gastro-intestinal (GI) mucosal sites and are involved in the innate immune response to extracellular bacteria and the containment of intestinal commensals (9, 10). ILC3s express CD127 and

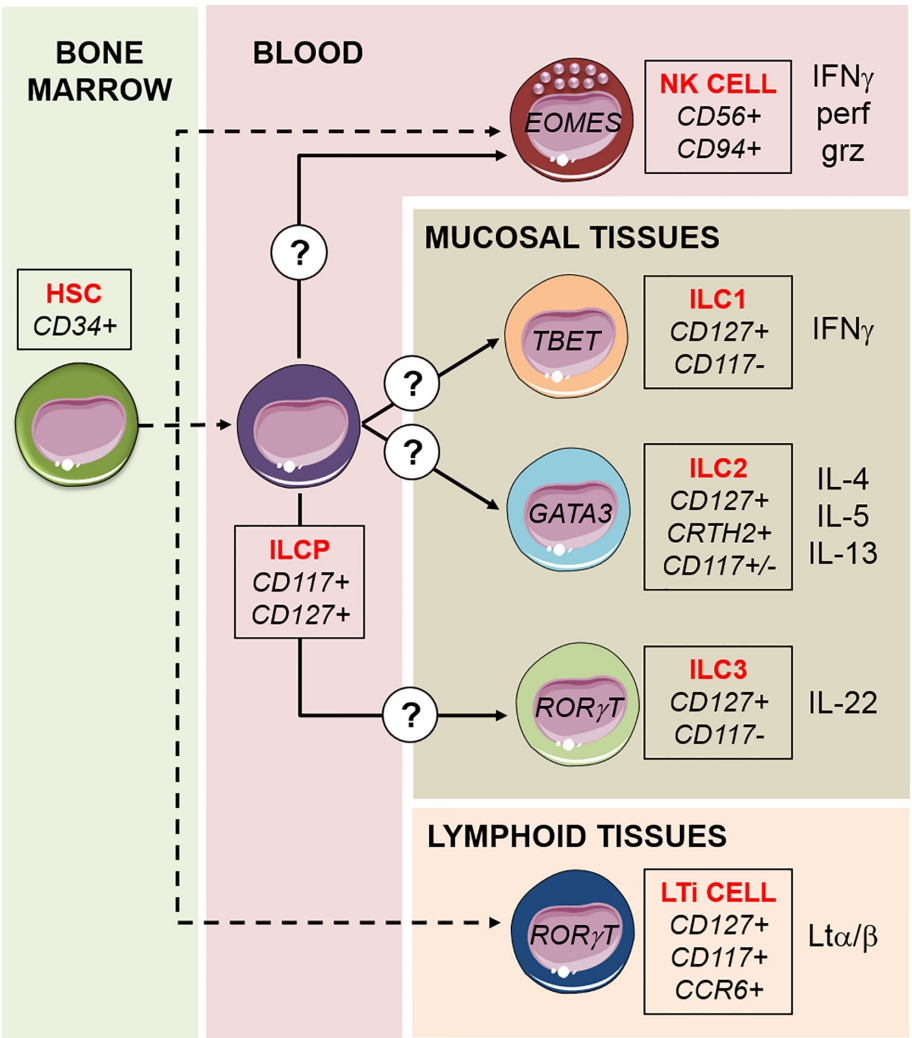


**FIGURE 1** | Schematic model of ILCs development. ILCs differentiation proceeds by steps and begins with the hematopoietic stem cell (HSC) in the bone marrow, via intermediate lymphoid restricted precursors (dashed lines). A common ILC precursor (ILCP) circulating in the peripheral blood harbors multipotent capacity to generate NK cells, ILC1s, ILC2, and ILC3s, in response to not completely understood signals. LTi cells are a fifth subset of ILCs, they have a different developmental path compared to the other ILCs and have a crucial role during embryonic development for the formation of lymphoid structures. For each subset, transcription factors, surface markers, and cytokines released are indicated. NK cells are the only "cytotoxic" ILCs, capable of killing target cells through the exocytosis of lytic granules containing perforin (perf) and granzyme (grz).

CD117, produce IL-22 as the predominant homeostatic cytokine, and they are strictly dependent on the transcription factor RORγt (11) (**Figure 1**). ILC3s can be further divided in two subsets on the basis of the cell surface expression of the Natural Cytotoxicity Receptor (NCR), NKp46 in mice and NKp44 in humans (1). Like ILC3s, also a fifth ILC subset, namely LTi cells, is dependent on RORγt and was initially considered to belong to the "group 3" of ILCs. However, LTi cells have a different developmental path compared to other ILCs, and have a crucial role during embryonic development for the formation of secondary lymph nodes and Peyer's patches, through the action of lymphotoxin (12) (**Figure 1**). LTi cells express CD117 and CCR6, but not NCRs, and are difficult to separate on the basis of marker expression from the postnatal $NCR^-$ ILC3s. Indeed, postnatal $NCR^-$ ILC3s residing in lymphoid tissues can mediate the formation of tertiary lymphoid structures, and for this reason are sometimes referred to as "LTi-like" ILC3s (13).

A peculiar property of hILCs is that they display a high degree of plasticity, not only during development but also in their mature compartments (1). The plasticity from one subset to the other requires polarizing signals in the tissue in which conversion occurs, together with the expression of cognate cytokine receptors and key transcription factors. For example, IL-22-producing ILC3s conversion into IFN-γ-producing ILC1-like has been documented *in vivo* in mice gut and in the human setting, *in vitro* (14–16). This conversion requires RORγt downregulation, and T-bet and Notch signaling upregulation (14, 17, 18). Also ILC2s can convert into IFN-γ-producing ILC1s both *in vitro* and *in vivo*, upon induction of T-bet and the IL-12 receptor (19, 20). This well-documented hILC subsets plasticity suggests that under the influence of soluble factors or cellular interactions the identity of each subset may not persist *in vivo* upon adoptive transfer, and implies that hILCs functional capability may change substantially in response to the microenvironment.

## HELPER ILCs IN HEMATOLOGICAL MALIGNANCIES

Differently from NK cells, whose anti-tumor function has been extensively studied over the last decades (21), the contribution of hILCs in the immune responses against tumors is less clear. From studies investigating hILCs role in solid cancers it seems that they are protective as they can respond rapidly to cytokine stimulation, but their response must be tightly regulated because excessive inflammation can lead to damage and favor tumorigenesis (22). Similarly, sustained secretion of cytokines that promote tissue repair (such as IL-22) can have pathological consequences during chronic activation, inducing epithelial hyperproliferation. Therefore, hILCs act as a double edged sword, and the inflammatory and anti-inflammatory reparative responses that arise during disease must be tightly balanced to prevent tumor development (23).

Much less is known regarding hILCs contribution to hematological malignancies. The data available come mainly from studies analyzing the abundance and function of hILC subsets in patients. For example, in monoclonal gammopathies of undetermined significance (MGUS, representing the earliest lesions leading to multiple myeloma, MM) an increased proportion of ILC1s in the bone marrow (BM) was observed (24). High expression of Ikzf3 in ILC1s together with the high IFN-γ production by ILC1s isolated from pomalidomide-treated patients, suggest that this is among the earliest cell subsets enriched in the tumor microenvironment during evolution of monoclonal gammopathies, and that ILC1s may be a target for immunomodulatory drugs (24). In acute myeloid leukemia (AML), an analysis of the PB from patients before treatment showed a general reduction of hILCs numbers with an increase in the frequency of ILC1s (25). Conversely, in chronic lymphocytic leukemia (CLL) PB hILCs counts are increased and, in particular, ILC1 subset shows a functional impairment, analogous to what was shown for NK cells (26, 27). Also an involvement of ILC2s in hematologic tumors has been reported. Thus, in acute promyelocytic leukemia (APL), elevated tumor-derived prostaglandin-2 (PGD2) and B7H6 induce increases and hyperactivation of ILC2s through binding to CRTH2 and NKp30, respectively. It has been reported that, by releasing IL-13, ILC2 activate myeloid-derived suppressor cells (MDSC) inducing an immunosuppressive pathway (28). Also in AML patients, ILC2s were found to expand in response to the high levels of PGD2 secreted by mesenchymal stromal cells (29). In particular, in this disease PGD2 was shown to induce ILC2s to release IL-5 that, in turn, acts on regulatory T cells stimulating the production of IL-10, promoting proliferation of normal and malignant hematopoietic stem and progenitor cells (29).

Form these evidences it can be concluded that ILC1 dysregulation, in terms of numbers and function, may be implicated in the pathogenesis and progression of diverse hematological malignancies, and that ILC2 induce a tolerogenic environment that favors tumor progression. However, it must be determined whether the phenotype and function reported for hILCs in these patients account for the disease, or they are a consequence of the malignancy itself.

Because of their role both in anti-tumor response in hematologic malignancies and maintenance of epithelial barrier integrity, hILCs represent an attractive tool to exploit in the treatment of these tumors through allogenic hematopoietic stem cell transplantation (allo-HSCT). Indeed, they may play a protective role in the first phase of treatment, when the preparative regimen that precedes transplantation causes mucosal damages. Helper ILCs may then represent a cellular component of the graft and contribute to the defense from infections while the donor-derived immune system develops. Finally, hILCs may be important in the protection from graft *vs* host disease (GvHD), and participate to the immune response that prevents leukemia relapse. Since hILCs have been discovered very recently, the actual contribution of hILCs in these steps has not been fully elucidated. We will summarize in the following paragraphs the evidence that came out of this recent field of investigation, and highlight some interesting aspects that suggest possible advantages of exploiting hILCs in HSCT.

## ALLOGENIC HSCT TO TREAT HEMATOLOGICAL MALIGNANCIES

In allo-HSCT, donor-derived HSCs engraft the BM of the recipient and differentiate into mature immune cells, thus reconstituting the recipient lympho/hemopoiesis compromised by either disease or myeloablative therapy. Allo-HSCT is used primarily for hematologic and lymphoid tumors. In adults, the majority of allo-HSCTs are performed for the treatment of acute leukemias, in particular AML. Other major indications include myelodysplastic syndrome or lymphoma (predominantly non-Hodgkin) and, to a lesser extent, MM, chronic myeloid leukemia (CML), and CLL (30). In pediatric patients, allo-HSCT is used also in non-malignant conditions, such as many genetic diseases, including severe combined immunodeficiency (SCID), the Wiskott–Aldrich syndrome, sickle cell anemia, and thalassemia (31).

Prior to transplantation, patients receive conditioning chemotherapy and radiotherapy in order to kill malignant cells and to deplete non-malignant recipient immune cells to avoid rejection (31). Although improving engraftment, preparative regimens cause damages to the mucosae, which favor infections in the mouth, gut, and skin. These damages accentuate and possibly stimulate the occurrence of one of the main complications of allo-HSCT, that is, GvHD (32). In GvHD, the injury is primarily confined to the GI tract, where high dose preparative regimens compromise the barrier function and induce the release of microbial and necrotic-cell elements into adjacent tissues and in the bloodstream (30, 33) (**Figure 2**). A critical role is played by the inflammatory cytokines, such as TNF-α, IL-1, IL-12, and IL-6, that recruit and activate innate immune cells, responsible for antigen presentation and subsequent allo-antigenic response by donor T cells (32). (See also paragraph "*Helper ILCs in graft vs host disease*").

The predominant source of HSCs for hematologic transplantation is represented by granulocyte colony stimulating factor (G-CSF)-mobilized PB stem cells. In donors who fail to mobilize, G-CSF is administered in combination with plerixafor, a CXCR4 antagonist, inducing an increase of $CD34^+$ HSCs in the PB (34). Of note, the *in vitro* development of NK cells and hILCs is influenced by the source of $CD34^+$ HSCs and G-CSF treatment (35). Although a major advantage of allogenic grafts is represented by histocompatibility-related immune reactions against tumor cells (Graft *versus* tumor effect), by recognizing recipient antigens, donor T cells also cause GvHD, as mentioned above. Another significant challenge in providing access to HSCT is represented by the availability of a suitable HLA-matched donor. HSCT from a haploidentical donor (haplo-HSCT) offers the option of immediate transplantation virtually to any patients in need of an allograft and lacking suitable HLA-matched donor. To prevent both GvHD and graft failure (36), positive selection of $CD34^+$ HSCs and administration of $CD34^+$ "megadoses" has been employed for many years (37). However, removal of lymphoid cells and committed hematopoietic progenitors from the graft entails prolonged lymphopenia and delayed immune reconstitution, resulting in an increased risk of non-relapse mortality, mainly from opportunistic infections. A promising approach to

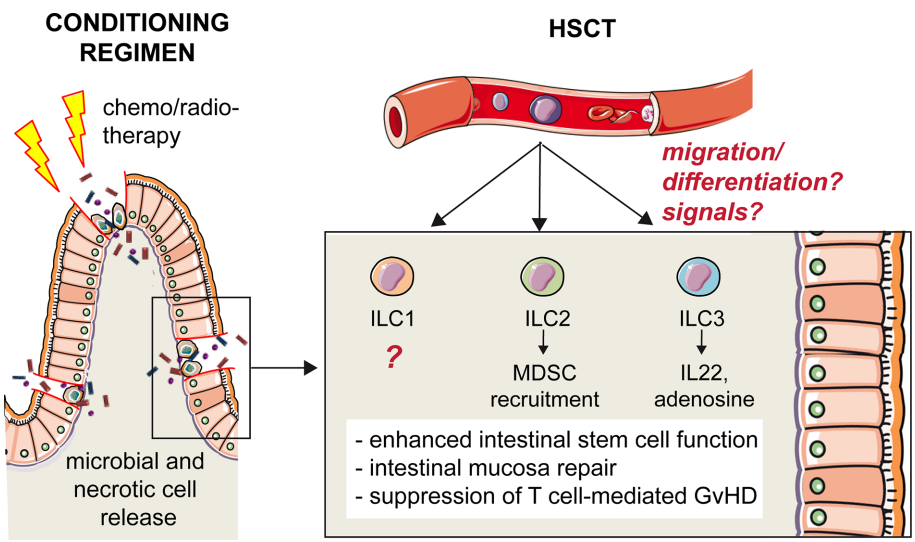


**FIGURE 2** | Protective role of hILCs from intestinal GvHD. High-dose preparative regimens that precede HSCT compromise the barrier function of intestinal epithelium and induce the release of microbial and necrotic-cell elements into adjacent tissues and in the bloodstream. The disruption of the intestinal mucosal barrier increases the incidence and severity of GvHD after HSCT. Helper ILCs play a protective role, although it is still not clear what are the signals that trigger these cells and what is their origin. One hypothesis is that a common ILC precursor is recruited from the peripheral blood to the inflamed tissue, and that differentiation occurs locally in response to environmental signals. The role of ILC1s in intestinal GvHD is unknown. ILC2s recruit MDSC that, in turn, suppress T cell-mediated GvHD. ILC3s secrete IL-22 enhancing intestinal stem cell function and promoting repair, and release adenosine, suppressing T cell proliferation.

circumvent this delay in immune recovery is represented by a method of graft manipulation based on selective depletion of αβ T lymphocytes, responsible for GvHD, and of B cells, from which post-transplant lymphoproliferative disease can arise (38–41). Through this approach it is possible to transfer to the recipient not only donor HSCs but also committed precursors as well as mature NK and γδ T cells, both capable of exerting a protective effect against tumor cell relapse and life threatening infections (42). The role of NK cells in this setting of transplantation has been extensively studied, since it involves an alloreactive mechanism dependent on NK cell expression of inhibitory receptors that interact with class I HLA epitopes (43, 44). The balance between the inhibitory signals from self epitopes and activating signals from ligands expressed by tumor cells improves the chances of engraftment and reduces the risk of GvHD (45). Donor NK alloreactivity can be predicted by analyzing donor KIR phenotype and genotype and HLA class I typing in both donor and recipient, and correlates with a better clinical outcome in both adult AML patients and pediatric ALL and AML patients (42, 46–48). On the other hand, hILCs lack either the expression of KIRs and cytotoxic activity and are therefore unable to exert a direct anti-leukemia effect. Nevertheless, they may indirectly influence the anti-leukemia activity of the other immune cells present in the graft by releasing cytokines. For example, ILC2s may activate a tolerogenic pathway and favor MDSC-dependent suppression of NK function (49), analogously to what was shown in APL (28). Moreover, like NK cells, through cytokine secretion, hILCs may play an important role in the protection against infections during the early time window following HSCT, when the donor-derived immune system is not yet reconstituted.

Although the number of circulating hILC is very low compared, for example, to NK cells, the presence and the function of mature circulating donor-derived hILCs in the graft in HSCT has not been investigated to date. It would be interesting to evaluate in retrospective studies whether there is a correlation between the abundance of infused hILCs in the graft and the clinical outcome of the patient in terms of both relapse and incidence of infections.

More information is available regarding the reconstitution of hILC compartment following HSCT and the role of these cells in GvHD, which are the topics of the following paragraphs.

## RECONSTITUTION OF THE HELPER ILC COMPARTMENT

Given the recent discovery of ILCs, our knowledge on their development derives from studies done in the last 10 years. For NK cells, it is well established that development occurs through discrete steps, from stage 1 $CD34^+$ NK cell progenitors to stage 4 $CD56^{bright}$ NK cells (50). By *in vitro* experiments using $CD34^+$ HSC, it was shown that $CD117^{high}CD56^+CD94^-$ stage 3 NK progenitor cells (51) were capable of generating both $CD94^+CD56^+LFA1^+$ NK cells and $CD56^+CD117^{high}LFA1^-$ ILCs, producing IL-8 and IL-22 (52, 53). IL22-producing ILCs were thus recognized as a separate cell lineage but developmentally related to NK cells. The "stage 3" common progenitor cell population was found to depend on the expression of the transcription factor RORγt (54, 55). Subsequently, it was demonstrated that RORγt expression broadly identifies a $CD34^+CD45RA^+CD117^+IL\text{-}1R1^+$ progenitor population exclusively found in secondary lymphoid tissues (SLTs: tonsils, lymph nodes, spleen) capable of generating all human ILC subsets, including NK cells (56). More recently, a human ILC precursor circulating in the PB has been identified, which displays properties in common with the multipotent ILC precursor (ILCP) previously found in SLTs. These circulating ILCPs characterized by a $CD127^+CD117^+$ phenotype were previously proposed to represent PB ILC3s (57), but it is now recognized that this cell population is enriched in multipotent ILCPs that can give rise to all hILC subsets as well as NK cells (58). It was shown that the expression of CD56 by this progenitor marks the divergence of a shared NK/ILC3 common developmental pathway from ILC2s (59). In addition, NKp46 was identified as a marker that clearly defines the ILC3-potential, while KLRG1 expression indicates a bias towards ILC2 (60). It was demonstrated *in vivo* that this PB ILCP originates from $CD34^+$ HSCP (58), but it remains to be studied what are the intermediate steps in this differentiation trajectory (**Figure 1**). A model of differentiation of ILCP towards ILCs is currently proposed and is defined "ILC-poiesis" (61, 62). According to this model, the presence of circulating $CD117^+$ ILCPs that eventually develop into mature ILCs ensures a rapid and localized generation of mature ILCs in the tissues in response to environmental signals. The precise mechanisms remain to be fully clarified, however it is clear that important factors in this process are cytokines that drive the trajectories of differentiation (such as IL-1β, IL-23, IL-12, IL-23, IL-25, IL-33, TSLP), and that maintain activated or dividing cells (IL-2, IL-15, IL-7) (63). Any local inflammation associated to infection or tumor transformation would trigger the cellular sources of these cytokines that is, stromal cells, epithelial cells, and other innate immune cells. How this localized ILC differentiation occurs in physiological condition, and how reconstitution of tissue resident ILC compartment occurs in different tissue environments is unclear, but it is likely to recapitulate what happens in infection and inflammation.

Very few studies investigated ILC reconstitution after HSCT. Vely et al. in 2016 studied a cohort of adult patients with SCID who underwent HSCT (64). They found that SCID patients were ILC deficient, and continued to display ILC deficiency after HSCT in the absence of a conditioning regimen to induce myeloablation, possibly because of competition with endogenous progenitors in the appropriate niches. Interestingly, the complete lack of ILCs was not associated to higher susceptibility to diseases, suggesting that, in the conditions of modern medical care and hygiene and in the presence of a functional adaptive immune system, ILCs may be redundant (64). Upon myeloablation, circulating and tissue resident ILCs of donor origin were detected. NK cell differentiation from HSCs requires 2–3 weeks to reach the maturation stage of $NKG2A^+KIR^-$ cells, and the first appearance of $KIR^+$, cytolytic, and potentially alloreactive NK

cells requires 4–6 additional weeks (45). Conversely, ILC reconstitution is much slower and is incomplete 6 months after allo-HSCT (65). Vely et al. propose that, analogously to tissue-resident macrophages that originate both from yolk sack progenitors and BM HSCs (66), ILCs may originate from a dual pathway: precursors that seed in tissues (probably SLT) during embryonic life, responsible for self-renewal, and BM after birth (64). It would be interesting to verify this hypothesis, to promote hILC reconstitution in tissues from BM precursors and improve protection from GvHD in HSCT.

## HELPER ILCs IN GRAFT *VS* HOST DISEASE

Because of the clinical manifestations of GvHD at the mucosal barriers and in particular in the gut, a role for ILC3s in GvHD immunity was hypothesized. Indeed, the critical involvement of hILCs was evidenced for the first time in a mouse model of acute intestinal GvHD (67). In this allo-HSCT model IL-22 producing ILC3 enhanced intestinal stem cell functions, and IL-22 deficiency resulted in increased incidence and severity of GvHD with excessive epithelial cell apoptosis and disrupted intestinal mucosal barrier (67). The main source of IL-22 were NKp46$^-$ ILC3s that, importantly, were of recipient origin, as they persisted following lethal conditioning radiotherapy, BM transplantation and even after T cell reconstitution in the *lamina propria* (67). IL-22-mediated epithelium protection by ILC3s is important also in the thymus upon transplantation and GvHD. Indeed, thymus is extremely sensitive to alloreactive damage, mediated by donor-derived T cells expressing IL-21 receptor (68, 69). ILC3s in the thymus are depleted upon GvHD, and it was shown that, preventing ILC3 loss, thymic regeneration and T cell reconstitution are enhanced (69). Thus, through IL-22 production, ILC3s not only favor epithelial regeneration protecting the recipient from GvHD, but also contribute to the restoration of adaptive immunity, which is a critical determinant of successful outcomes in allogenic HSCT.

Apart from maintaining and repairing epithelial barrier integrity through IL-22 secretion, ILC3s have additional modes of action to protect against GvHD. This is suggested by the identification of a novel subset of human ILC3 in the oral-GI tract and in the BM, co-expressing the ectoenzymes CD39 and CD73 (ecto ILC3s) (70). These cells are immunosuppressive because they release adenosine, suppress T cell proliferation and are depleted in patients with GvHD (70).

Different from ILC3s, it was shown that ILC2s in the GI tract but not in the lungs are highly sensitive to conditioning therapy prior to allo-HSCT in a murine model, and their reconstitution from donor BM is quite limited (71). In addition, in this model, co-transfusion of IL-33- activated ILC2s and T cells led to the prevention of GvHD, through the recruitment of MDSC in the GI tract (71). Of note, intravenously infused donor-derived ILC2 could migrate to the GI tract and reduce GvHD without affecting the beneficial T cell-dependent Graft *vs* Leukemia (71). Although it did not directly concern the transplantation context, another study demonstrated that ILC2s promote the renewal of intestinal stem cells through IL-13 secretion, activating the β-catenin pathway (72). This suggests that, analogously to ILC3s, also ILC2s may contribute to epithelial regeneration in the gut and GvHD prevention.

In humans, it was shown that ILCs are depleted from the blood of adult patients suffering from ALL and AML who undergo conditioning therapy before allo-HSCT (65). Patients with a relatively rapid recovery of ILC numbers after induction chemotherapy, before allo-HSCT, experienced less mucositis and less acute GvHD after allo-HSCT, as compared to patients with slower ILC reconstitution dynamics (65). Importantly, lower GvHD incidence was associated to higher proportions of activated CD69$^+$ ILCs, expressing tissue homing markers for gut (α4β7, CCR6) and skin (CCR10 and CLA) (65). Notably, 12 weeks after HSCT, the donor-derived circulating NCR$^+$ ILC3 count was higher in patients who did not develop GvHD. These cells may actually represent the CD117$^+$ ILCP identified later by Di Santo group (58), suggesting that an expansion of the ILC precursors can eventually be protective from GvHD, thanks to the ability of these cells to migrate to the damaged mucosa in response to inflammatory cytokines and give rise to the specialized ILC subsets. Further studies are needed to understand if this is the case, and to precisely describe the steps in the generation of tissue-resident ILC subsets after HSCT.

## FUTURE DIRECTIONS

While the role of hILCs in the immune response against hematologic malignancies is still controversial and seems to be dependent on the subset and the type of tumor, it is clear that hILCs are relevant in the protection from GvHD. The newly identified circulating ILCPs may represent an attractive cellular target to exploit to further improve the HSCT clinical outcome, thanks to its ability to provide a rapid substrate for the generation of all ILC subsets in response to specific sets of cytokines. For example, ILCPs generated *in vitro* from HSCs present in the graft might be adoptively transferred to patients receiving HSCT and suffering from GvHD with mucosal tissue lesions to facilitate epithelial regeneration. It would be interesting to verify whether tissue resident hILC reconstitution occurs from circulating ILCPs and study it in parallel with reconstitution in the PB. Indeed, although it has been shown that ILC3s in the thymus and GI tract are resistant to radiation injury (67, 68), it remains to be understood to what extent myeloablative regimens and mucosal damage can lead to tissue-resident hILCs depletion in humans.

Besides increasing the risk of GvHD, mucosal damage induced by conditioning regimens may increase the risk of infections, which occur frequently within the first 3 months after transplantation (73). Therefore, the preservation of hILCs (including ILCPs) in the manipulation of the graft can be envisaged as an efficient strategy to protect the recipient from infections, analogously to what has been done for NK cells and γδ T cells. Moreover, while to date there are no studies comparing

the effect of T cell depletion on hILC reconstitution, a positive role for hILCs has been shown in the recovery of the adaptive T cell compartment, through protection of thymic epithelium (69). This protective role may ideally be extended to secondary lymphoid tissues (such as lymph nodes) that are damaged by chemotherapy and irradiation used before allo-HSCT. LTi-like ILC3s identified in these adult lymphoid tissues may contribute to their repair, thus indirectly enhancing the recovery of efficient antigen-specific immune response and reducing the risks of opportunistic infection and relapse. Although it is very difficult to study these LTi-like ILC3s in humans, recent findings in mice showed that embryonic LTi cells are replaced in adult lymphoid tissues by HSC-derived cells (74). Since LTi cells in mice have a specific role in the restoration of spleen integrity after infection (75), it is possible that LTi-like ILC3s deriving from HSCs may contribute to lymphoid tissue regeneration upon transplantation.

In conclusion, the ability of hILCs to rapidly secrete an array of different cytokines in a subset-dependent manner makes them a promising tool to exploit to improve allogenic HSCT outcome, by protecting the recipient from GvHD and infections, and enhancing adaptive immune response reconstitution. The strategies aimed at exploiting the properties of these cells may be: (i) to preserve hILCs present in the graft and infuse them in the recipient; (ii) to generate and expand *in vitro* ILCPs from HSCs in the graft in order to adoptively transfer them in recipient; (iii) to accelerate in the recipient the differentiation of hILC subsets potentially useful for the treatment of severe mucosal damages. To do so, further studies on hILCs differentiation are clearly needed, especially to understand how the signals from the tissue microenvironment "tune" the generation of the appropriate ILC subset from a common HSC-derived precursor.

## AUTHOR CONTRIBUTIONS

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

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# ERBB2-CAR-Engineered Cytokine-Induced Killer Cells Exhibit Both CAR-Mediated and Innate Immunity Against High-Risk Rhabdomyosarcoma

*Michael Merker[1,2,3], Juliane Wagner[4], Hermann Kreyenberg[1], Catrin Heim[1], Laura M. Moser[1], Winfried S. Wels[2,3,5], Halvard Bonig[6,7], Zoltán Ivics[4], Evelyn Ullrich[1,2,8], Thomas Klingebiel[1,2,3], Peter Bader[1,2,3] and Eva Rettinger[1,2,3]**

[1] Division for Stem Cell Transplantation, Immunology, and Intensive Care Medicine, Department for Children and Adolescents, University Hospital Frankfurt, Goethe University Frankfurt, Frankfurt, Germany, [2] German Cancer Consortium (DKTK), Partner Site Frankfurt/Mainz, Frankfurt, Germany, [3] Frankfurt Cancer Institute, Goethe University, Frankfurt, Germany, [4] Division of Medical Biotechnology, Paul-Ehrlich-Institute, Federal Institute for Vaccines and Biomedicines, Langen, Germany, [5] Georg-Speyer-Haus, Institute for Tumor Biology and Experimental Therapy, Frankfurt, Germany, [6] Department of Cellular Therapeutics/Cell Processing (Good Manufacturing Practice, GMP), Institute for Transfusion Medicine and Immunotherapy, Goethe University, Frankfurt, Germany, [7] Division of Hematology, Department of Medicine, University of Washington, Seattle, WA, United States, [8] Experimental Immunology, Department for Children and Adolescents, University Hospital Frankfurt, Goethe University, Frankfurt, Germany

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Weidong Han, People's Liberation Army General Hospital, China

***Correspondence:**
Eva Rettinger
eva.rettinger@kgu.de

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High-risk rhabdomyosarcoma (RMS) occurring in childhood to young adulthood is associated with a poor prognosis; especially children above the age of 10 with advanced stage alveolar RMS still succumb to the disease within a median of 2 years. The advent of chimeric antigen receptor (CAR)-engineered T cells marked significant progress in the treatment of refractory B cell malignancies, but experience for solid tumors has proven challenging. We speculate that this is at least in part due to the poor quality of the patient's own T cells and therefore propose using CAR-modified cytokine-induced killer (CIK) cells as effector cells. CIK cells are a heterogeneous population of polyclonal T cells that acquire phenotypic and cytotoxic properties of natural killer (NK) cells through the cultivation process, becoming so-called T-NK cells. CIK cells can be genetically modified to express CARs. They are minimally alloreactive and can therefore be acquired from haploidentical first-degree relatives. Here, we explored the potential of ERBB2-CAR-modified random-donor CIK cells as a treatment for RMS in xenotolerant mice bearing disseminated high-risk RMS tumors. In otherwise untreated mice, RMS tumors engrafted 13–35 days after intravenous tumor cell injection, as shown by *in vivo* bioluminescence imaging, immunohistochemistry, and polymerase chain reaction for human gDNA, and mice died shortly thereafter (median/range: 62/56–66 days, $n = 5$). Wild-type (WT) CIK cells given at an early stage delayed and eliminated RMS engraftment in 4 of 6 (67%) mice, while ERBB2-CAR CIK cells inhibited initial tumor load in 8 of 8 (100%) mice. WT CIK cells were detectable but not as active as CAR CIK cells at distant tumor sites. CIK cell therapies during advanced RMS delayed but did not inhibit tumor

progression compared to untreated controls. ERBB2-CAR CIK cell therapy also supported innate immunity as evidenced by selective accumulation of NK and T-NK cell subpopulations in disseminated RMS tumors, which was not observed for WT CIK cells. Our data underscore the power of heterogenous immune cell populations (T, NK, and T-NK cells) to control solid tumors, which can be further enhanced with CARs, suggesting ERBB2-CAR CIK cells as a potential treatment for high-risk RMS.

**Keywords: cellular therapy, cytokine-induced killer cells, chimeric antigen receptor, rhabdomyosarcoma, ERBB2 (HER2/neu)**

## INTRODUCTION

The immune system recognizes and destroys tumor cells through a process known as immunosurveillance. However, especially in advanced disease, tumors escape immunosurveillance by cancer immunoediting and an immunosuppressive tumor microenvironment (TME). Despite improvements in surgical and radiotherapy techniques, new chemotherapy regimens, and the use of allogeneic stem cell transplantation, children, and young adults with metastatic alveolar rhabdomyosarcoma (RMS)—except those younger than 10 years of age—still succumb to their disease within a median of 2 years (1–8). Thus, all treatment advances made over the last three decades have not translated into improved outcomes in high-risk RMS patients.

In recent years, targeted immunotherapies have emerged as a therapeutic strategy that interferes with cancer cell growth and spread or triggers antitumor immunity. By directly transferring cell products with specific antitumor properties, innate and adoptive immune responses against tumors and tumor-associated antigens (TAAs) can be triggered or enhanced. In this context, adoptive cell therapy (ACT) with chimeric antigen receptor (CAR)-modified patient immune cells is attracting growing interest.

In this fast-moving field, a growing number of CAR-engineered cell products have emerged, although most involve autologous T cells targeting hematopoietic malignancies. Only a few approaches are used in targeting solid cancer (9–18). As surface expression of ERBB2 is detectable in a substantial subset of alveolar RMS and other tumor entities (19, 20), the use of CAR T cells targeting ERBB2 was developed in the context of ACT for soft tissue sarcoma (STS). This treatment was found to be safe in a phase I/II clinical trial, but induction of long-lasting immune responses was only possible in a minority of patients (9, 21, 22). We expect that this is at least in part due to the poor quality of autologous T cells after chemotherapy pretreatment and therefore propose using CAR-modified cytokine-induced killer (CIK) cells derived from healthy donors or patients' own apheresis prior to chemotherapy treatment.

CIK cells, which are generated from peripheral blood mononuclear cells (PBMC) in the presence of defined cytokines *in vitro*, are a heterogeneous cell population characterized by $CD3^+$ T cells with a $CD56^+$ natural killer (NK) cell phenotype, a high proliferative rate *in vitro*, and strong lytic activity against a broad spectrum of cancers (23, 24). CIK cell cytotoxicity is mostly attributed to the $CD3^+CD56^+$ T-NK cell fraction. T-NK cells are terminally differentiated non-proliferating cells derived from proliferating progenitor T cells in *in vitro* cultures. Pievani et al. reported that T-NK cells have a dual functional capability by preserving T cell receptor (TCR)-mediated specific cytotoxicity and acquiring non-major histocompatibility complex (MHC) restricted, inherently broader NK cell function (25). The NK cell-like cytotoxic capacity of CIK cells mediated *via* several receptors, such as NKp30, DNAM-1, and LFA-1, has mainly been ascribed to NKG2D, an activating NK cell receptor. The first reports by Schmidt-Wolf et al. documented the efficacy and safety of CIK cell treatment in different cancers (23, 26, 27). Since then, a wide variety of phase I/II clinical trials recorded in the International Registry on CIK cells (IRCC) have shown that adjuvant CIK cell therapy with or without chemotherapy or other therapeutic regimens, may prevent disease recurrence, improve progression-free and overall survival, and enhance the quality of life of cancer patients with only minimal and manageable toxicity and side effects (28–30).

We previously showed that CIK cells, which are already capable of NK cell-like antitumor function, can be supplemented with an ERBB2-CAR construct that provided synergistic activities *in vitro* (31). The alveolar RMS cell line RH30 which was established from the bone marrow (BM) metastasis of a 17-year-old male patient was used for preclinical *in vivo* analysis. Here we present an ACT approach targeting CIK cells to ERBB2 with a second-generation CAR for the treatment of primarily disseminated high-risk alveolar RMS in a complete new xenograft model.

## MATERIALS AND METHODS

### Generation of Wild-Type (WT) CIK Cells

WT IL-15-activated CIK cells were generated from the PBMCs of healthy volunteers after written informed consent and the study was approved by the Ethics Review Board of the Medical Faculty of the University Hospital Frankfurt/Main, Germany (Geschäfts-Nr. 413/15).

CIK cells were generated from PBMCs after standard Ficoll separation as previously described (32). In brief, cells were resuspended at $3 \times 10^6$ cells/mL in RPMI 1640 medium supplemented with 10% FCS, L-glutamine, antibiotics and 1,000 U/mL IFN-γ. On day 1 of culture, 100 ng/mL anti-CD3 antibody (MACS GMP CD3 pure, Miltenyi Biotech, Bergisch Gladbach, Germany) and 500 U/mL IL-2 were added. Starting at day 3 of

culture, cells were resuspended at $1 \times 10^6$ cells/mL and expanded in the presence of 50 ng/mL IL-15 (PeproTech, Hamburg, Germany). On day 4 to day 7 of culture, WT and ERBB2-CAR-engineered CIK cells (described below), were both cultured at $\sim 5 \times 10^5$ cells/2 mL in 6-well plates. On day 7 of culture, cell products were again transferred to culture flasks, resuspended at $1 \times 10^6$ cells/mL and supplemented with 50 ng/mL IL-15 every 3 days. On day 12 of culture, cell products were harvested and used for *in vitro* and *in vivo* analysis.

## CAR Engineering Using the ERBB2-Specific Lentiviral CAR Vector pS-5.28.z-IEW

The lentiviral CAR vector pS-5.28.z-IEW, which encodes an ERBB2-specific second-generation CAR, was described previously (33). The codon-optimized CAR sequence consists of an IgG heavy-chain signal peptide, an ERBB2-specific scFv antibody fragment (FRP5), and a modified CD8α hinge region, as well as CD28 transmembrane and intracellular domains and a CD3ζ intracellular domain (CAR 5.28.z), and was inserted into a pHR'SIN-cPPT-SIEW (pSIEW) (34) lentiviral transfer plasmid upstream of the IRES and enhanced green fluorescent protein (eGFP) sequences. eGFP was used as a fluorescent marker. Vesicular stomatitis virus G (VSV-G) protein pseudotyped lentiviral vector particles were produced using the lentiviral transfer plasmid with the packaging and envelope plasmids pCMVΔR8.91 and pMD2.G as described previously (35).

Transduction of CIK cells with lentiviral vector-containing supernatant was carried out at day 4 of the expansion culture, 24 h after the first stimulation with IL-15, as described previously (31). The culture was then adjusted to $1 \times 10^6$ cells/mL every 3–4 days and supplemented with 50 ng/mL IL-15 (described above). On day 12 of culture, the cells were harvested and used for *in vitro* and *in vivo* analysis.

## Generation of a Luciferase-Expressing RH30 Cell Line

The alveolar RMS cell line RH30 was purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) and cultured according to the manufacturer's instructions. The RH30 cell line, which was established from the BM metastasis of a heavily pretreated 17-year-old male patient with refractory alveolar RMS (p53 mutation- and Pax3/FKHR fusion protein-positive), was selected in regard to clinical translatability (36–38).

To image the *in vivo* trafficking of tumor cells, GFP/luciferase-expressing RH30 cells ($RH30^{GFP/Luc}$) were generated *via* lentiviral transduction using vector particles pseudotyped with the VSV-G protein that were produced using the transfer plasmid pSEW-luc2, which encodes firefly luciferase and eGFP linked *via* a 2A peptide (39). GFP-positive cells were enriched by fluorescence-activated cell sorting (FACS) using a FACSAria II™ instrument (BD Biosciences, San Jose, CA, USA).

## Preclinical Human RMS Mouse Model

NOD/SCID/*IL-2Rγc*$^{-/-}$ (NSG) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained in the animal facilities of Georg-Speyer-Haus, Institute for Tumor Biology and Experimental Therapy, Frankfurt/Main, Germany. The described research was approved by the appropriate government committee (Regierungspräsidium Darmstadt, Germany; Gen.-Nr. TVA FK/1000) and conducted in accordance with the requirements of the German Animal Welfare Act.

To establish a completely new disseminated human RMS model in mice best mimicking the clinical situation of residual circulating tumor cells and refractory tumors following chemo- and radiotherapy, 6- to 8-week-old female NSG mice were sublethally irradiated with 250 cGy (Biobeam 2000, Eckert & Ziegler, Bebig, Germany) 24 h (day −1) prior to intravenous injection of $1 \times 10^5$ luciferase-expressing $RH30^{GFP/Luc}$ cells applied in a total volume of 100 µL per mouse *via* the tail vein (day 0) (**Figure 1A**).

As this study includes a comparisons of mice without and with WT or ERBB2-CAR CIK cell therapies which were given preemptively (day +1 and day +36) to mice at risk for tumor progression as well as to mice with established tumors (day +22 and day +57), 28 mice were randomly divided into 5 different treatment groups:

- Control: Dulbecco's phosphate-buffered saline (DPBS) on day +1, $n = 5$
- WT, preemptive: $2.5 \times 10^6$ WT CIK cells on day +1 and day +36 (5 weeks, if possible), $n = 6$
- WT, established tumor: $2.5 \times 10^6$ WT CIK cells on day +22 (3 weeks) and day +57 (8 weeks, if possible), $n = 3$
- CAR-CIK, preemptive: $2.5 \times 10^6$ ERBB2-CAR CIK cells on day +1 and day +36 (5 weeks, if possible), $n = 8$
- CAR-CIK, established tumor: $2.5 \times 10^6$ ERBB2-CAR CIK cells on day +22 (3 weeks) and day +57 (8 weeks, if possible), $n = 6$

All mice treated preemptively with WT or ERBB2-CAR CIK cells had minimal residual, but already active disease at the time of immune cell infusions, thereby considering them as having an imminent risk for disease progression with limited treatment options.

During the course of the experiment, mice were screened for symptoms of disease and adverse side effects like xenogeneic graft-vs.-host disease (xGVHD) and cytokine-release-syndrom (CRS) at least twice daily for a maximum of 100 days. Mice showing visible signs of poor health or physical abnormalities were painlessly euthanized with carbon dioxide asphyxiation followed by cervical dislocation. All animals were sacrificed after a maximum of 100 days and tumors as well as potential tumor- and xGVHD-targeted organs were excised for further analysis.

## Bioluminescence Imaging

Tumor growth was monitored weekly by bioluminescence imaging (BLI) using an IVIS Lumina II *in vivo* imaging system (Perkin Elmer, Waltham, MA, USA). Mice were anesthetized by isoflurane inhalation and subcutaneously injected with 150 µg of *in vivo*-grade VivoGlo™ luciferin (Promega, Madison, WI, USA) dissolved in 100 µL of DPBS per mouse. Images were acquired

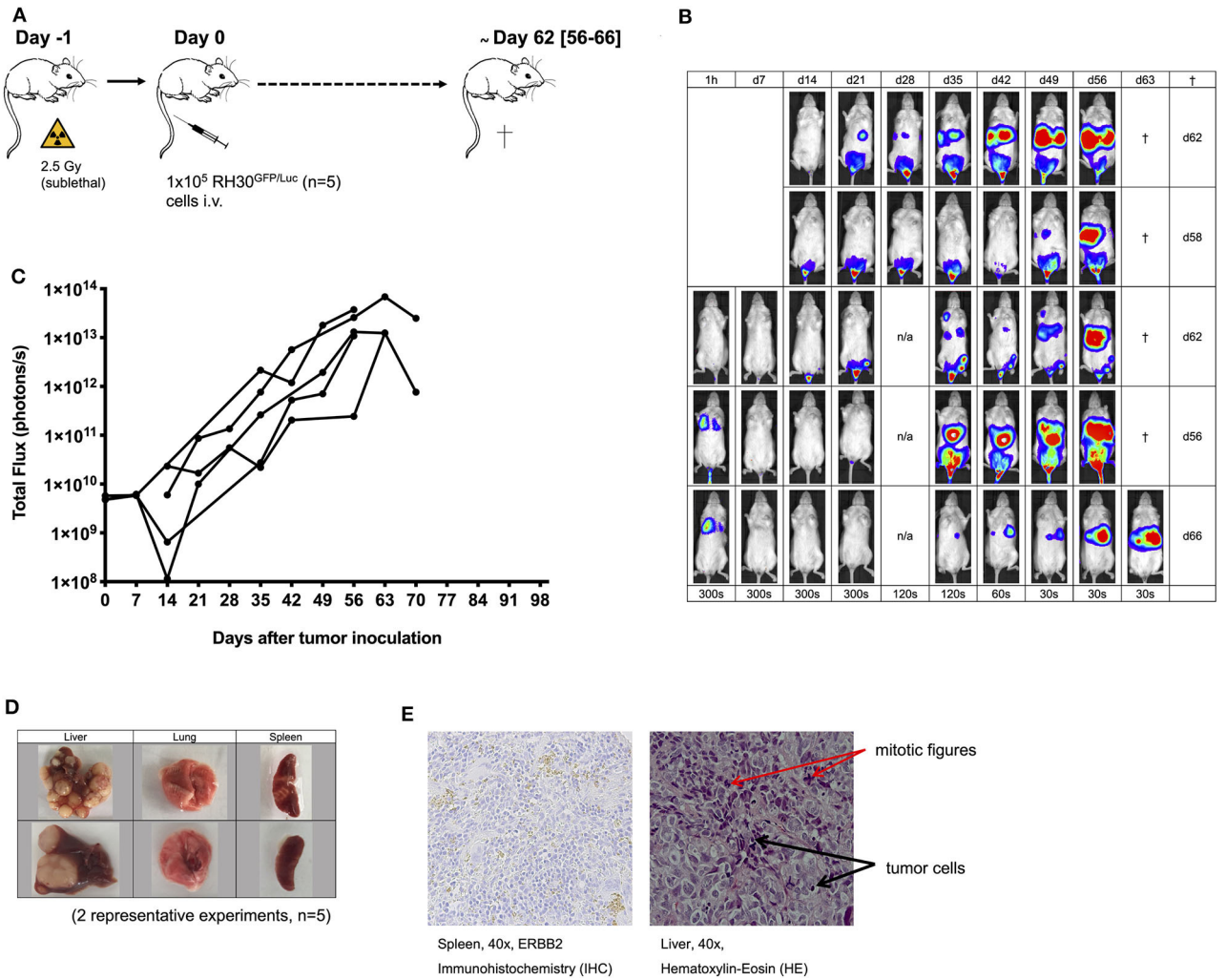


**FIGURE 1 |** Establishment of the human RH30$^{GFP/Luc}$ mouse tumor model. The establishment of a human luciferase-expressing RH30$^{GFP/Luc}$ cell line **(A)** was monitored by bioluminescence imaging (BLI; **B,C**). Tumor engraftment was confirmed by macroscopic examination (**D**, two representative experiments shown), histology [hematoxylin-eosin (HE) staining; **E**], immunohistochemistry (IHC; staining for human ERBB2; **E**), and PCR analysis (**Figure 2F**).

after an incubation time of 15 min. Data were recorded and analyzed using Living Image *in vivo* Imaging Software (Perkin Elmer). Total flux (photons/s) was used for measurement and statistical analysis of the tumor burden using a uniform region of interest in all mice.

## Harvest of Human Cells From the Organs of NSG Mice

Peripheral blood (PB), BM, lung, liver, gut, and spleen samples were excised and analyzed for occurrence of tumor and immune effector cells at the end of experiments. For this purpose, cell suspensions were prepared from the PB, BM, lung, liver, and spleen. Briefly, BM cells were collected from each tibia and femur by flushing the bones with culture medium. Mouse erythrocytes within BM and PB samples were lysed with lysis buffer (Mouse Erythrocyte Lysing Kit, R&D Systems, Wiesbaden, Germany) and washed once with washing buffer according to the manufacturer's instructions. Cell suspensions prepared from mouse organs digested with collagenase were filtered through a 100-μM cell strainer and washed with PBS. Aliquots of cell suspensions were analyzed by flow cytometry and quantitative polymerase chain reaction (qPCR).

## Flow Cytometry

WT and ERBB2-CAR CIK cells were characterized by flow cytometry prior to intravenous injection and at the end of experiments, if applicable after being harvested from organs of mice. Cells were washed once in PBS, resuspended in 100 μL of PBS, and stained with fluorescein peridinin chlorophyll (PerCP)-conjugated anti-human CD3, phycoerythrin (PE)-conjugated anti-human CD4, phycoerythrin-cyanin 7 (PE/Cy7)-conjugated anti-human CD56, allophycocyanin (APC)-conjugated anti-human CD8 or CD45RO, and allophycocyanin-cyanin 7 (APC/Cy7)-conjugated anti-human

CD45 or CD8 antibodies or pacific blue-conjugated anti-human CD62L antibody.

To detect the cell surface expression of ERBB2-CARs, we labeled the CIK cells with an ERBB2 fusion protein as the primary reagent (ERBB2-IgG-Fc chimera, Sino Biological Inc., Beijing, P.R. China) after unspecific Fc-receptor blocking using TruStain FcX (Fc-Receptor Blocking Solution, BioLegend, San Diego, CA, USA). A secondary anti-IgG- Fc monoclonal antibody conjugated with APC was used to detect the primary ERBB2-IgG-Fc chimera (31).

All the antibodies were obtained from BioLegend (San Diego, CA, USA) unless otherwise specified. Isotype-matched fluorochrome-conjugated IgGs were used as controls.

Gates were set on viable lymphocytes, and the data were used for further analysis if at least 4 x $10^4$ $CD45^+/CD3^+$ events were acquired using a BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA) with FACSDiva software (Version 6.1.3, BD Biosciences). Analyses were performed using FlowJo X software (Version 10.6.2, Tree Star Inc., Ashland, OR, USA). All multicolor flow cytometry assays with two or more colors were adjusted for spectral overlap.

## Chimerism Analysis

Genomic DNA was extracted using a QIAamp blood and tissue kit (Qiagen, Hilden, Germany). As a first step, a quantitative real-time PCR approach was used to assess the number of human cells in each tissue sample by specific amplification of the human albumin gene (40, 41). For each reaction, 50 ng of DNA was processed. This assay could detect one human cell in 1,000 murine cells. As a second step—within the human cell fraction—the proportions of CIK (WT and ERBB2-CAR) and tumor (RH30) cells were discriminated by a human-specific STR genotyping approach, similar to chimerism analyses (42). The tumor burden of each mouse was determined by evaluating tumor-specific STR signals per organ. Primers and probes were obtained from Eurofins (Eurofins MWG GmbH, Ebersberg, Germany) and genotyping of cell lines was performed using the STR multiplex PCR system Powerplex 16 (Promega GmbH, Mannheim, Germany).

## Histology and Immunohistochemistry

Histopathology of the tumor- and xGVHD-targeted internal organs of mice injected with WT or ERBB2-CAR CIK cells was performed by an external laboratory (mfd diagnostics GmbH, Wendelsheim, Germany) to assess antitumor capacity and alloreactivity. Experimenters were blinded to the treatment the mice had received. Tissue was fixed in 4% buffered formalin, paraffin embedded, sectioned, and stained with hematoxylin-eosin (HE) or immunohistochemistry (IHC) antibodies targeting human CD3 or ERBB2. A Zeiss AXIO Imager A1/M1 was used for microscopic examination of tumors and immune cell infiltration, as well as evaluation of xGVHD criteria.

## Statistics

Immune effectors cells (WT and ERBB2-CAR CIK cells) unless otherwise published were analyzed for surface markers, CAR expression, anti-tumor ability, and proliferation *in vitro*.

Groups of mice without and with immune cell therapies—which were given to mice at risk for tumor progression as well as to mice with established tumors—were compared regarding disease occurrence and immune cell infiltration as well as for adverse side effects and xGVHD. Animals were observed for 100 days and sacrificed for further analyses at day +100 of the experiment.

Differences between groups were evaluated by one-way ANOVA using the Holm-Sidak or Bonferroni-Dunn (non-parametric) method. A $p < 0.05$ was considered significant. Statistical analyses were performed using GraphPad Prism software (version 8.4, GraphPad Software, La Jolla, CA). Results are presented as disease free survival curves until day +100 or mean values ± standard errors of the mean (figures) and mean values ± standard deviations (results).

# RESULTS

## Establishment of a Human RMS Xenograft Model in Immunodeficient Mice

Establishment of alveolar human RMS xenografts using $1 \times 10^5$ luciferase-expressing $RH30^{GFP/Luc}$ cells (**Figure 1A**) was feasible in all mice ($n = 5$). Engraftment and organ distribution of $RH30^{GFP/Luc}$ cells could be monitored by BLI between days +13 and +35 (**Figure 1B**) after tumor inoculation. Tumor engraftment was reliable, rapid, and life limiting at a median of 62 days after intravenous injection of tumor cells. The pattern of tumor growth was exponential and established in the BM, lung, liver, and spleen (**Figures 1B–E**). Mice had to be sacrificed because of visible signs of poor health (e.g., paralysis or visible tumor burden) between days +56 and +66 (median +62 days, **Figures 1A,B**). Macroscopic engraftment of human RMS (**Figure 1D**) was confirmed histologically by HE and IHC staining for human ERBB2 as well as by PCR (**Figures 1E**, **2F,G**). High amounts of tumor cells were observed in the liver tissues of mice, whereas BM, lung, and spleen samples showed much lower human RMS engraftment. Human ERBB2 could be identified on tumor cells from spleens *via* IHC, whereas detection in intrahepatic cancer tissue (with more mitotic activity) was not possible due to autofluorescence of the liver tissue and bile.

## Generation, Expansion, and Characterization of the WT and ERBB2-CAR CIK Cells *in vitro*

WT and ERBB2-CAR CIK cells were generated from PBMCs of the same (single) healthy donor for immediate *in vivo* use after quality assurance (phenotypic characterization, proliferation, and viability). Applied batches of WT or ERBB2-CAR CIK cells did not differ in quality.

CIK cells were generated by stimulation with IFN-γ, IL-2, and anti-CD3 antibody, followed by addition of IL-15 starting at day 3 of the culture. On day 4, CIK cells were transduced

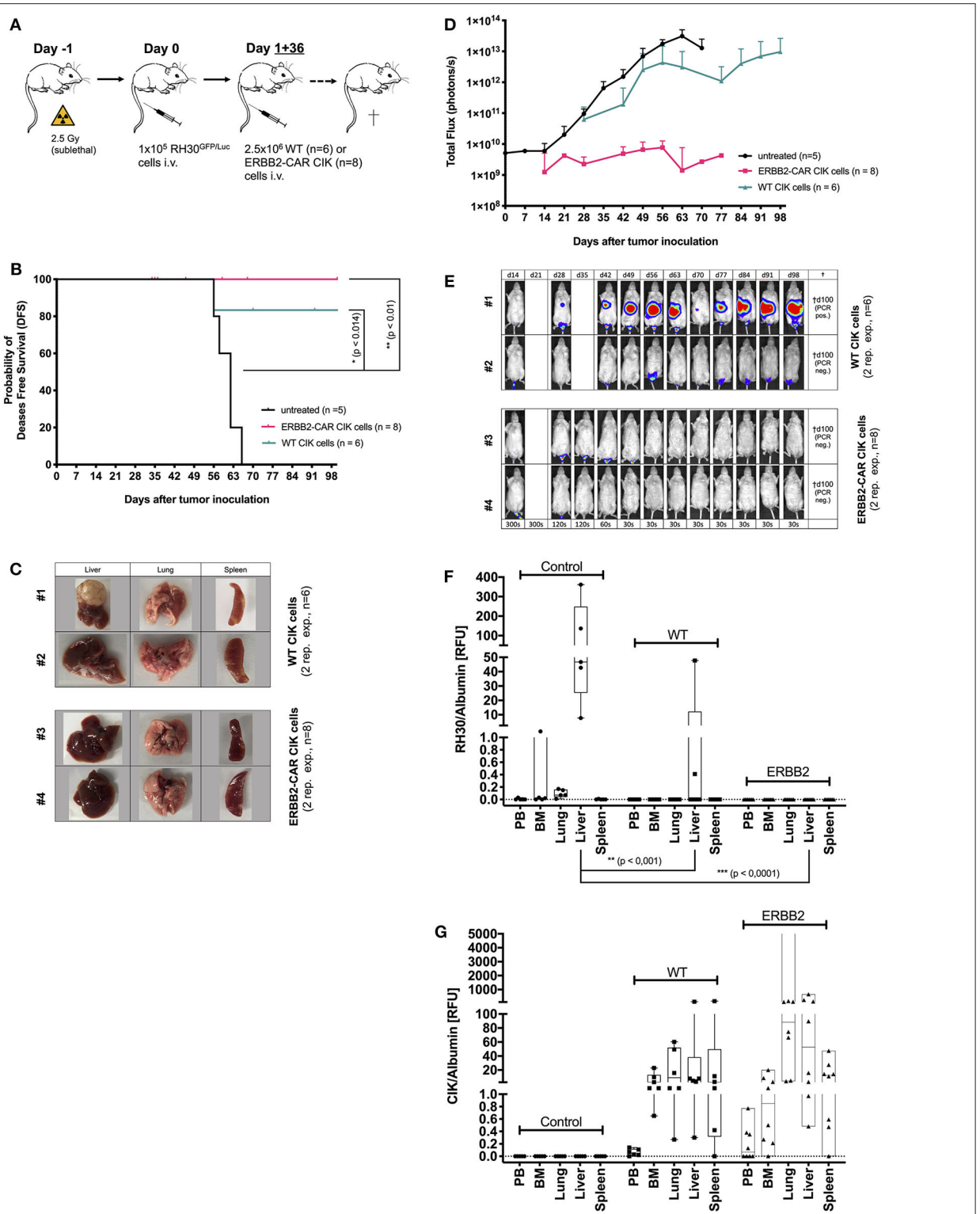


**FIGURE 2 |** Cytotoxicity of WT and ERBB2-CAR CIK cells against minimal residual disease. The cytotoxicity of WT and ERBB2-CAR CIK cells against mice with minimal residual disease established with the human RH30$^{GFP/Luc}$ cell line (preemptive treatment) was analyzed *in vivo* **(A)**. The disease-free survival **(B)** and tumor burden (evaluated by BLI) of WT and ERBB2-CAR CIK cell-treated mice (**D, E**; WT, $n = 6$ and ERBB2-CAR CIK, $n = 8$, respectively) were monitored. Tumor clearance was confirmed macroscopically (**C**, two representative experiments per group are shown) and tumor engraftment was analyzed by PCR **(F)**. CD3-positive human effector cells were identified in WT and ERBB2-CAR CIK cell-treated mice by immunohistochemistry (**Table 1**) and PCR **(G)**.

with the lentiviral CAR vector. After incubation for 12 days, cell numbers of WT and ERBB2-CAR CIK cells increased up to 36.5-fold, SD ±23.4 and 13.4-fold, SD ±6.6.) with over >90% cell viability. Differences between WT and ERBB2-CAR CIK cells were not significant ($p > 0.06$). Furthermore, no difference in the proportions of $CD3^+CD56^-$ T cells (93.3% SD ±2.1 and 93.84% SD ±1.4; $p > 0.95$), $CD3^+CD56^+$ T-NK cells (4.8% SD ±2.0 and 4.1% SD ±1.4; $p > 0.28$), and $CD3^-CD56^+$ NK cells (0.71% SD ±0.18 and 0.79% SD ±0.26; $p > 0.73$) between WT and ERBB2-CAR CIK cells were found during a 12 day culture period. In contrast, the proportion of $CD4^+$ (33.9% SD ±2.5 and 14.4% SD ±3.8; $p < 0.009$) and $CD8^+$ (59.9% SD ±3.1 and 75.4 SD ±4.2; $p < 0.03$) subpopulations differed significantly between WT and ERBB2-CAR CIK cells (**Supplementary Figure 1A**). Interestingly, prior to infusion, ERBB2-CAR CIK cells displayed significantly more helper T cells with effector memory phenotype compared to WT CIK cells, whereas the latter contained more naïve T cells (**Supplementary Figures 1C,D**). Mean transduction efficiency determined *via* eGFP expression of transduced CIK cells was 21.1% (SD ±8.8%, $n = 7$), with 84.3% (SD ±6.9%, $n = 3$) of these cells also displaying high CAR expression on the cell surface (**Supplementary Figures 1A,B**).

## Preemptive WT and ERBB2-CAR CIK Cell Treatment in RMS Xenografts

After reliably and successfully establishing the xenograft RMS mouse model as a control, the potential of WT and ERBB2-CAR CIK cells to reach and eliminate inoculated ERBB2-positive tumors was investigated. At first, immune cell therapies were given preemptively (day +1 and day +36 after $RH30^{GFP/Luc}$ tumor cell injection) (**Figure 2A**). All mice treated preemptively with WT or ERBB2-CAR CIK cells had minimal residual, but already active disease at the time of immune cell infusions, which translated into an imminent risk for disease progression with limited treatment options in a clinical setting. Hence, all mice without immune cell therapies showed progressive tumor growth and died within 56–66 days. Remaining animals were sacrificed at day +100 of the experiment and organs were excised for further analysis. Preemptive WT and ERBB2-CAR CIK cell treatment significantly improved disease-free survival until day +100 when compared to PBS-treated controls ($p < 0.014$ and $p < 0.01$), respectively, without differences between WT and ERBB2-CAR CIK cells (**Figure 2B**). In addition, BLI confirmed sustained inhibition of tumor engraftment in all ERBB2-CAR CIK cell-treated mice ($n = 8$), whereas mice treated with WT CIK cells only transiently responded, but ultimately progressed in 2 of 6 (33%) cases (**Figures 2D,E**, WT, $n = 6$ and ERBB2-CAR CIK, $n = 8$). Tumors were macroscopically cleared in all mice treated with ERBB2-CAR CIK cells, while small macroscopic lesions were observed in some of the mice treated with WT CIK cells (**Figure 2C**). Accordingly, 4 of 6 (67%) animals treated with WT and all 8 of 8 (100%) animals treated with ERBB2-CAR CIK cells were in complete molecular remission as determined by PCR analysis (**Figure 2F**). In contrast, CD3-positive human immune effector cells were identified in high numbers in the spleen of WT and ERBB2-CAR CIK cell-treated mice and in lower numbers in the liver but were not detectable by IHC in xGVHD-targeted organs, such as the guts and lungs (**Figure 2G** and **Table 1**). Whereas, weak signals were detectable by IHC for both cell types, only low amounts of WT CIK cells but high amounts of ERBB2-CAR CIK cells were detected by PCR. These data demonstrate that ERBB2-CAR CIK cells retain target cell specificity *in vivo*, and either exhibit increased tissue migration or improved persistence compared to WT CIK cells (**Figure 2G**).

## WT and ERBB2-CAR CIK Cell Treatment of Established RMS Tumors

To model WT and ERBB2-CAR CIK cell therapy during advanced RMS relapse, WT and ERBB2-CAR CIK cell infusions were applied in another group of mice already engrafted with human $RH30^{GFP/Luc}$ tumors (**Figure 3A**). Mice with delayed ERBB2-CAR CIK cell treatment on days +22 and +57 after tumor cell injection experienced significantly improved survival compared to untreated controls ($p < 0.01$, **Figure 3B**), whereas WT CIK cell-treated mice only showed a trend toward improved survival ($p > 0.07$). BLI, autopsy, and PCR analysis showed a short delay, but not an abrogation of tumor growth in WT and ERBB2-CAR CIK cell-treated mice compared to untreated controls. Here, antitumor responses were more pronounced in ERBB2-CAR CIK cell-treated mice than in WT CIK cell-treated mice (**Figures 3C–E**). Of note, ERBB2-CAR CIK cells, but not WT CIK cells were detectable by PCR at tumor sites (**Figure 3F**), albeit at lower levels compared to preemptive ERBB2-CAR CIK cell therapy (**Figure 2G**).

## Human ERBB2 Surface Expression of RMS Tumors

At the time of infusion, ERBB2 molecule expression on RH30 cells was identified by flow cytometry. However, expression levels were consistently low, not allowing detection of ERBB2 by standard IHC in all tumor-targeted organs (**Figure 1E**). Interestingly, IHC analysis of established tumors in spleens confirmed the surface expression of ERBB2 molecules (**Figure 1E**).

## Biodistribution and Toxicity

Preemptive ERBB2-CAR CIK cells and, to a lesser extent, WT CIK cells produced an effective antitumor response associated with the presence and prolonged persistence of immune cells at tumor sites. Interestingly, tumor eradication induced by only two infusions of ERBB2-CAR CIK cells enabled survival of the ERBB2-CAR CIK cells, but also led to the expansion and persistence of immune cell subpopulations, namely $CD3^+CD4^+$ helper T cells, $CD3^-CD56^+$ NK cells, and $CD3^+CD56^+$ T-NK cells, which had only been found in very low numbers within the adoptively transferred heterogeneous CIK cell population at the time of infusion (**Figures 4A,B**, **Supplementary Figures 1E,F**).

CIK cell engraftment also took place in potential xGVHD-target organs where ERBB2-expressing tumors were present, such as the spleen and lungs. There were no significant differences in numbers or phenotype (**Supplementary Figure 1**) between

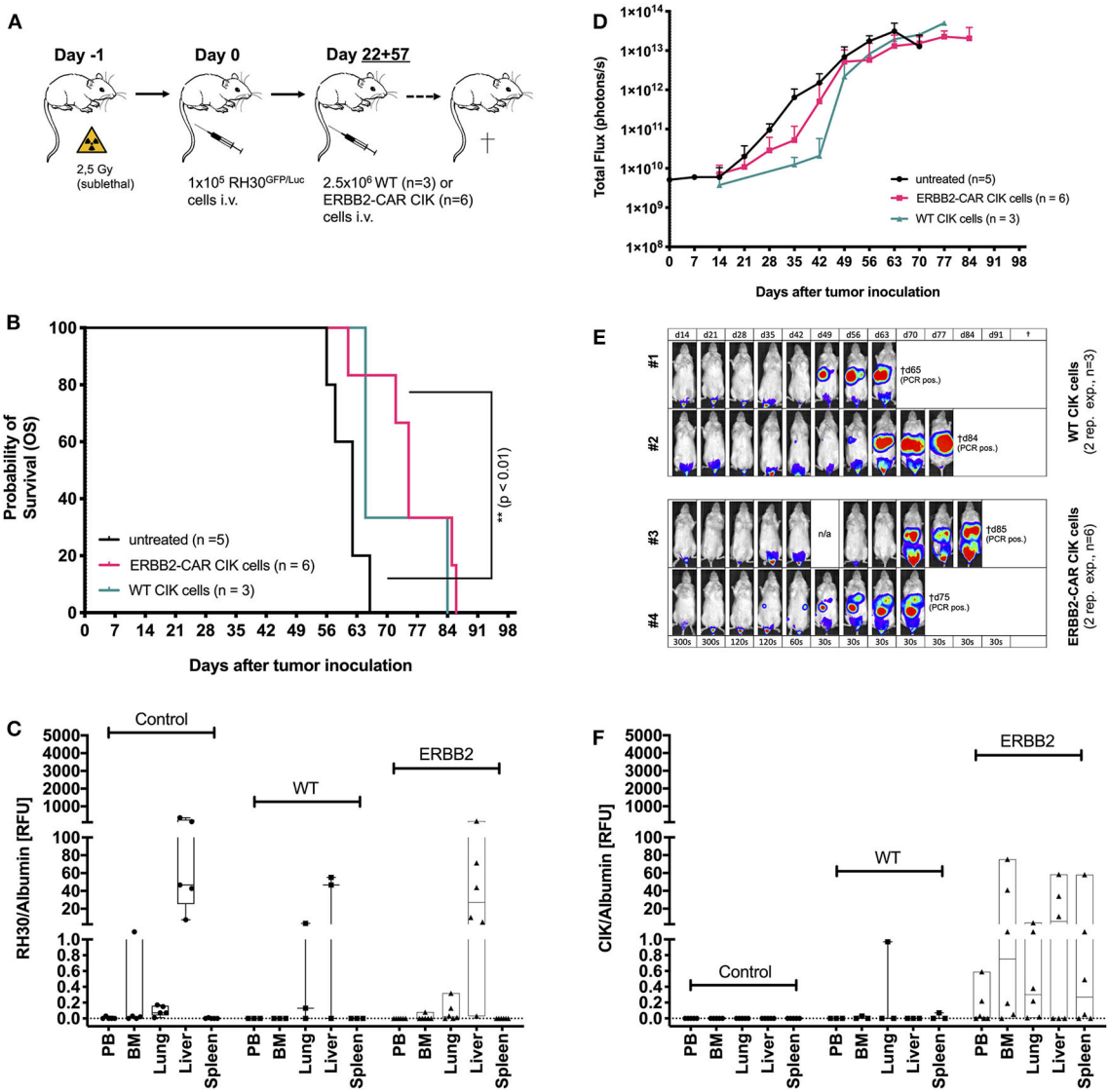


**FIGURE 3 |** Cytotoxicity of WT and ERBB2-CAR CIK cells against established human RH30$^{GFP/Luc}$ tumors. WT and ERBB2-CAR CIK cell infusions were analyzed for activity against established human RH30$^{GFP/Luc}$ tumors **(A)**. ERBB2-CAR CIK cell treatment significantly improved survival compared to no treatment ($p < 0.01$, **B**). Survival **(B)**, BLI **(D,E)**, and PCR results **(C)** were used to assess tumor engraftment. Of note, ERBB2-CAR CIK cells but not WT CIK cells were detectable by PCR at tumor sites **(F)**.

WT CIK cells and ERBB2-CAR CIK cells present in the spleen or lungs (**Figure 2G**). Histopathology of gut and lung tissue of mice injected preemptively with WT or CAR CIK cells showed no infiltration of CD3-positive human lymphocytes and no signs of tissue damage. Lymphocyte infiltration was moderate in livers and was highest in spleens. Histopathological analysis of these organs showed no signs of tissue damage, necrotic hepatocytes, or fibrosis (**Tables 1**, **2**). Furthermore, immune cell engraftment did not lead to severe CRS or xGVHD, but enhanced graft-vs.-tumor effects toward ERBB2-expressing RMS cells (**Table 2**). Altogether, the infusion of ERBB2-CAR CIK cells was found to be effective, safe, and well-tolerated.

## DISCUSSION

Preclinical studies using CAR T cells from healthy donors have shown that these cells are effective when targeting solid tumors, but this approach is limited when using patient's own T cells for CAR modification (9). In a phase I/II clinical trial, ERBB2-CAR T cells persisted for 6 weeks but ultimately failed to improve the outcome of high-risk sarcoma patients. However, treatment efficacy was improved by lymphodepleting conditioning prior to adaptive transfer of ACT, which further enhanced ERBB2-CAR T cell expansion and persistence *in vivo* (21).

CIK cells—which have documented safety in the autologous and allogeneic setting—may be considered an alternative

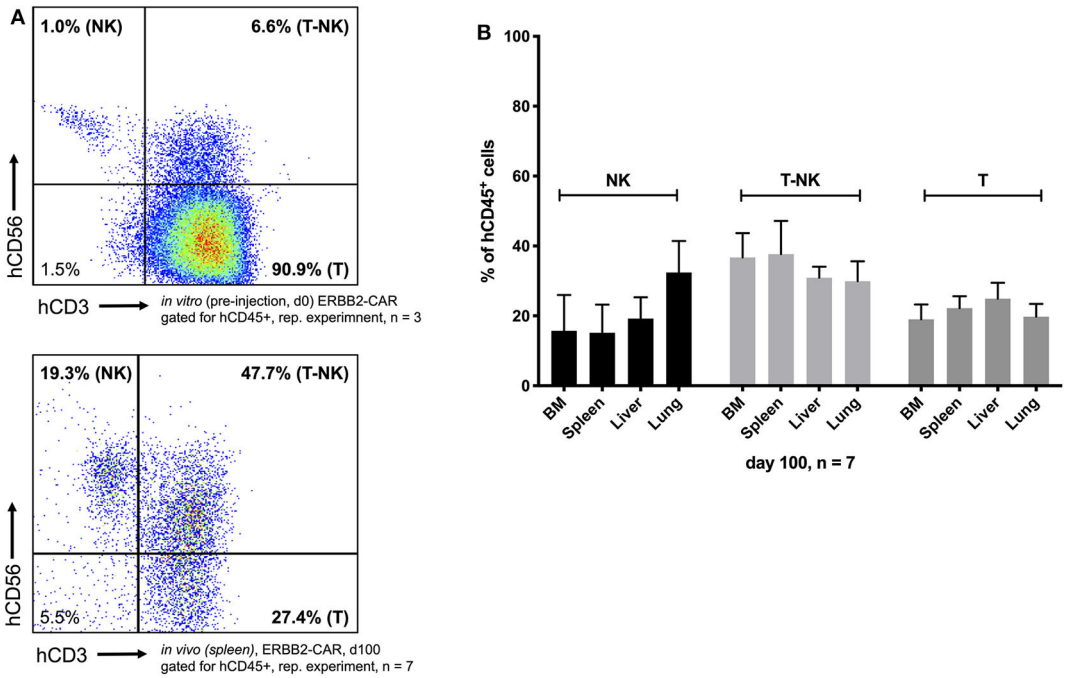


**FIGURE 4 |** Biodistribution and toxicity model. Two infusions of ERBB2-CAR CIK cells enabled survival of the ERBB2-CAR CIK cells and persistence of innate immune cells within the adoptively transferred heterogeneous CIK cell population, in particular $CD3^{-}CD56^{+}$ NK and $CD3^{+}CD56^{+}$ T-NK cells (**A** bottom and **B**), which accounted for only 1 and 6.6% of the cells at the time of infusion, respectively (**A** top). ERBB2-CAR and WT CIK cell engraftment took place in all analyzed organs (**B**, **Figures 2G**, **3F**), but did not lead to xGVHD (confirmed by histology, **Table 2**).

**TABLE 1 |** Biodistribution of WT and ErbB2-CAR CIK cells following preemptive treatment.

| | WT CIK ($n = 6$) | | | | | | | ErbB2-CAR CIK ($n = 8$) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Liver | + | + | − | + | − | − | − | − | + | − | − | + | − | − |
| Lung | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| Spleen | ++ | + | ++ | + | ++ | ++ | n/a | +++ | ++ | +++ | +++ | − | + | + |
| Gut | − | − | − | − | − | − | − | − | − | n/a | n/a | − | − | − |

*IHC staining of CD3-positive immune effector cells: –, negative; +, low grade; ++, medium; +++, high grade; n/a, not available.*

immune cell source for CAR modification, as patient- and even healthy donor-derived-CIK cells may be used for adoptive immunotherapy of high-risk RMS. Mutations affecting the receptor tyrosine kinase/RAS/PIK3CA pathway, such as mutations in the tyrosine kinase genes *FGFR4*, *PDGFRA*, and *ERBB2,* are the most common mutations observed in RMS and could be targeted by approved therapeutics (19, 20). Once preclinical *in vivo* safety and efficacy against RMS are demonstrated, ERBB2-CAR CIK cells may enable the rapid preparation of a subsequent planned clinical trial urgently needed for the treatment of high-risk patients with alveolar RMS.

Here, we present an ACT approach targeting CIK cells to ERBB2 with a second-generation CAR for the potential treatment of refractory human alveolar RMS in a completely new xenograft model. In this model, NSG mice carrying small or large tumor burden of $RH30^{GFP/Luc}$ cells that had already spread to BM, liver, lung and spleen at the time of treatment mimic the clinical situation of high-risk patients. The novelty of our study is based on the use of CIK cells rather than T cells targeting ERBB2 *in vivo* in the context of preemptive ACT and during alveolar RMS in its advanced form.

**TABLE 2 |** XGVHD after preemptive treatment with WT and ERBB2-CAR CIK cells.

| | WT CIK ($n = 6$) | | | | | | ErbB2-CAR CI*K* ($n = 8$) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Liver | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| Lung | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| Spleen | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| Gut | – | – | – | – | – | – | – | – | – | – | – | – | – | – |

*Signs of tissue damage, necrosis or fibrosis assessed by HE staining: –, no signs; +, low signs; ++, medium signs of xGVHD.*

Establishment of human alveolar RMS xenografts was feasible in 100% of mice following tail vein injection of tumor cells. Tumor engraftment was detectable after 13 days by BLI imaging. Samples taken from the PB, BM, lung, liver, and spleen of mice with visible signs of poor health after a median of 62 days (range 56–66 days) reassured human alveolar RMS engraftment. IHC staining confirmed ERBB2 target molecule expression on small tumor lesions but not on established tumors in the liver, probably due to the autofluorescence of the liver tissue and bile.

It is widely recognized that CAR-engineered T cell products lose *in vivo* function during culture, driving the development of ever-shorter manufacturing protocols (43–45). In previous work, we employed CIK cells after *ex vivo* activation with IL-15 for 10 days, which resulted in more rapid generation of CIK cells with stronger cytotoxicity compared to conventional CIK cells expanded in the absence of IL-15 over 3–4 weeks. (32, 46, 47). The main fraction in this short-term, IL-15-activated CIK cell culture is the $CD3^+$ T cell population. Modification of IL-15 activated CIK cells by lentiviral CAR transduction and its influence on proliferation, phenotype, anti-tumor ability, cytokine secretion, and alloreactivity *in vitro* was previously published by our group (31). CAR CIK cells efficiently and selectively lysed ERBB2-positive tumor cells, which only showed minimal sensitivity to WT CIK cells, whereas parental ERBB2-negative tumor cells remained unaffected. However, cytotoxicity triggered by interaction of their activating NK receptors with stress ligands expressed by tumor cells was retained by ERBB2-CAR-CIK cells. Target cell recognition triggered secretion of pro-inflammatory cytokines and chemokines such as IFN-γ, TNFα, and MIP-1α/CCL3 as well as release of granzyme B, while production of IL-6 or IL-10—which is associated with CRS—was not observed. Moreover, when co-cultured with freshly isolated PMBCs from HLA-mismatched donors, neither WT CIK nor CAR CIK cells displayed significant alloreactivity *in vitro*. In the actual study, no differences in proliferation nor in the proportions of T, T-NK, and NK cells were found, whereas ERBB2-CAR CIK cells before infusion contained significantly more helper T cells with an effector memory phenotype compared to WT CIK cells which showed more naïve T cells. This may be responsible for the improved overall antitumor capacity of ERBB2-CAR CIK cells compared to WT CIK cells. Mean transduction efficiency determined *via* eGFP expression of transduced CIK cells was 21.1 $\pm$ 8.8%, with 84.3 $\pm$ 6.9% of these cells also displaying high CAR expression on the cell surface.

Only partial tumor growth inhibition was achieved in WT CIK cell-treated mice, while the *in vivo* antitumor functions of ERBB2-CAR CIK cells resulted in complete elimination of human alveolar RMS in the setting of preemptive therapy. ERBB2-CAR CIK cells showed sustainably migration to distant tumor sites, were capable of penetrating tumor tissues, and showed long-lasting persistence and activity, which we did not observe for WT CIK cells. The transferred ERBB2-CAR CIK cell population was composed of low numbers of NK and T-NK cells and high numbers of T cells at the time of infusion, where at the ERBB2-CAR was exclusively expressed by the T and T-NK cell compartments. Hence, a comprehensive comparison of the *in vivo* antitumor functions of CIK cells to those of young T cells engineered with ERBB2-CAR might be interesting as a means of deciphering whether it is the use of CIK cells that is the basis of the observed activity of this approach. However, the T cells among CIK cells are non-classical terminally differentiated T lymphocytes with an NK cell phenotype representing diverse T and NK cell receptor specificities, which are not comparable to young T cells (31, 48). Furthermore, CIK cells include classical NK cells as well as $CD4^+$ and $CD8^+$ T lymphocytes with naïve T, effector memory, and central memory T phenotypes, and this may represent an advantage with respect to the unselected CAR-transduced T cell populations used so far, which contain significant amounts of $CD4^+$ T cells that may contribute to the dramatic cytokine storm sometimes observed *in vivo* (49). In contrast to the high specificity and memory of CAR T cells, CIK cells are capable of eliminating tumor cells by recognizing pathogen patterns through a variety of receptors (DNAM-1, NKG2D, NKp30, and TCR/CD3) (24), suggesting that ERBB2-CAR CIK cells may provide NK cell-like activities, mainly NKG2D-mediated functions, and specific anti-ERBB2-mediated cytotoxicity in combination, as indicated by NK and T-NK cells which were present at potential tumor sites in all ERBB2-CAR CIK cell-treated mice. However, due to the strong autofluorescence properties of analyzed organs, which interfered with eGFP emission signals, CAR expression was not assessable. Usually, NK and T-NK cells cannot be tracked *in vivo* (50), but infiltrating NK cells and NK cell-mediated antitumor responses have been described to be associated with a relatively good prognosis (51, 52). Here, we showed that multi-specific ERBB2-CAR CIK cell therapy could eliminate low tumor load and that ERBB2-CAR CIK cells could be used as a vehicle for delivering preferentially $T_H$-1 cytokines and chemokines directly to tumor sites, which are involved in regulating innate and adaptive immunities and may have allowed T, NK, and T-NK cells to proliferate, persist, and survive *in vivo,* which is in contrast to IL-15 activated WT CIK cells and conventional IL-2-activated CIK cells reported by others (53).

However, ERBB2-CAR CIK cell therapy was not as effective in the treatment of relapsed or progressive disease, even though the immune effector cells reached tumor sites and were increased in numbers compared with WT CIK cells. The tumor microenvironment and tumor cells themselves can shed target molecules, thereby limiting the potential of CAR-modified immune cells when targeting solid tumors (54, 55). Hence, overcoming the general resistance of solid tumors to immunotherapy appears highly warranted. Therefore, ERBB2-CAR CIK cell treatment may be used preferentially in the preemptive therapy setting given in repetitive doses. Furthermore, combination therapies with PD-1/PD-L1 inhibitors or histone deacetylase inhibitors (HDACis) might be considered to increase the antitumor efficacy of ERBB2-CAR CIK cells in patients with resistant alveolar RMS.

Of particular concern in clinical trials is the risk of toxicity. Hence, cytotoxicity is likely to occur even with low levels of the ERBB2 antigen, which may exist on normal tissues, such as those in the respiratory tract. In addition, numerous examples of more severe toxicity occurring with cellular therapy approaches that can kill normal cells with low-level antigen expression have been

reported. However, Ahmed et al. previously demonstrated the safety and activity of ERBB2-CAR T cell therapy in 17 patients with sarcoma in a phase I/II clinical trial using a very similar second-generation CAR with the same targeting domain as that employed in our study (9). Due to the non-MHC-restricted NK cell-like cytotoxicity of CIK cells and more importantly, based on the preclinical and clinical observations that CIK cells almost completely lack toxicity, we considered CIK cells as immune effectors for our analyses (28–30). Indeed, our treatment approach was well-tolerated, GVHD-targeted organs of mice showed only minimal infiltration of cytotoxic T lymphocytes, while no signs of tissue damage were observed. However, mice are not a good model for assessing toxicity, as they do not fully recapitulate human toxicity or provide insight into on-target toxicity due to the lack of cross-reactivity between anti-human CARs and homologous murine antigens.

Altogether, in biodistribution and toxicity analyses, ERBB2-CAR CIK cells were safe, well-tolerated, and effective, especially in the model with low tumor burden, which was not likewise observed for WT CIK cells. Thus, CIK cells engineered with lentiviral vectors, with their dual roles as targeted killers and modulators of innate immunity, appear to represent a platform with considerable potential to improve the outcomes of children and young adults with ERBB2-positive high-risk alveolar RMS.

## DATA AVAILABILITY STATEMENT

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

## ETHICS STATEMENT

The animal study was reviewed and approved by Regierungspräsidium Darmstadt, Germany Gen.-Nr. TVA FK/1000.

## AUTHOR CONTRIBUTIONS

MM, JW, WSW, EU, and ER: conceived and designed the experiments. MM, JW, HK, and ER: performed the experiments. MM, HK, CH, LMM, WSW, PB, and ER: analyzed the data. JW, HK, WSW, HB, ZI, and EU: contributed to reagents, materials, and analysis tools. MM and ER: wrote the manuscript. HK, CH, LMM, WSW, HB, ZI, EU, TK, and PB: revised the manuscript. PB and TK: supervised the research. All authors: approved the final version of the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.581468/full#supplementary-material

**Supplementary Figure 1 |** Phenotypic characterization of the WT and ERBB2-CAR CIK cells *in vitro* and *in vivo*. The development of CIK cell subpopulations during expansion of unmodified CIK cells and CIK cells transduced with the lentiviral CAR vector was investigated by flow cytometric analysis of the surface markers CD3 and CD56 **(A)**, as well as CD4 and CD8 **(A)**, and their memory phenotypes **(C,D)**. Results obtained with CIK cells before infusion are shown, including the rate of CIK cells with EGFP marker expression **(A)**. Cell surface expression of ERBB2-CARs was confirmed using a secondary anti-IgG-Fc monoclonal FACS antibody against an ERBB2-IgG-Fc chimera (**B**), one representative experiment shown, $n = 3$. Additionally, the biodistribution of WT and ERBB2-CAR CIK cells with helper T/CD4$^+$ and cytotoxic T/CD8$^+$ phenotype at the end of the *in vivo* experiment (preemptive treatment group) was assessed by flow cytometry **(E,F)**.

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REVIEW
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# How to Make an Immune System and a Foreign Host Quickly Cohabit in Peace? The Challenge of Acute Graft-*Versus*-Host Disease Prevention After Allogeneic Hematopoietic Cell Transplantation

*Benoît Vandenhove*[1†], *Lorenzo Canti*[1†], *Hélène Schoemans*[2], *Yves Beguin*[1,3], *Frédéric Baron*[1,3], *Carlos Graux*[4], *Tessa Kerre*[5‡] *and Sophie Servais*[1,3*‡]

[1] *Laboratory of Hematology, GIGA-I3, GIGA Institute, University of Liège, Liège, Belgium,* [2] *Department of Clinical Hematology, University Hospitals Leuven, KU Leuven, Leuven, Belgium,* [3] *Department of Clinical Hematology, CHU of Liège, University of Liège, Liège, Belgium,* [4] *Department of Clinical Hematology, CHU UCL Namur (Godinne), Université Catholique de Louvain, Yvoir, Belgium,* [5] *Hematology Department, Ghent University Hospital, Ghent University, Ghent, Belgium*

Allogeneic hematopoietic cell transplantation (alloHCT) has been used as cellular immunotherapy against hematological cancers for more than six decades. Its therapeutic efficacy relies on the cytoreductive effects of the conditioning regimen but also on potent graft-*versus*-tumor (GVT) reactions mediated by donor-derived immune cells. However, beneficial GVT effects may be counterbalanced by acute GVHD (aGVHD), a systemic syndrome in which donor immune cells attack healthy tissues of the recipient, resulting in severe inflammatory lesions mainly of the skin, gut, and liver. Despite standard prophylaxis regimens, aGVHD still occurs in approximately 20–50% of alloHCT recipients and remains a leading cause of transplant-related mortality. Over the past two decades, advances in the understanding its pathophysiology have helped to redefine aGVHD reactions and clinical presentations as well as developing novel strategies to optimize its prevention. In this review, we provide a brief overview of current knowledge on aGVHD immunopathology and discuss current approaches and novel strategies being developed and evaluated in clinical trials for aGVHD prevention. Optimal prophylaxis of aGVHD would prevent the development of clinically significant aGVHD, while preserving sufficient immune responsiveness to maintain beneficial GVT effects and immune defenses against pathogens.

**Keywords: allogeneic stem cell transplantation, acute graft-*versus*-host disease, T cells, alloreactivity, immune tolerance, tissue tolerance**

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***Correspondence:**
*Sophie Servais*
*s.servais@chuliege.be*

[†]*These authors share first authorship*

[‡]*These authors share last authorship*

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## INTRODUCTION

For almost 6 decades, allogeneic hematopoietic cell transplantation (alloHCT) has been the cornerstone of poor risk hematological cancer therapy. Although novel sophisticated cellular therapies (such as those with CAR T cells) have emerged and appear to be occupying a growing place in the modern therapeutic arsenal in hematology, their long-term effects on disease control and survival are still unclear. Therefore, alloHCT still remains standard of care in a variety of high risk hematological disorders, often offering the only curative option for these diseases (1). The persisting major role of alloHCT in current medicine is documented by the constant increase in the annual number of stem cell transplants performed worldwide, with >19,000 alloHCT procedures in Europe and associated countries in 2018 (701 centers in 50 countries) (2). In adults, the most frequent indications for alloHCT remain acute leukemia (more than 50% of all alloHCT), followed by myelodysplastic syndromes and non-Hodgkin lymphoma (2). In addition, an acceptable donor can currently be found for almost all patients, mainly due to the recent development of innovative platforms for alloHCT with HLA-haploidentical family donors (mismatched for one of the two HLA haplotypes).

The therapeutic efficacy of alloHCT against hematological cancers relies on the cytoreductive effects of the conditioning regimen but also (and mainly) on potent graft-*versus*-tumor (GVT) reactions, defined as immune-mediated reactions by donor cells against tumor cells. However, beneficial GVT effects may be counterbalanced by acute GVHD (aGVHD), a systemic syndrome in which donor immune cells attack healthy tissues of the recipient, resulting in severe inflammatory lesions mainly of the skin, gut and liver. Despite more than 6 decades of preclinical and clinical researches, the immunological requirements necessary to achieve GVT effects without promoting aGVHD have not been fully established.

Despite standard prophylaxis regimens, aGVHD occurs in approximately 20–50% of transplanted patients and is a major cause of treatment failure and mortality after alloHCT. Therefore, the prevention of aGVHD after alloHCT represents an unmet medical need in the modern era of cancer immunotherapy and research must continue in this field. Here, we provide a brief overview of criteria for aGVHD diagnosis and grading as well as current knowledge on aGVHD immunopathology. Then, we discuss current approaches and novel strategies being developed and evaluated in clinical trials for aGVHD prevention.

## WHAT IS AGVHD? THE CLINICAL POINT OF VIEW

GVHD is separated into two syndromes, historically defined according to the time frame of occurrence of symptoms: acute GVHD (aGVHD) occurring within the first 100 days after transplantation and chronic GVHD (cGVHD) developing thereafter. Although simple, this classification based only on empirical observations and did not rely on actual biological or clinical bases. More recent classification systems have emphasized differentiating a- and cGVHD based on pathophysiological mechanisms and clinical manifestations (3, 4).

In 2018, a consortium of GVHD experts from the European Society for Blood and Marrow Transplantation (EBMT), the National Institutes of Health (NIH) and the Center for International Blood and Marrow Transplant Research (CIBMTR) reviewed the terminology and guidelines for GVHD diagnosis and scoring (5). Clinically, aGVHD typically presents with inflammatory lesions, the three main organs involved being: the skin (erythematous and pruriginous maculopapular skin rash), the gastro-intestinal (GI) tract (nausea, vomiting, and anorexia with weight loss in the upper tract; and/or watery or bloody diarrhea, crampy abdominal pain and/or ileus in the lower tract), and the liver (cholestasis with hyperbilirubinemia) (5, 6). Typical aGVHD is defined by the presence of these exclusive inflammatory manifestations, without any other sign consistent with cGVHD. Ideally, the diagnosis of aGVHD should be confirmed by positive histological findings, but this is not formally required (5). AGVHD can be categorized as "*classic aGVHD*" in the setting of typical aGVHD manifestations occurring less than 100 days after alloHCT or donor lymphocyte infusion (DLI), and "*late, recurrent or persistent aGVHD*" in patients with typical aGVHD signs experienced later than 100 days after alloHCT/DLI (5, 7).

Grading aGVHD is essential because it is predictive of non-relapse mortality and it guides therapeutic management. Several scoring systems have been developed during the past decades, including the original Glucksberg classification (first established in the 1970s), the "Modified Glucksberg" or "Keystone", the IBMTR and the "MAGIC" scoring systems (8–11). Each of them proposes a 4-grade scale, integrating the individual stage of each target organ (skin, GI tract, and liver), with or without the general *Performance Status*. Recently, the EBMT–NIH–CIBMTR Task Force Consortium recommended the MAGIC criteria as the most accurate and detailed clinical criteria for diagnosis and grading the severity of aGVHD (5). A web-application has also been developed based on this position statement (*eGvHDApp; https://www.uzleuven.be/egvhd*) and has been found to be helpful in improving aGVHD and cGVHD scoring consistency and compliance with guidelines (12, 13).

In addition to the typical manifestations of aGVHD in the skin, GI, and liver, there is accumulated evidence that aGVHD may also affect other tissues, including the cellular niches in the bone marrow (BM), thymus and secondary lymphoid organs (14). Although lesions in these organs are hardly clinically detectable, they can severely impact outcome by impairing hematopoiesis, compromising T- and B-cell reconstitution and predisposing to the development of subsequent cGVHD (15). It has also been suggested that aGVHD can cause damages to the endovascular endothelium and can be the trigger of endothelitis-related complications after alloHCT, such as transplant-associated microangiopathy, diffuse alveolar hemorrhage, idiopathic pneumonia syndrome (16). Finally, over the past

decade, experimental data (17, 18) and clinical case reports (19) have gradually accumulated suggesting that the central nervous system may also be a potential target of aGVHD. Although they are not considered in current standard aGVHD diagnosis criteria and grading systems, alloreactive lesions to these tissues can be associated with significant morbidity.

Despite conventional prophylactic measures, it is estimated that 20–50% of transplanted patients develop clinically significant grades II–IV aGVHD after alloHCT. Known risk factors include the stem cell source (G-SCF mobilized peripheral blood stem cells, PBSC), the donor type (unrelated, female donor for a male recipient), the degree of donor/recipient HLA-mismatch, the intensity of the conditioning regimen (myeloablative regimen), the occurrence of severe infections during the peri-transplant period and administration of DLI (7, 20, 21).

The standard first-line of treatment for grades II–IV aGVHD is high-dose systemic corticosteroids. However, aGVHD fails to respond to steroids in approximately 30–50% of patients (the risk increasing with increasing grade), therefore requiring subsequent lines of immunosuppressive therapies (22, 23). Outcomes of patients with steroid refractory aGVHD have been dismal (up to 60–85% of non-relapse mortality at 2 years), partly due to aGVHD by itself, but also to cumulative toxicity and increasing susceptibility to infections and relapse incurred with additional immunosuppressive therapy (22, 24). Hopefully, research is constantly developing in the field and two recent large phase III studies have provided significant benefit in efficacy outcomes with two novel strategies for the treatment of steroid refractory aGVHD. First, Socie et al. reported better long-term overall survival with inolimomab (an anti-CD25 monoclonal antibody) in comparison with anti-T cell globulin (25). The second phase III study demonstrated higher response rate with ruxolitinib (a JAK 1–2 inhibitor) compared to the investigator's therapy of choice (26). Nevertheless, aGVHD remains a severe complication and one of the major cause of early post-transplant mortality (27).

## WHAT IS AGVHD? THE IMMUNOLOGICAL POINT OF VIEW

Despite significant improvements in the field over the past 20 years, the complex immunobiology of aGVHD still remains only partially elucidated. Here, we present a simplified overview of the main basic immunological concepts on aGVHD biology, with the aim of providing readers with some clues for understanding the rationale of both current and emerging preventive approaches. For more detailed information about aGVHD pathophysiology, readers are referred to several outstanding reviews (6, 28–30).

### Donor T Cells as Drivers, Amplificators, and Effectors of aGVHD Responses

AGVHD after alloHCT mainly results from donor T-cell alloreactivity against the recipient's tissues, as evidenced by the low incidence of GVHD observed in patients transplanted with a T-cell depleted allograft (31). After alloHCT, transferred donor T cells are able to recognize structurally dissimilar allogeneic peptide/HLA complexes in the recipient, reacting against either polymorphic HLA molecules (in case of alloHCT with HLA-mismatched donor/recipient pair) and/or peptides (minor histocompatibility antigens) presented by either shared or dissimilar HLA molecules (in the setting of alloHCT with HLA-matched or mismatched donor/recipient pair, respectively) (32–34).

In general, three types of signals are required to generate full alloreactive T-cell responses after alloHCT (**Figure 1**) (6, 28–30, 35). The first triggering event that makes a donor T cell alloreactive is the activation of its TCR by the peptide/HLA complex (signal 1). TCR engagement leads in the activation of a series of intracellular downstream signaling pathways that ultimately result in the nuclear translocation of key transcription factors such as *nuclear factor-kappa B* (NF-κB), *Adaptor-related Protein complex 1* (AP1), and *nuclear factor of activated T cell* (NFAT), whose coordinated activity orchestrates the complete activation of the T cell, its proliferation and its synthesis of cytokines and cytokine receptors, such as IL-2 and CD25 (the $\alpha$ subunit of the high affinity $\alpha\beta\gamma$ forms the IL-2 receptor) (36). Besides the basic biology, the blockade of one of these TCR-downstream signaling pathways, namely the NFAT calcium/calcineurin-dependent transduction pathway, was one of the first strategies explored to repress alloreactive T-cell activation after alloHCT in pioneered preclinical and clinical studies (37) and is still currently universally used as a standard approach for aGVHD prophylaxis (see below). Inhibition of the NF-κB pathway was also demonstrated to be efficacious for reducing proliferation, survival, cytotoxic functions and production of cytokines in alloreactive T cells during aGVHD (38–40).

Along with TCR activation, additional positive costimulation (*signal 2*) is required to allow complete T-cell activation and avoid anergy or apoptosis (41). Multiple T-cell positive costimulatory molecules have been identified to play role in aGVHD, such as CD28, *inducible co-stimulator* (ICOS), OX40, and 4-1BB [nicely reviewed in (41, 42)] (**Figure 1**). Their cognate ligands [namely B7 ligands (CD86 or CD80), *B7-related protein-1* (B7RP-1), OX40L and 4-1BBL, respectively] are highly expressed at the surface of mature antigen presenting cells (APCs). Among all of the T-cell costimulatory receptors, the most extensively studied is CD28, which is constitutively expressed at the surface of naive T cells. Another B7 receptor, induced with T-cell activation, is *cytotoxic T-lymphocyte-associated protein 4* (CTLA-4) that has similar structure to CD28 and acts as a competitor for CD80 and CD86 ligation, resulting in dowregulation of T-cell responses. Blockade of CD28/B7 interactions has been shown to attenuate alloreactive T-cell activation, induce tolerance to host alloantigens and to reduce aGVHD in *in vitro* studies and animal models of alloHCT (43–46). One of these approaches consists in using fusion proteins of the Fc region of human immunoglobulin with the extracellular domain of CTLA4 (CTLA4-Ig) (43, 45) and is tested for aGVHD prevention in clinical trials (see below).

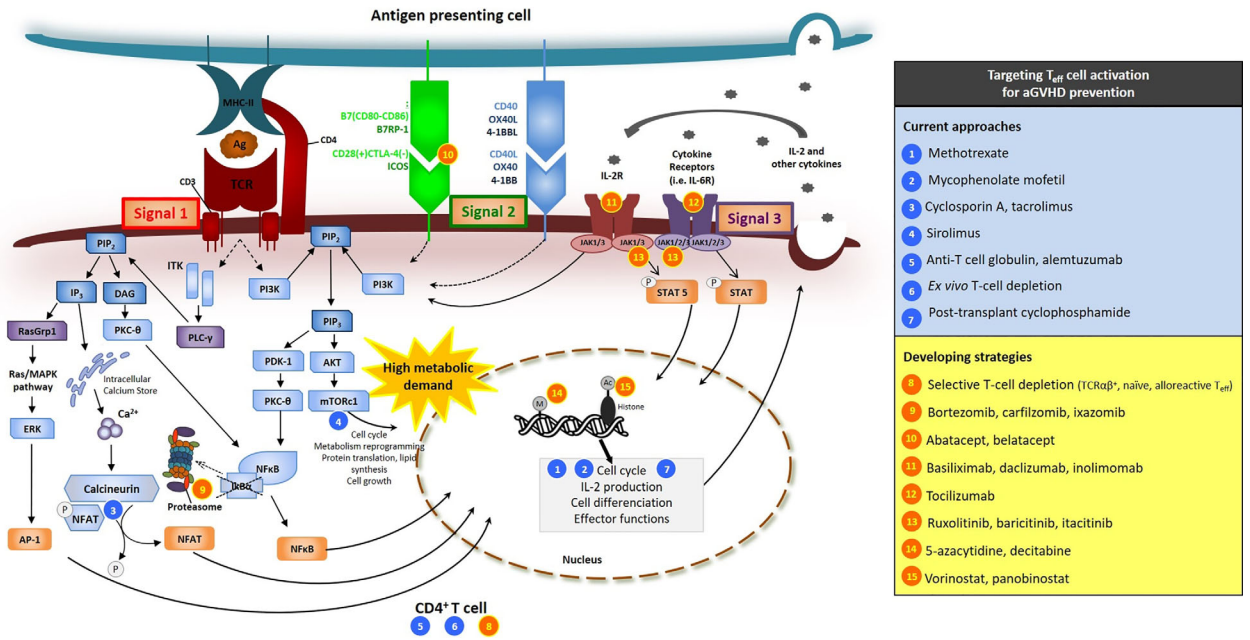


**FIGURE 1** | Signals 1, 2, 3 of T-cell activation and sites of action of several molecules used or tested in clinical trials for aGVHD prevention. Sites of action of current approaches (blues circles) and developing strategies (orange circles) are presented. Beyond their effects on $T_{eff}$, several of these molecules have also effects on other cell types (see text). [adapted from (35)].

The third signal for sustained T-cell activation, acquisition of effector functions and survival is provided by cytokines [*signal 3*, nicely reviewed in the context of aGVHD in (42, 47)]. Among all, IL-2 is a key cytokine for alloreactive T-cell proliferation and survival. Produced by activated T cells, IL-2 acts through paracrine and autocrine signaling, further providing a self-activation loop. Among others, IL-2 receptor intracellular signaling in T cells include JAK (janus kinase)/STAT (*signal transducers and activators of transcription*) pathways (namely JAK1 and JAK3/STAT5 in particular) (**Figure 1**) (48, 49). JAK1/2 pathways are also involved in signal transduction downstream the receptors of multiple other cytokines (such as IL-6, IL-12, ...) and play major role in T-cell proliferation, polarization, and metabolic reprogramming (49). Pharmacological inhibition of JAK1/2 pathways was demonstrated to reduce aGVHD in preclinical models (50) and to be efficacious for the treatment of patients with steroid refractory-aGVHD (see above, *INTRODUCTION*) (26). Mechanisms of JAK1/2 inhibition on aGVHD reactions include at least decreased Th1 and Th17 differentiation, but also broad anti-inflammatory and immunosuppressive effects on multiple immune cell types [reviewed in (50)]. It is commonly accepted that pathogenic Th1 and Th17 cells as well as their polarizing cytokines [IL-12 and interferon gamma (IFN$\gamma$); IL-6, IL-1$\beta$, IL-21 and IL-23, respectively] play important role during aGVHD (42, 47, 51). Among all cytokines, IL-6 is the hallmark of pro-inflammatory cytokines and increased systemic IL-6 levels were reported in patients early after alloHCT (52, 53). IL-6 signaling in donor T cells is critical for the polarization of donor naive T cells towards Th17/Tc17, but IL-6 also exerts many other effects (such as several on DC and regulatory cells) (54, 55).

*Mechanistic/mammalian target of rapamycin* (mTOR) is another key signaling kinase in T cells that integrate an array of activating signals (including the three aforementioned signals of T-cell activation) and environmental cues to regulate cell survival, growth, proliferation, differentiation, and metabolism (56). Inhibition of mTOR Complex 1 (mTORC1) has demonstrated efficacy against aGVHD in preclinical models (56–58) and has been explored as GVHD prevention in clinical trials for several years (see below).

Over the past decade, it has become increasingly clear that metabolic reprogramming of the T cell is required to enable the transition from a naive T cell to a proliferative and differentiated T cell that will drive immune effector functions and mediate aGVHD. Studies have reported that effector T cells use multiple metabolic pathways (glycolysis, oxidative phosphorylation, fatty acid oxidation, glutaminolysis) to keep the pace with high energy demands during aGVHD, (59, 60). Furthermore, the metabolic demand of different T cell subsets is likely not identical.

A key event in the initiation phase of aGVHD is the interaction of $CD4^+$ and $CD8^+$ donor T cells with activated APCs (*via* cross-presentation for the latter) that provide the three aforementioned signals. During the initiation phase of aGVHD, most of the APCs are host-derived hematopoietic APCs and host non-hematopoietic APCs (intestinal epithelial cells, keratinocytes, myofibroblasts...) (61, 62). By expressing

pattern recognition receptors (PRR) such as Toll-like (TLR) and nucleotide oligomerization domain (NOD)-like receptors, innate immune cells and some epithelial cells are able to detect danger signals such as sterile DAMP (*damage-associated molecular pattern* molecules, which are released from dying cells or disrupted extracellular matrix) and PAMP (*pathogen-associated molecular pattern* molecules, which can be released from invasive bacteria, fungi or viruses at the epithelial surfaces). After alloHCT, an increased number of DAMP and PAMP molecules can be released as a consequence of cytotoxic conditioning regimen or aGVHD [reviewed in (63)]. After alloHCT, several studies have demonstrated that host exposure to gut microbial flora and PAMPs due to disrupted intestinal barrier can be an important initiating event in aGVHD reactions (64–67). Mechanisms include the recruitment and activation of host neutrophils (which further contribute to tissue damage and inflammation) as well as inflammatory macrophages, dendritic cells and non hematopietic APCs (which further prime T cells) (61, 67–69).

Beyond T-cell activation and clonal expansion, T-cell chemotaxis towards secondary lymphoid organs and target tissues are also important in aGVHD immunobiology [nicely reviewed in (70)]. For example, among the so-called "homing receptors", the chemokine-receptor CCR7 and the L-selectin (CD62L) are expressed at the surface of naive and central memory T cells and direct them to secondary lymphoid organs in which they can be primed and activated by professional APCs. This raises the hypothesis that T cells may contribute differently to aGVHD according to their differentiation status, with naive $CD4^+$ T cells being more prone to cause aGVHD than (late) effector memory $CD4^+$ T cells (71, 72). In addition, T-cell migration towards GVHD target organs is also crucial to cause aGVHD. Namely, the chemokine receptor CCR5 is involved in T-cell migration towards lymph nodes, the GI tract and the liver. Hence, CCR5-chemotaxis blockade was reported to limit aGVHD in some murine models (73, 74). Integrins also participate in T-cell migration to target organs and the specific tissue expression of some of them may make their study interesting in the context of aGVHD. Several mouse studies have indeed suggested that $\alpha4\beta7$ integrin on donor T cells was important for T-cell migration into gut-associated lymphoid tissues and for the development of GI aGVHD (75, 76).

After being primed by APCs in secondary lymphoid organs, activated and differentiated donor T cells migrate to target organs where they generate effector T cell ($T_{eff}$) responses (effector phase of aGVHD). Cytotoxic T cells can cause direct target tissue cell death *via* diverse cytolytic pathways that involve the release of granzyme B and perforin and the expression of members of the *tumor necrosis factor* (TNF) family (including FasL). Immune activation and tissue lesions lead to a cytokine storm that further recruits multiple cellular effectors (*e.g.* other T cells, neutrophils, and activated macrophages) and brings molecular effectors (*e.g.* TNF-$\alpha$, IFN-$\gamma$, complement molecules, reactive oxygen species, ...), further intensifying tissue lesions and inflammatory responses (amplification phase) and thus leading to sustained aGVHD reactions and severe end-organ damages.

## Mechanisms Establishing Immune Cell and Tissue Tolerance During aGVHD

As mentioned above, $T_{eff}$ cell activation and proliferation are negatively regulated by co-inhibitory signals. In addition to these T-cell intrinsic pathways, peripheral immune tolerance can also be achieved by the intervention of several anti-inflammatory molecules as well as tolerogenic cells. In the context of aGVHD, all of these components can help restraining the destructive machinery of immune cell and limiting tissue damages.

Numerous investigations have focused on regulatory T cells ($T_{regs}$), which can exert multiple tolerogenic and anti-inflammatory effects [nicely reviewed in (77–79)]. $T_{regs}$ are characterized by the expression of the master *forkhead box protein 3* transcription factor (FoxP3) and their constitutive expression of the surface receptor CD25, the high affinity IL-2R $\alpha$-chain (in contrast to Teff in which CD25 expression starts upon the TCR activation) (80). Hence, in steady-state conditions (low dose of IL-2), $T_{regs}$ capture all the IL-2 molecules in the milieu, therefore quenching spurious activation of $T_{eff}$. There are several types of $CD4^+$ $T_{reg}$: (1) "*natural thymus-derived* $T_{reg}$*"* ($nT_{reg}$ or $tT_{reg}$), generated from lymphoid precursors in the thymus; and (2) "peripheral $T_{reg}$" ($pT_{reg}$), derived from the differentiation of conventional naive T cells in secondary lymphoid organs in the context of low-dose or tolerogenic antigen exposure and upon IL-10 and TGF-$\beta$ stimulation. $pT_{reg}$ can also be generated *in vitro* and in this case are referred as "*induced* $T_{reg}$" ($iT_{reg}$). Interestingly, preclinical studies in mice have shown that co-transplanting high doses of $CD4^+$ $iT_{reg}$ or infusing fewer freshly isolated $T_{reg}$ from donor peripheral blood (likely containing a mixture of t- and $pT_{reg}$) several days prior to alloHCT in lymphopenic conditions was effective for mitigating allogeneic and human xenogeneic GVHD (81, 82). However, one issue with $T_{reg}$ adoptive transfer could be their phenotypic and functional instability in the context of prolonged inflammation (such as during aGVHD), causing them to lose their immunosuppressive properties and even acquire pro- inflammatory functions. Such observations were made in mice (83, 84) but also with human $T_{reg}$ in the context of xenogeneic GVHD (85).In comparison to $tT_{reg}$, the expression of FoxP3 is more unstable in $iT_{reg,}$ since they lack the locked-in gene expression signature of transcription factors implicated in FoxP3 activity stabilization. Specifically, hypermethylation of FoxP3 gene/promoter in $iT_{reg}$ was reported to destabilize their phenotype (86). By contrast, phenotypic and functional stabilization of $T_{reg}$ cells has been demonstrated with hypomethylating agents in a model of xenogeneic GVHD (87).

Type 1 regulatory T cells (Tr1) are another subset of suppressive peripheral T cells, still suppressing immune response similarly to t- and $pT_{reg}$ but characteristically lacking CD25 and Foxp3 lineage marker expression (88). Although if this subpopulation has been only partly unraveled so far, Tr1-like cells are being considered more and more important for immune response homeostasis. Similarly to $iT_{reg}$, Tr1-like cells can be induced *in vitro* (88, 89), and a recent preclinical co-transfer study has shown promising results for suppressing GVHD (89).

Other cell types that have been reported to exert immunoregulatory properties during aGVHD, include invariant natural killer T cells (iNKT), natural killer cells (NK), innate lymphoid cells (ILC), tolerogenic dendritic cells, various myeloid suppressor populations of hematopoietic [*e.g.*, myeloid-derived suppressor cells {MDSCs}, CD34+ regulatory monocytes] and stromal origin [*e.g.*, mesenchymal stromal cells (MSCs)] (90–92). In particular, iNKT cells are under increased investigation, owing to their reported suppressive activity against GVHD in preclinical models (93, 94).

In addition to these tolerogenic immune cell subtypes, other non-immune cells and components of the damaged organs can also reveal protective properties in the context of aggression, through several mechanisms including the up-regulation of anti-inflammatory surface receptors, release of tolerogenic soluble factors and activation of repairing mechanisms (a concept known as "tissue-tolerance"). This concept has been described in recent nice articles (95, 96). Among others, IL-22, keratinocyte growth factor (KGF), R-spondin-1 (R-Spo1) and glucagon-like peptide 2 (GLP-2) were reported to be protective against GI manifestations of aGVHD (*e.g.* by preserving and/or enhancing the regeneration of intestinal epithelial cells, intestinal stem cells and/or Paneth cells) (97–101). Paneth cell secretion of antimicrobial peptides (*e.g.* $\alpha$-defensin) is also critical for maintaining the GI microbial ecosystem (97).

There is also growing evidence that the commensal microbiota at mucosal and cutaneous surfaces plays important role in tissue homeostasis and immune tolerance after alloHCT. This concept has been particularly studied at the intestinal interface [nicely reviewed in (102)]. It was recently reported that the bacterial and viral gut microbiota is altered (with loss of diversity and dominance of some taxa) after alloHCT and that such dysbiosis may be associated with aGVHD outcomes (103–107). Regarding bacteria, low intestinal abundance of gut commensals belonging to the *Lactobacillales*, *Clostridiales* and *Blautia* genus was reported to be associated with and increased incidence of lethal aGVHD and poor survival (104, 108). Consistent with this, increased risk of aGVHD-related death was also reported with the use of some anti-anaerobic or broad-spectrum antibiotics in mice and in patients (104, 109–111). However, most of the aforementioned studies were based on associations, and the causations as well as the precise mechanisms of how the microbiota can influence immune and tissue tolerance post-allo-HCT remain to be determined. Recent data suggested that an important way could be through microbiota-derived metabolites (112, 113). A recent elegant work has indeed highlighted significant variations in microbiota-derived metabolites (especially aryl hydrocarbon receptor ligands, bile acids and plasmalogens) at the onset of aGVHD in patients (114). A significant reduction in fecal levels of butyrate [a short-chain fatty acid (SCFA) generated by the fermentation of non-digestible carbohydrates by certain anaerobic commensal bacteria] in patients after alloHCT was also alloHCT reported by another group (114). Interestingly, in a mouse model, restoring butyrate levels, either by direct administration of butyrate or by changing the composition of intestinal microbiota towards an increase in butyrogenic bacteria (*e.g.* selected strains of *Clostridia*) mitigated aGVHD and improved survival (115). Understanding the precise effects of all these metabolites on host tissues and immunity is the subject of intense current research, with some data already suggesting various potential roles in enhancing the trophicity and regenerative properties of the intestinal epithelium as well as in modulating innate and adaptive immune responses (102, 112, 113). Overall, these findings highlight the likely major role of the microbiome-metabolome axis in aGVHD, which may offer potential new targeted strategies to explore for improving aGVHD prophylaxis or treatment.

# HOW TO PREVENT AGVHD AFTER ALLOHCT? WHEN THE CLINICIAN MEETS THE IMMUNOLOGIST

## Conventional Strategies for aGVHD Prevention

Currently, there is no standardized aGVHD preventive approach. However, the backbone of most conventional prophylactic regimens is based on T-cell immunosuppression, by the pharmacological inhibition of their clonal expansion and activation and/or by their direct depletion (116). Here, we provide a short overview of the current standard regimens for aGVHD prevention and briefly describe their biological rationale. For detailed clinical considerations, readers are referred to the recently published 2019 EBMT consensus recommendations for aGVHD prophylaxis and treatment (117).

Since the mid-1980s (37), the most commonly adopted GVHD prophylaxis regimens among patients given alloHCT with BM or PBSC from HLA-matched sibling or unrelated donor consist in the combination of an anti-metabolite [either short course of methotrexate (MTX) or mycophenolate mofetil (MMF)] with a calcineurin inhibitor [CNI, either cyclosporin A (CSA) or tacrolimus (FK506, tacro)]. The former (MTX or MMF) delete proliferating T cells, while the second (CNI) blocks TCR-induced T-cell activation (*signal 1*) by interfering with NFAT nuclear translocation thereby reducing transcription of IL-2 (**Figure 1**).

Several other alternative regimens have also been explored with the aim of improving the control of aGVHD and/or reducing drug toxicity. Among them, administration of mTOR inhibitors [of which sirolimus (siro) is the most widely studied molecule] has been tested for several years (**Figure 1**). Unlike CNIs which, by reducing IL-2 production, limit $T_{eff}$ activation but with a concomitant negative impact on IL-2-dependent $T_{reg}$, inhibition of the mTOR signaling pathway precludes the activation of $T_{eff}$ while preserving $T_{reg}$ activity (which are less dependent on the mTOR/Akt pathway) (58, 118). Several randomized phase III trials have addressed the effects of siro either as a substitution of MTX (tacro + siro *vs.* tacro + MTX) in myeloablative TBI-based alloHCT (119) or in addition to the standard prophylaxis (tacro + MMF + siro triplet regimen) after

non-myeloablative/RIC-alloHCT (120). Although these studies provided encouraging results, clinical data and experience with siro are still considered insufficient to recommend its routine use as part of the prophylactic regimen (117). Moreover, a warning has been issued with the use of siro after high dose busulfan-based conditioning regimens due to the increased risk of sinusoidal obstruction syndrome (121).

For almost two decades, *in vivo* T-cell depletion using serotherapies with rabbit anti-T-cell globulin (ATG, ATG-Thymoglobulin® or ATG-Grafalon®) (122–126) or alemtuzumab (ALEM, an anti-CD52 monoclonal IgG1 antibody) (127) has also been used to prevent GVHD. Both of these antibody preparations have a long half-life in the human plasma and therefore, once administered as part of the conditioning regimen, they exert their biological effects for several weeks after the graft infusion and induce profound depletion of both host and donor immune cells (128, 129). Moreover, besides the pan T-cell depletion (**Figure 1**), ATG and ALEM also mediated a variety of other immune effects [detailed in other informative reviews (130, 131)]. Several large randomized phase 3 trials have demonstrated the benefit on both a- and cGVHD incidence of adding ATG to standard prophylaxis in the setting of MAC-alloHCT with PBSC (122–126). In a related approach, *ex-vivo* immune cell depletion of the graft (*e.g.* by immunomagnetic positive selection of $CD34^+$ stem cells or ALEM in the bag) was also evaluated and proved to be effective to prevent GVHD (132–134). However, a major concern with such an approach is its negative impact on GVL effects and immune recovery.

In recent years, there has been an exponential increase in the number of haplo-alloHCT performed worldwide. This was made possible thanks to the development of innovative platforms for GVHD prevention in this peculiar high alloreactivity setting. Among them, the advent of post-transplant cyclophosphamide (PTCy) has revolutionized this procedure and can be considered as one of the major advances in the field of alloHCT over the past two decades (135–139). This approach, designed by the John Hopkins University group in Baltimore, consists in the administration of (one or) two boluses of high dose cyclophosphamide (Cy, a nitrogen mustard alkylating agent) shortly after alloHCT (day +3 and/or +4) followed by MMF/tacro prophylaxis (starting from day +5). The initial rationale of this strategy mostly assumed to be a cytotoxic and selective depletion of highly proliferative $T_{eff}$ (supposed to be the newly primed alloreactive T cell clones during the first days after the graft infusion) (**Figure 1**), while preserving resting hematopoietic stem cells and non-alloreactive T cells (such as anti-infectious memory T cells) (135). Additional researches further demonstrated that PTCy also induces central tolerance by additional intrathymic clonal deletion of alloreactive T cell precursors (140, 141). Moreover, it was recently suggested that beyond these effects on $T_{eff}$, PTCy-mediated protection against GVHD also (and mainly) relies on the promotion of $T_{reg}$ and the induction of tolerance (135, 141). $T_{reg}$ are indeed less sensitive than $T_{eff}$ to the Cy cytotoxic effects due to their higher expression of aldehyde dehydrogenase (the major detoxifying enzyme for cyclophosphamide) (142). In murine PTCy haplo-alloHCT models, Kanakry et al. showed that PTCy does not completely eliminate alloreactive $T_{eff}$, but instead alters T-cell response to alloantigens and induces the rapid and preferential recovery and expansion of $T_{reg}$ (142). Evidence for the pivotal role of $T_{reg}$ in PTCy-mediated immune tolerance is also illustrated by the development of severe and fatal GVHD when FoxP3+ $T_{reg}$ are depleted (143). Going back to clinical studies, the pioneer pilot trial with the PTCy strategy led by the Baltimore group reported a very low incidence of grade III–IV aGVHD (10%) in patients transplanted with HLA-haploidentical BM after non-myeloablative conditioning regimen (138). Similar encouraging results were further observed by numerous other groups, even using PBSC as the stem cell source and more intensive conditioning regimens (139, 144). Beyond haplo-alloHCT, PTCy recently starts gaining popularity in other settings, including HLA-matched sibling/unrelated donor and HLA-mismatched unrelated donor alloHCT (145). Recently, in a large multicenter phase III trial comparing several novel immunosuppressive prophylactic regimens (PTCy + tacro +MMF; tacro + MMF + bortezomib; tacro + MMF + maraviroc) with the contemporary standard tacro + MTX scheme after RIC-alloHCT, PTCy + tacro + MMF appeared to be the most promising intervention, yielding the best GvHD-free, relapse-free survival (GRFS) (146). It is currently unknown whether another combination (*i.e.* MMF/siro) can be as effective as MMF/CNI in addition to PTCy in haplo-alloHCT, or even if PTCy can be safely used as a single agent after HLA-identical sibling transplantation. It is the subject of numerous investigations.

## Developing Strategies for aGVHD Prevention

The deeper understanding of aGVHD immunobiology has facilitated the diversification of preventive strategies, and many novel approaches are currently under investigation (6, 29, 116). The concrete clinical goal of aGVHD prophylaxis after alloHCT is to prevent or at least to significantly reduce the damage to target tissues induced by alloreactive immune responses in order to decrease the risk of clinically relevant organ dysfunction leading to "clinical aGVHD". To achieve this objective, the current strategies being developed/under investigation for limiting aGVHD after alloHCT can be categorized according to three main areas of intervention: (1) limitation of donor-derived immune cell alloreactivity, (2) promotion of immune tolerance, and (3) modulation of the target tissue environment to make it less prone to but rather more resistant to aGVHD immunopathology and to improve regenerative properties. Given the large number of strategies under development, it is difficult to cover them all. Here, we have chosen to present some of those which have already reached clinical trials and which seem to be the most promising in our opinion (**Table 1**).

### Strategies Aimed at Limiting Alloreactivity of Donor Immune Cells (Mainly T Cells) Against Host Tissues

As donor $T_{eff}$ are main causative agents of aGVHD, huge efforts have been made to optimize and refine donor $T_{eff}$ depleting

**TABLE 1** | Developing strategies for aGVHD prevention.

| Developing strategies | Ongoing clinical trial | $T_{eff}$ depletion | Inhibition of signal 1 of $T_{eff}$ activation | Inhibition of signal 2 of $T_{eff}$ activation | Inhibition of signal 3 of $T_{eff}$ activation | Inhibition of $T_{eff}$ trafficking | Promotion of immune tolerance | Modulation of microenvironment |
|---|---|---|---|---|---|---|---|---|
| | | Main putative mechanisms of action | | | | | | |
| *Ex vivo* depletion of TCR$\alpha\beta$+/CD19+ donor cells [phase I–II (147)] | NCT04088760 (phase II)<br>NCT02508038 (phase I) | X | | | | | | |
| *Ex vivo* depletion of CD45RA$^+$ naive T cells [phase II (148)] | | X | | | | | | |
| *Ex vivo* photodepletion of anti-host reactive donor T cells (Kiadis) [phase II (149)] | NCT02999854 (phase III) | X | | | | | X | |
| Proteasome inhibitors (bortezomib) [phase I–II (146, 150, 151)] | NCT03945591 (phase II)<br>NCT03082677 (phase II)<br>NCT01991301 (phase I)<br>NCT02145403 (phase I–II) | | X | | | X | X | X |
| $\alpha$-CTLA-4 Ig (abatacept, belatacept) [phase II (152–156)] | NCT02867800 (phase I)<br>NCT01743131 (phase II)<br>NCT04380740 (phase II) | | | X | | | | |
| Anti-IL-6 receptor antibody (tocilizumab) [phase I–II (52, 53)] | NCT03434730 (phase II) | | | | X | | | X |
| Janus kinases inhibitors (anti-JAK1/2 ruxolitinib, and baricitinib; anti-JAK1 itacitinib) | NCT02806375 (phase I–II)<br>NCT04131738 (phase I)<br>NCT04127721 (phase II)<br>NCT03755414 (phase I)<br>NCT03320642 (phase I) | | | | X | | X | X |
| Demethylating agents (5-azacytidine, decitabine) [phase I–II (157)] | NCT00813124 (phase II)<br>NCT01758367 (phase I–II) | X | X | X | X | X | X | X |
| Histone deacetylase inhibitors (vorinostat, panobinostat) [phase I–II (158, 159)] | NCT03842696 (phase I–II)<br>NCT03842696 (phase I–II)<br>NCT02588339 (phase II) | X | X | X | X | X | X | X |
| CCR5 blocker (maraviroc) [phase I–II (74, 146, 160)] | NCT02799888 (phase II) | | | | | X | | |
| $\alpha 4\beta 7$ integrin blocker (vedolizumab) (phase I–II) (161) | NCT03657160 (phase III) | | | | | X | | |
| Low dose IL-2 [phase I (162, 163)] | NCT02659657 (phase II) | | | | | | X | |
| $T_{reg}$ infusion [phase I–II (reviewed in (164)] | NCT01795573 (phase I)<br>NCT03977103 (phase II)<br>NCT04013685 (phase I) | | | | | | X | |
| Mesenchymal stromal cells [phase I–II (reviewed in (165))]<br><br>iNKT cells [$\alpha$galCer, phase II (166) , TLI conditioning (129)] | NCT02270307 (phase II–III)<br>NCT01045382 (phase II)<br>NCT04247945 (phase II–III)<br>NCT03605953<br>NCT00631072 | | | | | | X<br><br>X | |

*(Continued)*

**TABLE 1** | Continued

| Developing strategies | Ongoing clinical trial | $T_{eff}$ depletion | Inhibition of signal 1 of $T_{eff}$ activation | Inhibition of signal 2 of $T_{eff}$ activation | Inhibition of signal 3 of $T_{eff}$ activation | Inhibition of $T_{eff}$ trafficking | Promotion of immune tolerance | Modulation of microenvironment |
|---|---|---|---|---|---|---|---|---|
| | | Main putative mechanisms of action | | | | | | |
| Recombinant urate-oxidase [phase I (167)] | – | | | | | | | X |
| Alpha-1-antitrypsin | NCT03805789 (phase II-III) | | | | | | | X |
| Keratinocyte growth factor [phase I-II (168, 169)] | – | | | | | | | X |
| Probiotics and fecal material transplantation [phase I-III (170–172)] | NCT03720392 (phase II) | | | | | | | X |
| Prebiotics | NCT02805075 (phase I) NCT02763033 (phase II) | | | | | | | X |

*Strategies Aimed at Limiting Alloreactivity of Donor Immune Cells (Mainly T Cells) Against Host Tissues (red); Strategies Aimed at Promoting Immune Tolerance (blue); Strategies Aimed at Modulating Target Tissue Environment (green).*

approaches, *e.g.* by selectively depleting specific T-cell subpopulations. In particular, the selective depletion of TCR$\alpha\beta$+ cells, of naive T cells, or even of activated alloreactive T cells (*e.g.* with *ex vivo* photodepletion of anti-host reactive donor T cells) has demonstrated encouraging results for aGVHD prevention (147–149). Besides $T_{eff}$ depleting approaches, strategies aimed at functionally interfering with $T_{eff}$ activation (signal 1, 2 and/or 3, **Figure 1**), intracellular signaling pathways, metabolism and homing properties are also developing as well as gene editing approaches.

As described above, signal transduction downstream of TCR activation (signal 1) in $T_{eff}$ occurs through multiple pathways that result in the nuclear translocation of key transcription factors, including NFAT, NF-$\kappa$B and AP1. Blockade of the NFAT calcium-dependent transduction pathway with CNI (CSA or tacro) is universally used as standard GVHD prophylaxis. Inhibition of the NF-$\kappa$B pathway also recently appeared as an interesting approach. Proteasome inhibitors, such as bortezomib (BOR), have been shown to suppress NF-$\kappa$B activation (in part by reducing the degradation of its inhibitory protein I$\kappa$B$\alpha$) and were reported to confer protection against GVHD in mouse models (39). Moreover, by reducing the degradation of many other intracellular proteins, blocking the proteasome also has an impact on T-cell chemotaxis, secretion of inflammatory cytokines, APC functions and promote $T_{reg}$ (173). Based on these observations, the early addition of short-course BOR (on days +1, +4, and +7 after alloHCT) to standard tacro/MTX has been assessed in phase I–II clinical trials and provided encouraging results (150). However, in a large open-label three-arm phase 2 randomized trial comparing conventional Tacro/MTX *vs.* BOR/Tacro/MTX and *vs.* BOR/Tacro/Siro after UD RIC-alloHCT, BOR-based regimens failed to show an improvement in day +180 aGVHD incidence (32.6, 31.1 and 21%, respectively) (151). Similarly, in another large prospective phase II study comparing several novel prophylactic regimens with contemporary MTX/tacro controls, the addition of BOR to standard MTX/tacro in RIC-alloHCT did not result in lower aGVHD incidence (146). Combination of BOR with other agents, such as PTCy, as well as use of other proteasome inhibitors (carfilzomib, ixazomib) is currently under investigation (**Table 1**).

Targeting costimulatory signals at the APC/T-cell interface (signal 2) has also been investigated as aGVHD prophylaxis for several years. Of all these strategies, the one that has reached the more advanced stage of development concerns CTLA4-Ig (abatacept, belatacept). Addition of abatacept to background CNI-based aGVHD prophylaxis in the setting of alloHCT with HLA-matched donor has produced promising results in phase I–II clinical trials (152, 153). Addition of abatacept to the PTCy platform is also under investigation in the setting of haplo-alloHCT for non-malignant disorders (154, 155). Moreover, unlike T-cell anergy, recent data have shown that NK cell cytotoxicity is not altered, but even enhanced in the presence of CTLA4-Ig. This makes the CTLA4-Ig approach an interesting

strategy for reinforcing GVT effects while still limiting aGVHD risks in the setting of HLA-mismatched donor (haplo) transplantation. This hypothesis prompted several groups to study the CTLA4Ig sequential primed donor lymphocyte (DLI) infusion protocols after PTCy-based haplo-alloHCT as adoptive immunotherapy in patients with advanced malignant disorders (156). Of note, one issue with targeting CD80/CD86 with CTLA4-Ig may be associated with concurrent undesired blockade of tolerogenic CTLA4-dependent signaling to $T_{reg}$ and APCs. Hence, CD28-specific inhibition is under investigation in preclinical studies (44).

Different strategies that target signal 3 of T-cell activation by blocking cytokines or their receptors were also tested in clinical studies (42, 47). Among them, blockade of IL-2 signaling with monoclonal antibodies binding to the IL-2 receptor α-chain CD25 (*e.g.* basiliximab, daclizumab, inolimomab) was unfortunately discouraged for controlling aGVHD since it was reported to be associated with increased GVHD-related mortality (174, 175). This is likely due to the negative impact of IL-2 blockade on suppressive $T_{reg}$ since IL-2 is not only crucial for $T_{eff}$ expansion but also for $T_{reg}$ homeostasis.

IL-6, TNF-$\alpha$, and IL-1$\beta$ are important pro-inflammatory cytokines in aGVHD pathogenesis. Addition of Tocilizumab (an anti-IL-6 receptor monoclonal antibody) to CNI/MTX prophylaxis has been tested in phase I–II studies and has been shown to be associated with a very low incidence of grades II–IV aGVHD (<15%) (52, 53). However, these promising results have to be confirmed in larger phase III studies. By contrast, inhibition of TNF-$\alpha$ or IL-1$\beta$ added to standard GVHD prophylaxis failed to prevent aGVHD (176, 177). Several additional cytokines (such as IL-12, IL-23, GM-CSF, *etc.*) have also been implicated in aGVHD pathogenesis, and their inhibition should also be evaluated in the future.

T cells respond to many inflammatory cytokines (including IL-6) through JAK/STAT pathways. As described above, several studies have shown that the inhibition of JAK1/2 pathways (*i.e.* with ruxolitinib and baricitinib, two JAK1/2 inhibitors, or with itacitinib, a selective JAK1 inhibitor) prevented aGVHD in preclinical model (50) and was efficacious for controlling steroid refractory-aGVHD in patients (26). Further ongoing studies are investigating the use of this molecule and other JAK1/JAK2 inhibitors for aGVHD prevention (**Table 1**). Itacitinib, which inhibits JAK1 while sparing JAK2, is expected to have reduced myelosuppressive activity compared to broader specificity JAK inhibitors.

Encouraging results also come from epigenetic modifiers [*e.g.* demethylating agents such as 5-azacytidine, decitabine, histone deacetylase inhibitors (HDACi)] which can exert pleiotropic effects on aGVHD reactions, not only on the fate of $T_{eff}$ but also on other immune cells (such as $T_{reg}$ and DCs) (157–159). For example, the addition of vorinostat (a HDACi) to standard GVHD prophylaxis after alloHCT with HLA-matched donors was examined in two phase 2 clinical trials (158, 159). Both studies showed that vorinostat was well tolerated and was associated with a low incidence of aGVHD (grade II-IV aGVHD less than 25%, and grades III–IV less than 10%). Additional advantages of such approaches lie in the fact that, besides their immunomodulatory effects, these molecules (demethylating agents and HDACi) can also exert anti-tumor activity, therefore offering opportunities for mitigating GVHD while enhancing anti-tumor effects.

Interfering with the homing of $T_{eff}$ towards target organs can be viewed as an additional strategy for preventing aGVHD. A phase I–II study indeed investigated the addition of maraviroc (a CCR5 antagonist) to standard tacro/MTX after RIC-alloHCT in adults and demonstrated a low incidence of visceral (GI and liver) grades II–IV aGVHD (14.7%) (74). Similar encouraging results were observed in pediatric patients (160). Nevertheless, a recent multicenter phase II trial comparing several new prophylactic regimens with contemporary MTX/tacro controls in RIC-alloHCT showed that, when added to standard MTX/tacro, maraviroc did not result in lower GVHD rates compared to PTCy or BOR (146). The redundant mechanisms in the signaling of chemokines/chemokine receptors may be an explanation for the limited effectiveness of strategies based on blocking just a single chemokine receptor. Integrins also represent attractive potential targets for novel preventive therapies against GVHD. Low incidences of grades II–IV overall and lower-intestinal aGVHD (19 and 14% at day 100, respectively) were recently observed in a phase Ib study in which patients received vedolizumab (an antibody directed againts $\alpha4\beta7$ integrin) in combination with standard tacro/MTX (161). A large phase III randomized placebo control trial evaluating vedolizumab added to standard aGvHD prophylaxis is currently recruiting (NCT03657160, **Table 1**).

Finally, since T cells consume a lot of energy during aGVHD, it can also be envisaged that targeting metabolic pathways and subverting the use of T-cell energy could offer other potential innovative preventive strategies to explore in the future. The challenge will be to make these molecules specific enough to avoid important toxicities.

## Strategies Aimed at Promoting Immune Tolerance

Rather than trying to decrease the reactivity of the donor immune cells, another way of preventing aGVHD after alloHCT may be through the promotion of tolerance between the donor immune cells and the recipient, by strengthening the tolerogenic arm of the immune system. Indeed, cell-based approaches to promote immune tolerance have shown encouraging results. In our view, the most promising are $T_{reg}$, iNKT, and MSC-based therapies.

Early clinical trials with $iT_{reg}$ infusion in patients have shown promising results for aGVHD prevention (178–180), [reviewed in (164)]. Nevertheless, the major problem with the clinical transfer of $T_{reg}$ is the difficulty of reaching a sufficient number of $T_{reg}$ with good purity to infuse and of ensuring that the transferred cells persist and retain their tolerogenic properties in the inflammatory context of aGVHD (181). Strategies aimed at promoting $T_{reg}$ proliferation in the donor before $T_{reg}$ donation, for example by pretreating the donor with TNF superfamily receptors DR3 agonists, have been reported to

be effective in murine models (182) but have not yet been explored in humans. The scientific community is currently focusing on examining approaches to promote *in vivo* $T_{reg}$ expansion and stability within the recipient. In particular, the high sensitivity of $T_{reg}$ to IL-2 (determined by their constitutive expression of CD25) makes treatment with low doses of this cytokine an interesting approach. A phase I–II study investigated the administration of ultra-low dose IL-2 (100,000–200,000 IU/$m^2$, 3 times/week) after alloHCT and reported promising results in terms of safety and low incidence of aGVHD (0/16 patients experienced grade II–IV aGVHD) (162). Another study using a similar approach is underway in China (NCT02659657, **Table 1**). However, in another trial administration of low doses of IL-2 in addition to tacro/siro for GVHD prophylaxis failed to prevent aGVHD despite resulting in higher $T_{reg}$ levels (163).

As with $iT_{reg}$, adoptive transfer of IL-10/TGF-$\beta$ producing Tr1 cells is gradually being seen as a new option for the prevention of aGVHD. A pilot phase 1 clinical trial evaluating the safety of Tr1 cell co-transplantation in pediatric patients in an HLA-mismatched donors setting is currently being planned (NCT03198234).

A high content of iNKT cells in the transplant has been reported to be associated with a reduced risk of aGVHD in clinical studies (183). Thus, protocols for promoting the expansion of iNKT cells (*e.g.* through *ex* or *in vivo* manipulations) appear as attractive novel strategies to explore in order to prevent aGVHD. Clinical studies involving the *ex vivo* expansion of iNKT cell populations are underway (NCT00631072, NCT03605953, **Table 1**). Recently, it was reported that RGI-2001, a CD1-binding synthetic derivative of alpha-galactosylceramide, activates and expands iNKT cells *in vivo* (166). Conditioning regimens that foster the induction of iNKT cells, such as total lymphoid irradiation, are also being considered (129).

MSCs are multipotent progenitor cells that reside within the BM microenvironnement and several other connective tissues such as the adipose tissue, the umbilical cord, and placenta membranes. Among a wide variety of functions, MSCs also have a multiplicity of immunomodulatory and anti-inflammatory properties, making them attractive candidates to consider as cell-based therapies to prevent aGVHD. Moreover, MSCs are hypoimmunogenic and can therefore be derived from third-party HLA-mismatched donors. A number of preclinical studies using various animal models have evaluated the effectiveness of MSCs in alleviating GVHD. However, results were mixed, with some studies reporting benefits (184), while others did not (185). Several factors, including MSC tissue of origin (BM, adipose tissue, cord blood, placental membranes), cell dose, timing of infusion and pre-activated MSC status likely influenced the results and caused heterogeneity between studies. Pilot clinical studies have also suggested a potential role for MSCs in preventing GVHD (186–188), [reviewed in (165)]. Further studies are currently underway to more precisely assess the impact of MSC co-transplantation on aGVHD (**Table 1**).

## Strategies Aimed at Modulating Target Tissue Environment

Beyond targeting $T_{eff}$ and promoting immune tolerance, approaches aimed at controlling target tissue environment to make it less pro-inflammatory and/or aimed at strengthening its mechanisms of resilience, repair and regeneration ("tissue tolerance") may be considered as complementary strategies to be exploited to mitigate aGVHD clinical severity.

Among others, molecules aimed at reducing danger signal production (*e.g.* recombinant urate-oxidase, alpha-1-antitrypsin) are currently under investigation (167, 189, 190).

Tissue-protective/regenerative approaches that promote the healing of aGVHD-related tissue damages have also emerged as promising complementary strategies to standard aGVHD immuno-prophylaxis. As mentioned above, KGF, R-Spo1, IL-22, and GLP-2 were reported to be protective during GI aGVHD (97–101). To the best of our knowledge, among all these molecules, only KGF has been tested to date in clinical trials for aGVHD prevention. Two phase 1/2 randomized, double-blind, placebo-controlled studies tested peri-transplant palifermin (KGF) administration in combination with standard prophylaxis (168, 169). Both of them failed to demonstrate benefit in terms of reduction of severe grades III–IV aGVHD. Clinical trials on IL-22 IgG2-Fc (NCT02406651) and GLP-2 (Teduglutide, NCT04290429) for the treatment of GI aGVHD are underway, and it is plausible that these drugs will soon be tested for aGVHD prophylaxis.

The accumulation of evidence on the involvement of the commensal microbiota in intestinal tissue homeostasis and immune tolerance post-alloHCT has also recently opened up the concept of manipulating the gut microbiota as an innovative approach to prevent aGVHD. Several strategies under study include careful risk-balanced use of broad-spectrum antibiotics, dietary or pharmaceutical interventions to limit growth of noxious bacterial taxa [*i.e.* eviction of lactose (191) or enteral immunoglobulin administration (192)] and direct transfer of living microbial species using fecal material transplantation (FMT) (170, 171) or selective transfer of microbial consortia (probiotics) (172, 193, 194). Some of them have already reached clinical trials (see **Table 1**). As such, FMT appears to be a promising approach to improve microbiota diversity in alloHCT patients and to limit aGVHD (170, 171). However, considering the highly immunocompromised status of alloHCT patients, safety of FMT should be carefully established in this specific population, particularly regarding risk of bacterial translocation, septicemia and norovirus infection (195, 196). The modulation of the microbiome–metabolome axis with prebiotic/postbiotic interventions is also under investigation. Among others, the microbiota-derived SCFA butyrate appears as an important metabolite for intestinal homeostasis and immune tolerance after alloHCT. Interestingly, one approach for stimulating microbial SCFA production could be *via* dietary supplementation with non-digestible carbohydrates that can be metabolized by selected commensal gut bacteria. Such strategy is currently being explored in clinical trials in alloHCT patients (**Table 1**). Among them, a phase II clinical trial is testing the

safety and early efficacy for GVHD prevention of an oral dietary supplement containing potato-based starch [which was reported to increase microbial butyrate production in healthy volunteers (197)] (NTC02763033). Besides SCFAs, roles of other microbial metabolites (such as indole derivatives, peptides derived from bile acids, aryl hydrocarbon receptor ligands, polyamine, plasmalogens) would also be interesting to explore in the future.

## CONCLUSION AND PERSPECTIVE

AGVHD is a severe complication after allogeneic stem cell transplantation. It results from a highly deregulated immune process, involving a complex network of multiple molecular and cellular mediators and effectors causing end-organ damages mainly to the skin, GI tract and/or liver. Despite prophylactic measures, aGVHD still develops in about 20–50% of transplanted patients, making it an unmet medical need in alloHCT survivorship research. Improved understanding of the pathology of aGVHD has led to the development of novel strategies to optimize its prevention, with some of them appearing particularly promising based on early data from clinical trials. However, these and other new strategies that will be developed in the future will have to be tested in prospective phase 3 trials before they can become standard. Standardization of aGVHD definition criteria and severity grading system using the validated MAGIC criteria will be vitally important to guarantee the quality, reproducibility and interpretation of these future clinical studies.

Theoretically, it would be logical to think that the combination of multiple approaches targeting several aGVHD immunopathological pathways would ultimately provide a complete suppression of aGVHD. However, the complete abrogation of donor-derived immunity after alloHCT is clinically irrelevant, as this would seriously compromise the engraftment, anti-infectious immune reconstitution as well as the beneficial GVT effects. The ideal step in the future would rather be to provide a personalized risk-stratified aGVHD prophylaxis regimen for each patient, reserving intensive immunosuppressive regimens for patients at high risk for aGVHD and avoiding excessive immunosuppression for those at a low risk for aGVHD. To make this approach feasible, the development of future algorithms to improve the accuracy of aGVHD risk prediction will be an essential prerequisite. Algorithms may be based on HLA disparities and other factors, including predictive biomarkers, clinical predictive factors and genetic variants associated with increased risk of aGVHD. Recipient and/or donor single nucleotide polymorphisms (SNPs) for chemokines, cytokines, costimulatory molecules, and micro-RNAs (miRNAs) would also likely allow transplant physicians to identify specific immune profiles predictors of aGVHD in the future. However, these analyses are not yet accessible for a routine assessment in daily clinical practice.

Unlike immunosuppressive strategies, approaches aimed at modulating the interactions between the host and gut microbiota and/or promoting the regenerative properties of the target tissue of aGVHD would likely not increase the risk of non-engraftment or relapse after alloHCT and would therefore appear to be interesting complementary approaches to combine with classical GVHD immunosuppressive prophylaxis. At present, little is known about the precise mechanisms of host–microbiota cross-talk and about tissue-specific tolerance to diseases, but it is a topic of growing interest and intense research.

## AUTHOR CONTRIBUTIONS

BV and LC are co-first authors. TK and SS are co-last authors. All authors contributed to the article and approved the submitted version.

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REVIEW
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# Off-the-Shelf Allogeneic T Cell Therapies for Cancer: Opportunities and Challenges Using Naturally Occurring "Universal" Donor T Cells

*Cynthia Perez, Isabelle Gruber and Caroline Arber**

*Department of Oncology UNIL CHUV, Ludwig Institute for Cancer Research Lausanne, Lausanne University Hospital and University of Lausanne, Lausanne, Switzerland*

Chimeric antigen receptor (CAR) engineered T cell therapies individually prepared for each patient with autologous T cells have recently changed clinical practice in the management of B cell malignancies. Even though CARs used to redirect polyclonal T cells to the tumor are not HLA restricted, CAR T cells are also characterized by their endogenous T cell receptor (TCR) repertoire. Tumor-antigen targeted TCR-based T cell therapies in clinical trials are thus far using "conventional" αβ-TCRs that recognize antigens presented as peptides in the context of the major histocompatibility complex. Thus, both CAR- and TCR-based adoptive T cell therapies (ACTs) are dictated by compatibility of the highly polymorphic HLA molecules between donors and recipients in order to avoid graft-versus-host disease and rejection. The development of third-party healthy donor derived well-characterized off-the-shelf cell therapy products that are readily available and broadly applicable is an intensive area of research. While genome engineering provides the tools to generate "universal" donor cells that can be redirected to cancers, we will focus our attention on third-party off-the-shelf strategies with T cells that are characterized by unique natural features and do not require genome editing for safe administration. Specifically, we will discuss the use of virus-specific T cells, lipid-restricted (CD1) T cells, MR1-restricted T cells, and γδ-TCR T cells. CD1- and MR1-restricted T cells are not HLA-restricted and have the potential to serve as a unique source of universal TCR sequences to be broadly applicable in TCR-based ACT as their targets are presented by the monomorphic CD1 or MR1 molecules on a wide variety of tumor types. For each cell type, we will summarize the stage of preclinical and clinical development and discuss opportunities and challenges to deliver off-the-shelf targeted cellular therapies against cancer.

**Keywords: allogeneic off-the-shelf T cells, virus-specific T cells, unconventional T cells, engineered, CD1, MR1, GVHD, rejection**

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*Banc de Sang i Teixits, Spain*

****Correspondence:***
*Caroline Arber*
*caroline.arber@unil.ch*

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## INTRODUCTION

Engineered T cell therapies using chimeric antigen receptors (CARs) against $CD19^+$ B cell malignancies have been commercialized and have changed clinical practice. Current commercial products are manufactured in a highly personalized way for each individual patient with autologous peripheral blood αβ-TCR T cells (1, 2). Challenges with the use of autologous products include

aspects related to previous chemotherapies or allogeneic hematopoietic cell transplantation (HCT) that can impact on the quantity and quality of the starting material, uncontrollable interpatient variability, and (too) long waiting times for the patients due to global manufacturing chains (3–5). Thus, the development of readily available off-the-shelf allogeneic immune effector cell (IEC) therapy products is an attractive alternative approach. Cell banks can be generated in advance; donors can be well-characterized according to the desired biological parameters of the final product. Major challenges to allogenic IEC therapies include the possibility of dual rejection: infused cells may produce graft-versus-host disease (GVHD), or the host immune system may reject the infused cells (4–6).

In this review, we will seek, evaluate and discuss challenges and opportunities for T cell-based IEC therapies, using naturally occurring "universal" donor T cells. These cells are either characterized by the recognition of well-defined HLA-restricted conventional αβ-TCR antigens, or are HLA-independent and recognize lipids, metabolites or phosphoantigens presented in the context of non-polymorphic receptors on target cells. By definition, these "universal" donor T cells do not produce GVHD, do not require genome editing for safe application as a therapeutic product, and have the capacity to potentially target a wide variety of cancers. We will focus our review on human preclinical and clinical developments including αβ-TCR T cells [virus specific T cells (VSTs), CD1-, and MR1-restricted T cells] as well as γδ-TCR T cells. Finally, we also discuss the potential use of universal TCRs that can be inserted as transgenes into IECs. Engineering of these "universal" donor T cells aims to combine and simultaneously exploit the endogenous natural properties of the cells with engineered properties that enhance the anti-tumor potential of the final product (e.g., recognition of tumor-derived lipids or metabolites by endogenous TCR and cell surface antigen by the introduced CAR).

# NATURALLY OCCURRING "UNIVERSAL" DONOR T CELLS AS PLATFORMS FOR T CELL ENGINEERING

## αβ-TCR T Cell Subsets

Conventional αβ-TCR T cells express HLA-restricted TCRs composed of an α- and a β-chain and recognize peptides presented by HLA molecules on the cell surface of target cells. Selected in the thymus, these T cells constitute the majority of the circulating T cells in the human body (7). Under physiologic conditions αβ-TCR T cells are polyclonal and express an extremely diverse TCR repertoire to cover a wide range of potential target antigens. This TCR diversity is reduced in memory T cell pools that form upon antigen specific expansion and clearance of a pathogen. VSTs directed against cytomegalovirus (CMV) for example are characterized by their oligoclonality, with a limited number of high avidity TCRs dominating the pool of memory VSTs that can re-expand upon repeated viral challenge (8–10). Unconventional αβ-TCR T cells are non-HLA-restricted and recognize non-peptide targets that are presented in the context of non-polymorphic molecules. In fact, several types of unconventional αβ-TCR T cells express semi-invariant TCRs, such as, for example, invariant natural killer T (iNKT) cells that recognize targets in the context of the monomorphic antigen-presenting molecule CD1, or mucosal-associated invariant T (MAIT) cells that recognize targets in the context of MR1 (**Figure 1**). Several of these αβ-TCR T cell subsets therefore harbor unique features that could potentially qualify them as universal donor cells for adoptive T cell therapy (ACT) (**Table 1**).

## Virus-Specific T Cells (VSTs)

### *Adoptive transfer of VSTs to prevent or treat infections and/or EBV-associated post-transplant lymphoproliferation*

Viral reactivations and infections after allogeneic HCT remain a major cause of morbidity and mortality (29). Current anti-viral drugs are associated with dose-limiting end organ toxicities or lack of efficacy due to primary or secondary resistance, and only virus-specific immune reconstitution can resolve the issue of recurrent infections. Thus, in patients that do not concomitantly present with GVHD, adoptive transfer of VSTs is a safe and efficient therapy to accelerate immune reconstitution. The various approaches of VST manufacturing for adoptive transfer as well as clinical trial results have recently been reviewed (30–32). Allogeneic HCT donor derived VSTs have shown significant clinical activity against Epstein-Barr Virus (EBV), CMV, adenovirus, BK virus, and Human Herpes Virus 6. Importantly, impressive anti-viral responses have been reported across studies, and significant alloreactivity or GVHD has only been described in a very limited number of patients. HCT donor derived Epstein-Barr Virus specific T cells (EBVSTs) are also active against post-transplant $EBV^+$ lymphoproliferative disorders in 65–85% of treated patients (11, 33, 34), and their long-term persistence was demonstrated in gene marking studies (11).

With the goal to facilitate rapid access to VSTs including for patients with seronegative donors, allogeneic third-party VST banks have been developed by several groups and institutions (12, 30, 35–37) and have entered commercialization. Safety and anti-viral activity of adoptively transferred allogeneic third-party donor VSTs are excellent, and the overall response rate (ORR) when treating viral infections can reach up to 92% (36, 38). When targeting $EBV^+$ lymphoproliferation, ORRs were 68% in HCT and 54% in solid organ transplant (SOT) patients (12). Because persistence is a key parameter for tumor targeted T cell therapies but has not been summarized in the recent review articles on VSTs, we selected clinical trials with information on persistence on the infused third-party VSTs and summarized the results in **Table 2**. Available information is quite scarce. Each group used different tracking methods, no gene-marking was available to easily detect the *in vivo* fate of the infused cells, and long-term follow-up is mostly lacking. Available data suggests that third-party VSTs do not engraft and persist as well as HLA-matched HCT donor-derived VSTs. The variability between patients and trials was broad. Gallot et al. for example were not able to detect significant levels of cells derived from the infused VST lines (41). But others found a correlation between detection of anti-viral activity (12, 38, 39, 42) or VST line derived TCR

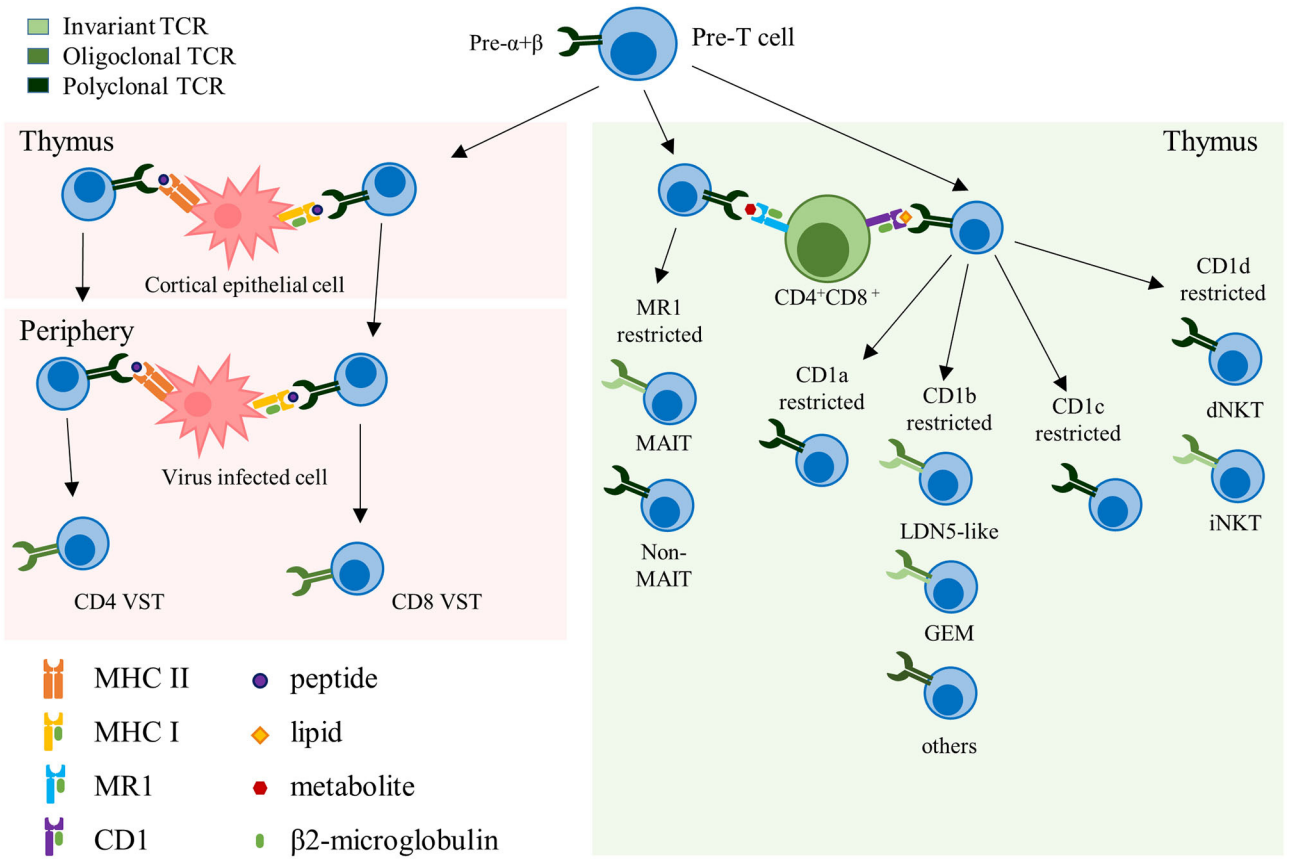


**FIGURE 1 |** Representation of the different αβ-TCR subsets. Schematic representation summarizing the different αβ-TCR subsets, their development and restricting elements. The color grading of the TCR represents its clonality, from light to dark green (monoclonal to polyclonal, respectively). TCR, T cell receptor; VST, virus-specific T cell; MHC, major histocompatibility complex; MAIT, mucosal-associated invariant T cell; GEM, germline-encoded mycolyl lipid-reactive; dNKT, diverse natural killer T cell; iNKT, invariant natural killer T cell.

sequences (37, 40) and viral clearance. The limited persistence is most likely due to rejection of the infused third party VSTs by the host immune system. Thus, in order to overcome this problem, novel types of chimeric alloimmune defense receptors (ADRs) have been developed. One strategy consists of a chimeric receptor using the extracellular part of human β2-microglobulin and signaling through CD3ζ and was shown to protect VSTs from alloreactive T cells *in vitro*, but this approach cannot protect from NK cell mediated rejection (43). More recently, a receptor recognizing 4-1BB (CD137)—temporarily upregulated on both activated T and NK cells—and signaling through CD3ζ was developed. 4-1BBζ ADR-engineered T cells were protected from T and NK cell mediated rejection *in vitro* and *in vivo* in a mouse xenograft model, and CARs retained their antitumor function when co-expressed with the 4-1BBζ ADR (44). Thus, ADRs have the potential to further enhance the persistence, efficacy, universality and safety of third-party engineered VSTs, and can be co-engineered with CARs. In the post-transplant setting, endogenous immune reconstitution also plays an important role in the establishment of long-term viral control.

We still need to learn more about the contributions of infused cells and endogenously reconstituted anti-viral immunity upon third-party VST adoptive transfer when targeting viral infections or EBV-associated malignancies. Systematic assessment of VST persistence with standardized methods across clinical trials would facilitate this understanding.

### *Engineered VSTs in clinical trials*

VSTs have been clinically validated as a cellular therapy platform to genetically redirect antigen specificity against tumor-associated antigens (**Table 3**). With this approach, endogenous anti-viral TCR specificities can be exploited for the *in vivo* expansion and stimulation of the transgenic VSTs or targeting of viral-associated malignancies. Indeed, autologous CAR-engineered VSTs have been evaluated in clinical trials targeting GD2 for neuroblastoma (45, 46), and allogeneic HCT donor-derived CAR$^+$ VSTs targeting CD19 in B-acute lymphoblastic leukemia (47–49). Reactivation of CMV in patients after allogeneic HCT and CD19 CAR$^+$ CMVST infusion for example led to significant *in vivo* re-expansion of the infused cells and CD19 directed cytotoxic activity with elimination of B cells (49). To assess whether vaccination could be used to *in vivo* re-expand CAR$^+$ VSTs, a clinical trial is underway to assess autologous GD2-CAR$^+$ Varizella Zoster

**TABLE 1 |** Features of universal donor cells.

| Cell type | Size of the TCR repertoire | Polymorphism of restricting element | TCR cross-pairing potential * | Difficulties of *ex vivo* expansion | Risk of off-tumor on target recognition | Reported or expected risk of GVHD | Universality score (lowest is best) | Intrinsic immune activity | References |
|---|---|---|---|---|---|---|---|---|---|
| **αβ-TCR T cells** | | | | | | | | | |
| VST | ++ | +++ | ++ | – | – | – | 7 | Anti-viral | (11, 12) |
| iNKT | + | + | + | – | + | – | 4 | Anti-tumoral, pro-inflammatory, protect from GVHD | (13–16) |
| dNKT | +++ | + | +++ | ++ | ++ | + | 12 | Immune suppressive | (7, 17, 18) |
| CD1a-restricted | +++ | + | +++ | + | ++ | + | 11 | Unknown | (19–21) |
| CD1b GEM | + | + | ++ | + | ++ | + | 8 | Unknown | (22, 23) |
| CD1b LDN5-like | + | + | ++ | + | ++ | + | 8 | Unknown | (22, 23) |
| CD1b-restricted | ++ | + | +++ | + | ++ | + | 10 | Unknown | (19–21) |
| CD1c-restricted | +++ | + | +++ | + | ++ | + | 11 | Unknown | (19–21) |
| MAIT | + | + | +++ | +++ | ++ | + | 11 | Unknown | |
| **γδ-TCR T cells** | | | | | | | | | |
| Vγ9Vδ2 | – | + | – | – | + | – | 2 | Anti-tumoral, pro-inflammatory, APC | (24–27) |
| Vδ1 | +++ | Unknown (non-HLA) | – | – | + | – | 4 | Anti-tumoral, pro-inflammatory | (28) |

*Table summarizing the different features restricting the use of a T cell subset as universal donor cells. The amplitude of each restricting feature is depicted as none/extremely low (–), low (+), intermediate (++) and high (+ + +). The addition of each (+) is reported in the "Universality score" column, which represents the universal potential of each T cell subset, with a lower score corresponding to higher universality.*

***Upon introduction of a transgenic TCR.***

*TCR, T cell receptor; VST, virus-specific T cell; MAIT, mucosal-associated invariant T cell; GEM, germline-encoded mycolyl lipid-reactive; dNKT, diverse natural killer T cells; iNKT, invariant natural killer T cells; HLA, human leukocyte antigen; GVHD, graft-versus-host disease; APC, antigen-presenting cell.*

**TABLE 2 |** *In vivo* persistence of third-party off-the-shelf VSTs.

| Targeted virus/es | Treatment indication | *N* patients/treatment | Persistence evaluated in *N* | Technique of detection | Result/persistence | References |
|---|---|---|---|---|---|---|
| EBV | EBV$^+$ lymphoma | 33 SOT | 5 | TCR spectratyping | Up to 7 days post-infusion<br>Trace of infusion product detected in 3/5 patients analyzed | (37) |
| EBV | EBV$^+$ lymphoma | 2, HCT (cord blood) | 2 | CTLp by LD | No durable engraftment, but transient CTLp increase 7–10 days after infusion | (39) |
| CMV, AdV, EBV | Infection and EBV$^+$ lymphoma | 50 HCT, 9 with EBV$^+$ lymphoma | 6 (4 responders, 2 non-responders) | TCR Vβ CDR3 sequencing | Clones derived from the VST line detectable in 4 responders up to 12 weeks | (40) |
| EBV | EBV$^+$ lymphoma | 6 HCT<br>3 SOT<br>2 non-transplant | 8 | STR on PBMCs | Signal barely detectable in 3/8 patients, up to day 10 | (41) |
| EBV, AdV, CMV, BKV, HHV6 | Infection and EBV$^+$ lymphoma | 38 HCT, 1 with EBV$^+$ lymphoma | 16 responders | IFN-γ ELISPOT with informative epitopes (VST line, patient or shared origin) | 11/16 (69%) persistence up to 12 weeks, HLA match at 2 or more alleles<br>Confirmed by STR in 1 case | (38) |
| CMV | Infection | 10 HCT | 8 | IFN-γ ELISPOT with informative epitopes | 5/8 activity of infused VST line, 8/8 activity against shared epitopes between line and patient | (42) |
| EBV | EBV$^+$ lymphoma | 33 HCT<br>13 SOT | 3 HCT<br>3 SOT | STR on *ex vivo* EBV restimulated T cells | HCT:<br>1: CR, 100% VST line derived (day 10)<br>1: SD, no VST line derived cells detected<br>1: CR, 100% VST line derived cells day 32<br>SOT:<br>1: no response, no persistence<br>1: durable PR, long-term persistence of VST line derived cells (24 months)<br>1: durable PR, no VST line derived cells but host reconstitution | (12) |

*EBV, Epstein-Barr Virus; CMV, Cytomegalovirus; AdV, Adenovirus; BKV, BK Virus; HHV6, Human Herpes Virus 6; SOT, Solid organ transplant; TCR, T cell receptor; HCT, hematopoietic cell transplant; CTLp, cytotoxic T lymphocyte precursors; LD, limiting dilution; STR, short tandem repeat; PBMCs, peripheral blood mononuclear cells; IFN, Interferon; ELISPOT, enzyme-linked immunospot; CR, complete response; PR, partial response; SD, stable disease.*

VST (VZVSTs) cell infusions in combination with vaccination (NCT01953900). Preclinical investigations had shown that anti-tumor function of GD2-CAR$^+$ VZVSTs could be rescued *in vitro* upon stimulation with VZV peptide-pulsed dendritic cells (DC) (50). A clinical trial with gene-modified third-party partially HLA-matched healthy donor-derived banked CAR$^+$ EBVSTs targeting CD30 in patients with EBV-associated CD30$^+$ lymphomas is in preparation at Baylor College of Medicine (NCT04288726).

Engineering VSTs with a tumor-targeted transgenic TCR has been more challenging than with CARs, as forced expression of a transgenic TCR leads to downregulation of the endogenous TCRs (51). Indeed, these findings were confirmed in TCR transgenic VSTs, where reduction of antiviral activity was reported in several preclinical studies (52–55) and in one clinical trial (56). Interestingly, one report showed that TCR$^+$ VSTs can shift their antigenic predominance depending on the type of antigenic exposure given to the cells (viral or tumor antigen) (55). The oligoclonal features of VSTs minimize the risk of cross-pairing between transgenic and endogenous TCR chains, and thus the use of VSTs to express a transgenic TCR is thought to reduce the risk of both off-target toxicities and GVHD. In the only clinical trial reported to date, a Wilms tumor antigen-1 (WT-1) specific TCR was expressed in single epitope specific EBVSTs generated from the HLA-matched HCT donor. The clinical responses in high-risk AML patients who received allogeneic HCT followed by prophylactic WT-1 TCR$^+$ EBVST infusions were impressive (56). Unfortunately, no viral reactivation occurred in the cohort of 12 treated patients, so the question whether the level of anti-viral specificity is sufficient to mediate *in vivo* re-expansion of TCR$^+$ VSTs upon viral reactivation and to protect against viral disease remains elusive. TCR transgenic third-party VSTs have not yet been evaluated clinically.

**TABLE 3 |** Overview of clinical development status.

| Cell type | Frequency in circulating T cells | Isolation | GMP-compatible expansion | Genetic engineering | Hypothetical number of cross-paired TCRs* | Status of clinical translation | Reference |
|---|---|---|---|---|---|---|---|
| **αβ-TCR T cells** | | | | | | | |
| VST | 0.01–0.2% | Antigen specific expansion, IFNγ-capture, multimer selection | Yes | CAR and TCR | Low | In phase 1 and 2 clinical trials | (45–56) |
| iNKT | 0.01–0.2% | α-GalCer-induced expansion | Yes | CAR and TCR | Low | In phase 1 clinical trials | (57–60) |
| dNKT | 1% | Not done | No | Not done | High | Not in clinical trial | (61) |
| CD1a-restricted | 0.1–10% | *In vitro* expansion with CD1-expressing cells | No | Not done | High | Not in clinical trial | (62) |
| CD1b GEM | < 0.01% | Tetramer | No | Not done | Low | Not in clinical trial | (22, 23) |
| CD1b LDN5-like | < 0.01% | Tetramer | No | Not done | Low | Not in clinical trial | (22, 23) |
| CD1b-restricted | 0.1–10% | Single cell sorting for the generation of T cell clones | No | Not done | High | Not in clinical trial | (63) |
| CD1c-restricted | 0.1–10% | Single cell sorting for the generation of T cell clones | No | Not done | High | Not in clinical trial | (63, 64) |
| MAIT | 5–10% | Tetramer | No | Not done | Low | Not in clinical trial | (65–67) |
| **γδ-TCR T cells** | | | | | | | |
| Vγ9Vδ2 | 1–5% | Zoledronate-induced expansion | Yes | CAR and TCR | None | In phase 1 clinical trials | (68–70) |
| Vδ1 | 0.1–1% | Beads selection and cytokine expansion | Yes | CAR and TCR | None | Clinical trials expected soon | (71, 72) |

**Upon introduction of a transgenic TCR. TCR, T cell receptor; VST, virus-specific T cell; MAIT, mucosal-associated invariant T cells; GEM, germline-encoded mycolyl lipid-reactive; dNKT, diverse natural killer T cells; iNKT, invariant natural killer T cells; HLA, human leukocyte antigen; GVHD, graft-versus-host disease; APC, antigen-presenting cell; CAR, chimeric antigen receptor; GMP, Good Manufacturing Practice.*

### *VSTs as platform for engineered ACT: how universal can they be?*

Third-party banked VSTs have been established as a safe and efficient ACT to treat infections and $EBV^+$ lymphoproliferation after allogeneic HCT or SOT. The oligoclonal nature of VSTs limits their capacity to induce GVHD in this patient population. Despite the polymorphic nature of HLA, VST banks can be built with a limited number of well-chosen and characterized donors to cover a highly diverse patient population (42). Furthermore, VSTs can efficiently be redirected to tumors with both CARs and TCRs for clinical use. Drawbacks of third-party VSTs are that (i) their use has so far been limited to HCT and SOT patients, (ii) the *in vivo* re-stimulation through the endogenous TCR depends on unpredictable endogenous viral reactivations or scheduled vaccinations, and (iii) their long-term persistence has not yet been conclusively assessed. The clinical development of engineering strategies such as incorporation of ADRs may overcome these limitations in the future and make VSTs safer and more universal.

## CD1-Restricted T Cells

### *Background on CD1 molecules*

The monomorphic CD1 family is constituted of five members, four extracellular (CD1a-CD1d) and one intracellular molecule (CD1e). CD1a, CD1b, and CD1c belong to the group 1 CD1, while group 2 is solely constituted of CD1d (7, 19). Circulating within the different secretory and endosomal compartments of the cells, CD1 molecules present a large array of lipid antigens to T cells (7, 73–75). So far, only a limited number of CD1-restricted antigens are known, consisting of lipids shared by multiple microorganisms and of self-lipids that accumulate during cellular stress, for example in cancer (19, 20, 76). CD1d expression is constitutive and present on all antigen-presenting cells (APCs), while the expression of CD1a/b/c is inducible and limited to a subset of APCs. For example, B cells express CD1c, Langerhans cells express CD1a and CD1e, while myeloid cells can express all five CD1 molecules (19, 20).

### *CD1d-Restricted T cells: their implication in tumor immunity*

CD1d-restricted T cells, also called NKT cells, are selected in the thymus after recognition of CD1d molecules expressed by $CD4^+CD8^+$ double positive thymocytes (13, 19, 77). CD1d-restricted T cells are classified into two distinct groups based on their ability to recognize α-galactosylceramide (α-GalCer), a glycosphingolipid originally derived from marine sponge. Type I NKT, or iNKT, express a semi-invariant TCR consisting of an invariant TCRα chain (TRAV10-TRAJ18 in human) paired with a limited number of TCRβ chains and recognize α-GalCer. Type II NKT, or dNKT, express a more diverse polyclonal TCR repertoire, and are unresponsive to α-GalCer [(7); **Figure 1**].

α-GalCer is produced by the gut flora and many mammalian tissues and acts as a potent iNKT cell stimulator (14, 78–80). Dysregulated lipid production in tumors is also a source of antigenic lipids capable of stimulating iNKT cells [e.g., GD3 and GM3 in melanoma (81, 82), or α-fucosylceramides in colorectal

and pancreatic adenocarcinomas (83)]. Upon TCR engagement, iNKTs rapidly secrete high levels of cytokines (e.g., IFNγ, TNFα, IL4, IL13, and IL17) and lytic granules (granzymes and perforin), and upregulate killing receptors such as Fas ligand and TRAIL (17, 84). Thus, iNKTs are rapidly cytotoxic and strongly modulate the tumor microenvironment by direct targeting of $CD1d^+$ tumors, tumor-associated macrophages and myeloid-derived suppressor cells (15, 85). Modulation of the immune response occurs by transactivating NK cells, licensing DCs and activating γδ-TCR T cells (16, 19, 86). This crosstalk leads to a strong activation of the endogenous adaptive immune system (87). That iNKTs play an important role in anti-tumor immunity is inferred from the facts that low frequency of iNKT cells in patients with hematologic or head and neck cancers correlated with poor prognosis (88–90), while higher iNKT cell infiltration in colorectal cancer correlated with longer survival (91). After allogeneic HCT, higher doses of iNKT cells contained in the graft were associated with protection from acute GVHD (92), and early donor-derived iNKT cell reconstitution post-transplant correlated with reduced acute GVHD and lower non-relapse mortality while maintaining graft-versus-leukemia effects (93).

dNKTs are less well characterized and thought to have a more immune regulatory role (17, 18, 94). In multiple myeloma for example, dNKTs have been involved in suppression of anti-tumor immunity in an IL-13-dependent manner (94). Potential immunotherapeutic applications for dNKT cells have been reviewed elsewhere (61).

### *Ex vivo expanded iNKT cells in clinical trials*

Due to their biology, iNKT cells are an attractive cell type to investigate for cancer immunotherapy (**Table 3**). However, establishing GMP compatible *ex vivo* expansion protocols for iNKT cells has been a hurdle to broader development. To date, results from two clinical trials assessing the safety of adoptively transferred *ex vivo* expanded autologous iNKT cells in cancer patients were reported (95, 96). In a lung cancer trial, autologous iNKT cells from 6 patients were expanded in the presence of α-GalCer and IL2, reaching 0.1–25% iNKT cells in the final products. Infusions were safe but no significant clinical responses were seen (96). In a melanoma trial, autologous iNKT cells were sorted from PBMCs and expanded *ex vivo* for 6–8 weeks with anti-CD3 and IL2. Purity post-expansion ranged from 13 to 87%. The nine treated patients had only minimal or no evidence of disease at time of infusion and were not lymphodepleted. A clear correlation between iNKT cell infusions, immune parameters and outcome could not be established (95).

Meanwhile, *ex vivo* expansion methods have been refined and now allow genetic engineering of iNKT cells (57, 58). Dual targeting by harnessing endogenous and engineered properties of iNKT cells produced very promising pre-clinical results in neuroblastoma with GD2-CAR iNKT cells also incorporating transgenic IL15 (57, 59) and in lymphoma with CD19-CAR iNKT cells expanded in media containing IL21 (58, 60). Both approaches have started phase I clinical evaluation at Baylor College of Medicine. Safety of autologous GD2-CAR.IL15 engineered iNKT cells is evaluated in patients with neuroblastoma (NCT03294954). Since iNKT cells are not alloreactive and clinical studies suggest that iNKTs can suppress GVHD (92, 93, 97), a clinical trial with third-party allogeneic off-the-shelf iNKT cells genetically engineered to express a CD19-CAR and IL15 is underway to assess safety, *in vivo* expansion and persistence, and responses in patients with B-cell malignancies (NCT03774654).

### *CD1d-Restricted T cells as platform for ACT*

iNKT cells have the ability to kill $CD1d^+$ tumor cells and immune suppressive cells in the tumor microenvironment through direct cytotoxicity, but also modulate the immune response of NK cells and DCs through cytokine secretion, producing enhanced anti-tumor responses of conventional endogenous T cells (16, 19, 86). In addition to its anti-tumor activity, iNKT cells can protect the patient from developing GVHD after allogeneic HCT, as better iNKT cell recovery correlated with a reduced risk of GVHD (92, 93, 97). With their lack of HLA-restriction, semi-invariant TCR and protective potential against GVHD, iNKT cells possess several unique features required for universal donor cells (**Table 1**). Their *in vivo* persistence will need to be analyzed. The field is currently moving toward evaluating the safe use of iNKT cells from allogeneic third-party universal donors in engineered ACT (NCT03774654).

### *CD1a/b/c-Restricted T cells and their implication in tumor immunity*

The current knowledge on T cells restricted to group 1 CD1 is limited, and mostly results from studies performed *in vitro* on human T cell clones (62). Recognizing diverse microbial and self-lipid antigens, group 1 CD1-restricted T cells are relatively abundant among circulating lymphocytes in healthy individuals (63), and the majority has a polyclonal TCR repertoire (19–21). Two subsets of CD1b-restricted T cells, the germline-encoded, mycolyl lipid–reactive (GEM), and the LDN5-like T cells, which recognize glucose monomycolate, a lipid antigen derived from *Mycobacterium tuberculosis,* express an invariant TCR (TRAV1-TRAJ9, and TRAV17-TRBV4-1, respectively) (22, 23).

Group 1 CD1-restricted T cells are thought to participate in immune surveillance of hematologic malignancies. Analysis of a limited number of patient samples revealed positivity for CD1c in 51% and CD1b in 54% of AML patients ($n = 33$), 71% of B-ALL samples expressed CD1c ($n = 7$), and 75% of pediatric T-ALL samples expressed CD1a and CD1b ($n = 8$) (64). Methyl-lysophosphatidic acid (mLPA) is a self-lipid antigen presented in the context of CD1c on hematological malignancies (64). T cell clones recognizing mLPA in the context of CD1c produced higher levels of IFNγ when stimulated with malignant than with normal hematopoietic cells. Intracellular accumulation of mLPA in tumor cells is thought to increase CD1c-restricted presentation of mLPA on the cell surface compared to normal cells (64), and therefore leading to differential recognition of malignant cells with mLPA-restricted T cell clones.

### *Group 1 CD1-Restricted T cells as platform for ACT*

Despite expression of CD1c on APC, tumor-reactive T cells differentially recognized CD1c-restricted mLPA presented by tumor cells, suggesting that CD1c-restricted lipid antigens

specifically accumulate in malignant cells but not normal APCs (64). If CD1c-restricted T cells are made amenable to genetic engineering, they could be an interesting population to investigate for ACT. Similar prospects apply to CD1a- and CD1b-restricted T cells, as both CD1a and CD1b expression is restricted to APCs (19, 20). Due to their polyclonal TCR repertoire, CAR engineering could be more straightforward than TCR engineering due to potential cross-pairing of transgenic and endogenous TCR chains and higher risk to produce off-target toxicities.

CD1b-restricted invariant TCR T cells (GEM and LDN5-like T cells) theoretically are top candidates as universal donor cells for both CAR and TCR-based ACT (**Table 1**). However, their frequency is extremely low in *M. tuberculosis* positive patients [<0.01% of peripheral blood T cells (22)], and has not yet been described in healthy donors.

## MR1-Restricted T Cells

### *General definition*

The MR1 molecule is an evolutionary conserved, monomorphic protein (98, 99). Ubiquitously expressed, MR1 cell surface expression is however modulated by antigen abundance. Under physiological conditions, MR1 is almost undetectable at the cell surface. Bound antigen is needed for its trafficking to the cell surface, and cell surface expression is further enhanced by exogenous antigen loading (19, 98, 100). Known to be involved in the immunity against bacterial and yeast infections (101, 102), MR1 presents small metabolites derived from the metabolic pathways of vitamin B9 (folate) or B2 (riboflavin) (103). Only a few antigens have been identified so far, but the list is growing, and includes small cyclic molecules utilized as pharmacological agents, such as the aspirin analog 3-FSA (3-formylsalicylic acid) or the non-steroidal anti-inflammatory drug diclofenac, which suggests that MR1 may be involved in drug hypersensitivity (104, 105). Even if self-derived MR1-restricted antigens have not been identified yet, several studies suggest that such antigens exist (106). For several years, mucosal-associated invariant T cells (MAIT) were the only known T cells with MR1 restriction. Now there is growing evidence that MR1-restricted non-MAIT cells exist (107–109), but much more needs to be learned.

### *MR1-Restricted MAIT cells*

MAIT cells develop upon interaction with $CD4^+CD8^+$ double positive cortical thymocytes, and continue to mature and expand after leaving the thymus [(105, 110, 111); **Figure 1**]. In humans, their numbers continuously increase during the first 25 years of life, and then slowly decrease with age (112, 113). Their expansion is thought to be dependent on stimulation with microbial antigens, as germ-free mice do not have any MAIT cells in the periphery, despite positive selection in the thymus (106).

Originally identified in the gut, MAIT cells are characterized by the expression of a semi-invariant TCR, constituted of an invariant TCR Vα chain (Vα7.2-Jα3.3) paired with a limited number of Vβ chains. While byproducts of the microbial riboflavin biosynthesis, such as 5-OP-RU (5-[2-oxopropylideneamino]-6-D-ribitylaminouracil), are known to strongly activate MAIT cells, the folate biosynthesis pathway generates molecules, such as 6-formyl-pterin, that exert inhibitory effects on MAIT cells (103, 114). Although MAIT cells constitute only 5% of the total T cell pool in humans, their frequency can greatly vary in different organs (105). MAIT cells are abundant in the liver, lung and gastro-intestinal tract, as well as in the blood. In the liver, for example, 45% of resident T cells are MAIT (115). In the periphery, MAIT cells constitute up to 10% of the circulating T cells (112). Similar to conventional T cells, MAIT activities can be modulated by the antigen recognized, the cytokines present in the microenvironment and the tissue to which they naturally home. In the colon, for example, MAIT cells preferentially display a Th1-type of cytokine secretion, and reside in the lamina propria and the intraepithelial compartment of the mucosa, while in lung and liver MAIT cells resemble tissue-resident memory T cells (116).

TCR engagement together with co-stimulation leads to rapid MAIT cell activation, in a memory-like manner (117, 118). Activated MAIT cells display both direct and indirect cytotoxic functions, through the secretion of granzyme B, perforin, and a large range of Th1 and Th17 type of cytokines (105). Together with their ability to home to infected sites, MAIT cells constitute an important player in anti-microbial defense.

### *MAIT cells in tumor immunity*

MAIT cells are part of the tumor infiltrating lymphocyte population in colorectal cancer (116, 119, 120), hepatocellular carcinoma (121, 122), or kidney and brain tumors (123). In some tumors, an inverse correlation between circulating and tumor infiltrating MAIT cells was observed, but it is not clear yet if tumor-infiltrating MAIT cells are pro- or anti-tumorigenic. In brain and kidney tumors, for example, MAIT cell infiltration was associated with higher levels of pro-inflammatory cytokines (123). On the other hand, MAIT cell infiltration in colorectal cancer and hepatocellular carcinoma was associated with unfavorable clinical outcome. Failing to produce pro-inflammatory cytokines such as IFNγ upon *ex vivo* stimulation, these tumor-infiltrating MAIT cells were functionally impaired (119, 124). One study even showed by transcriptome sequencing analyses that MAIT cells infiltrating hepatocellular carcinoma acquired a pro-tumorigenic phenotype (122). Surprisingly, peripheral MAIT cells seem to be unaffected and retain their ability to respond to bacterial antigens and even to tumor cells (116, 120, 124).

In patients after allogeneic HCT, robust peripheral blood MAIT cell reconstitution has been associated with a lower risk for the development of subsequent severe acute GVHD (118, 125), and activated MAIT cells suppressed proliferation of $CD4^+$ T cells *in vitro*. Correlations between gut microbiota composition, the related riboflavin pathway, and MAIT reconstitution exist (118, 126). Further investigations are necessary to decipher the precise role of MAIT cells in human GVHD and whether adoptively transferred *ex vivo* expanded MAIT cells could be immune suppressive.

### *MAIT cells as platform for ACT*

MAIT cells possess unique features that would make them interesting candidates as universal donor cells for ACT (**Table 1**),

and methods for their isolation and *ex vivo* expansion are being established (65–67). However, their precise role in anti-tumor immunity remains to be defined in more detail. Some of the burning questions include (i) if it is possible to reprogram them *in vitro* to express a stable Th1 profile, (ii) if adoptively transferred MAIT cells efficiently migrate to the tumor site and retain their anti-tumor function, and (iii) if they can be genetically engineered during *ex vivo* expansion. We are convinced that these and more questions will be answered soon, and MAIT cells will be investigated as universal donor cells.

## γδ-TCR T Cell Subsets

γδ-TCR T cells constitute 1–5% of total circulating T cells (14, 127). Selected in the thymus, the rearrangement process of γδ-TCR is highly complex and not fully understood yet. Constituted of a far smaller number of gene segments than αβ-TCRs, only 4–6 functional Vγ and 8 Vδ vs. 46 Vα and 48 Vβ chains, the potential of γδ-TCR diversity is however thought to surpass the diversity of αβ-TCRs (127, 128). Vδ4-Vδ7 gene segments rearrange with segments of the TCR α-chain, and have alternative TRAV names. Vδ1-Vδ2 preferentially rearrange with Dδ, Jδ, and Cδ to create TCR δ-chains, though few reports showed that Vδ1 and Vδ3 could also rearrange with segments of the TCRα locus, thus generating a δ/αβ-TCR T cell (129–132).

Vδ usage pre-determines γδ-TCR T cells function and localization: the majority of peripheral γδ-TCR T cells expresses Vδ2, while tissue-resident γδ-TCR T cells favor Vδ1 and Vδ3 (127). In humans, the majority of γδ-TCR T cells consists of Vδ1 and Vδ2 T cells (129). Their ligands are not well-characterized. The Vγ9Vδ2 T cell subset recognizes phosphorylated antigens, presented by the butyrophilin (BTN) molecules. Vδ1 γδ-TCR T cells can recognize antigens presented on CD1c, CD1d, and MR1 molecules. Both Vδ1 and Vδ2 γδ-TCR T cells are able to recognize stress-related molecules such as MIC A/B either via their TCR or via NK receptors such as NKG2D (129, 133, 134).

### BTN-Restricted Vγ9Vδ2 T Cells

#### *General background*

The Vγ9Vδ2 T cell subset is relatively abundant in circulating lymphocytes, and represents 1–5% of all T cells in healthy individuals, and 50–95% of γδ-TCR T cells (127, 135, 136). This T cell subset expresses an invariant TCR that recognizes phosphorylated isoprenoid metabolites, also called phosphoantigens, derived from the mevalonate pathway. These metabolites, such as the isopentenyl pyrophosphate (IPP), can accumulate in transformed and infected cells because of their dysregulated metabolism (127, 135, 137). IPP accumulates when the activity of the IPP-metabolizing enzyme farnesyl-diphosphate-synthase (FPPS) is blocked (135). The use of aminobiphosphonates, such as zoledronate, inhibits FFPS, which leads to an increase in intracellular level of IPP and the activation of Vγ9Vδ2 T cells (135, 138–140).

IPPs are presented to T cells by butyrophilin molecules. Belonging to the immunoglobulin superfamily, these glycoproteins are divided into three subfamilies (BTN1, BTN2, BTN3) (141). Only BTN3A (CD277) presents phosphoantigens to Vγ9Vδ2 T cells. Constituted of three isoforms, BTN3A molecules are expressed by the majority of human immune cells, including γδ-TCR T cells (142). Phosphoantigens bind the intracellular domain B30.2 of BTN3A, which induces conformational changes to the receptor and increases binding force of Vγ9Vδ2 TCR to BTN3A (129, 143, 144). Therefore, Vγ9Vδ2 T cells are able to recognize altered metabolites present in infected or cancer cells.

#### *Vγ 9Vδ2 T cells in tumor immunity*

Once activated, Vγ9Vδ2 T cells acquire similar effector functions as conventional αβ-TCR T cells: they directly kill tumor cells upon engagement of death receptors (e.g., FAS, TRAIL, NKG2D) or by secreting granzymes and perforins (24). In addition, Vγ9Vδ2 T cells produce various pro-inflammatory cytokines such as TNFα or IFNγ, and can modulate the immune response. For example, Vδ9Vδ2 T cells can license and accelerate DC maturation (145, 146) and provide help to B cells (147).

Human γδ-TCR T cells can be expanded *in vitro* to clinically relevant numbers, are able to migrate to and kill tumors, and are amenable to genetic engineering (68–70, 148, 149). Both HLA class I- and class II-restricted αβ-TCRs have been successfully introduced into Vγ9Vδ2 T cells and recognized the cognate peptide when co-transduced with CD4 or CD8 co-receptors (69, 70). TCR mispairing between α-/β-chains and γ-/δ-chains cannot occur. Thus, γδ-TCR T cells are optimal recipients for transgenic αβ-TCRs. αβ-TCR-transgenic γδ-TCR T cells express both αβ- and γδ-TCRs and mediate tumor cytotoxicity through both TCRs (69). Vγ9Vδ2 T cells were also engineered to transiently express a TCR or a CAR and exerted both endogenous and engineered properties (68).

One unique feature of Vγ9Vδ2 T cells is their capacity to differentiate into professional APCs upon IPP stimulation. Vγ9Vδ2 T cells can phagocytose cells and crosspresent antigens, leading to the proliferation of both $CD4^+$ and $CD8^+$ T cells (25, 26). This interesting feature is maintained in genetically engineered Vγ9Vδ2 T cells. For example, GD2-CAR-transduced Vγ9Vδ2 T cells killed $GD2^+$ neuroblastoma, while retaining their ability to endocytose long peptides derived from the melanoma antigen MART-1 and inducing the proliferation of autologous T cells transduced with a MART-1-specific TCR (27).

### Non-Vδ2 γδ-TCR T Cells

#### *General background*

Non-Vδ2 γδ-TCR T cells consist of Vδ1 and Vδ3 γδ-TCR T cells, and are mostly tissue-resident T cells present in barrier epithelium, though some of these cells are also circulating in blood (127). Between these two subsets, Vδ1 are the most abundant. From the original diverse repertoire present in neonates, only few Vδ1 γδ-TCR T cell clones will expand and ultimately dominate the adult Vδ1 repertoire (150). Even though the antigens recognized by Vδ1 γδ-TCR T cells are mostly unknown, they were shown to recognize ligands presented by

CD1a, CD1c, CD1d, and MR1 molecules as well as various stress-induced ligands (133, 151, 152).

### *CD1-Restricted Vδ1 γδ-TCR T cells*

CD1 molecules were among the first ligands identified for γδ-TCR T cells (153), but only few CD1-restricted lipids recognized by Vδ1 γδ-TCR T cells have been identified so far. Exogenous antigens comprise pollen-derived phospholipids and bacterial lipids, while known self-lipids consist of glycolipid sulfatides, which are present in tissues where Vδ1 γδ-TCR T cells reside (154–157). γδ-TCR T cells have been reported to be involved in tissue repair and homeostasis (158, 159), and predominate among γδ-TCR T cells that infiltrate various tumors (see below). However, the presence of CD1-resticted γδ-TCR T cells in tumors, and their involvement in tumor immunity, have not been investigated yet.

Crystal structure of CD1d-Vδ1 binding reveals a distinct TCR recognition: CD1d recognition by Vδ1 γδ-TCR T cells is solely mediated by the germline-encoded CDR1 loop, independently of the bound antigen. The antigen is in contact with the CDR3 region, which determines the antigen specificity (156, 160). Similarly, CD1c recognition was also shown to be dictated solely by Vδ1. Bound antigens modulate TCR recognition: some self-lipids were shown to permit TCR binding, while other self-lipids blocked it (154).

### *MR1-Restricted Vδ1 γδ-TCR T cells*

γδ-TCR T cells recognizing antigens presented by MR1 have only been recently identified (152). This novel γδ-TCR T cell subset is rare with a frequency between <0.001 and 0.1% of total $CD3^+$ T cells, or <0.1–5% of γδ-TCR T cells in blood of healthy donors, but has also been found in TILs of a Merkel cell carcinoma patient. MR1-restricted γδ-TCR T cells preferentially expressed Vδ1 (72% of the 76 TCRs analyzed). Structural studies revealed that TCR recognition occurred by binding to the MR1 α3 domain situated underneath the antigen-binding site and independently of the bound antigen (152) suggesting inherent autoreactivity of these cells.

### *Vδ1 γδ-TCR T cells in tumor immunity*

Consistent with their preferential localization in epithelial tissues, Vδ1 γδ-TCR T cells are the predominant γδ-TCR T cell population in the majority of solid tumors (28, 161–163). Vδ1 γδ-TCR T cell infiltration correlated with favorable prognosis in several cancer types, such as triple negative breast cancer (TNBC) or CLL (162, 164). Upon *in vitro* expansion, Vδ1 γδ-TCR T cells isolated from PBMCs of cancer patients displayed strong IFNγ secretion and cytotoxic responses against several autologous tumors including melanoma (165), TNBC (162), colon cancer (166), AML (71), CLL (164, 167), diffuse large B cell lymphoma (168), and multiple myeloma (169). *In vitro* expanded Vδ1 γδ-TCR T cells were able to kill autologous CLL *in vitro* and spare healthy B cells isolated from the same patient, thus showing their ability to distinguish transformed cells from healthy cells (164). Moreover, these Vδ1 γδ-TCR T cells restrained tumor growth and prolonged the survival of immunodeficient mice engrafted with either human colon cancer or AML (71, 166). In other tumors, such as breast cancer (161, 170), colorectal cancer (163), melanoma (171), or squamous cell carcinoma (172), Vδ1 γδ-TCR T cell infiltration was associated with poor prognosis, as these cells displayed an immunosuppressive phenotype promoting tumor growth (170, 171). Vδ1 γδ-TCR T cells were shown to differentiate into Th17-like T cells, producing elevated level of IL-17, and other immunosuppressive factors, such as IL-10, IL-18, and adenosine (161, 170). Cancer cells were directly responsible for the skewing of Vδ1 γδ-TCR T cells toward Th17 regulatory profile. Breast cancer, for example, secretes exosomes containing the IncRNA SNHG16, a long non-coding RNA inducing the expression of SMAD5, and therefore TGF-β1, in Vδ1 γδ-TCR T cells (161). In colorectal cancer patients, cancer stem cells directly promoted IL-17 production by Vδ1 γδ-TCR T cells by secreting immunomodulatory molecules. By multiplex analyses on 50 different cytokines, the authors identified IL-18 and VEGF as the two most promising candidates responsible for the skewing of Vδ1 γδ-TCR T cells toward IL-17-producing immunosuppressive cells (163).

So far, no clinical trials have investigated the safety and efficacy of Vδ1 γδ-TCR T cells as anti-tumor therapy (**Table 3**). However, several groups established GMP-compatible protocols to expand and genetically engineer Vδ1 γδ-TCR T cells *in vitro* with the goal to translate this approach to the clinic (27, 28, 71, 72). Vδ1 γδ-TCR T cells can be expanded from healthy donor or patient PBMCs using a cocktail of different cytokines and anti-CD3 antibody stimulation. Over 3 weeks of culture, cells expanded more than 3-log fold and differentiated into cytotoxic Th1-like T cells, capable of controlling tumor growth in xenograft mouse models (28, 71). Moreover, Vδ1 γδ-TCR T cells expanded from PBMCs could be transduced with an anti-GD2 CAR and killed GD2-positive neuroblastoma cells lines that were not naturally recognized by Vδ1 γδ-TCR T cells (27).

## γδ-TCR T Cells in Clinical Trials

Published and ongoing clinical trials investigating the safety and antitumor function of γδ-TCR T cells have recently been reviewed (173). Overall, the results of published trials outside the setting of allogeneic HCT have been disappointing, demonstrating safety but no efficacy. A major limitation lies in the lack of understanding of γδ-TCR diversity and their potential target antigens (**Table 3**). Some of the current and future clinical trials are trying to address these issues. We will focus our discussion on efforts in developing allogeneic γδ-TCR T cell therapies, with the overall goal to move their application to third-party banked off-the-shelf therapies. We identified five registered clinical trials, but more are expected to emerge. Three trials explore the safety of allogeneic *ex vivo* expanded adoptively transferred γδ-TCR T cells in hematologic malignancies. In one trial, γδ-TCR T cells are expanded from the haploidentical stem cell donor and infused to the patient post-transplant in combination with post-transplant cyclophosphamide treatment (NCT03533816, Incysus Therapeutics). Safety and effects on post-transplant GVHD are investigated. Another trial assesses the adoptive transfer of *ex vivo*

expanded γδ-TCR T cells derived from related haploidentical or HLA-matched donors in patients with relapsed/refractory AML after lymphodepleting chemotherapy (NCT03790072, TC Biopharm). Safety and efficacy are analyzed. Future prospects are to move to allogeneic third-party banked γδ-TCR T cell products, and to include genetic engineering with CARs. A third active trial is also investigating *ex vivo* expanded γδ-TCR T cells from allogeneic related donors in patients with relapsed/refractory AML (NCT04008381, Wuhan Hospital). For solid tumors, a randomized clinical trial investigates safety and efficacy of tumor reducing surgery alone or in combination with adoptive transfer of *ex vivo* expanded γδ-TCR T cells in patients with locally advanced pancreatic cancer. The source of the γδ-TCR T cells is not entirely clear (NCT03180437, Fuda Cancer Hospital). Finally, haploidentical NKG2DL-CAR engineered γδ-TCR T cells for a variety of relapsed or refractory solid tumors will be investigated in a phase I dose escalation trial (NCT04107142, Cytomed Therapeutics).

### γδ-TCR T Cells as Platform for ACT

γδ-TCR T cell expansion protocols allow the incorporation of genetic engineering to redirect γδ-TCR T cells to tumor-associated antigens recognized by CARs or αβ-TCRs (28, 68, 71, 72). Preclinical data suggest that γδ-TCR T cells are particularly suitable for αβ-TCR-based engineering, as the risk of TCR mispairing is inexistent. Both TCRs remain well-expressed, and the redirected T cells can exert anti-tumor functions through both TCRs. As γδ-TCR T cells are not HLA restricted (137), there is theoretically no risk of causing GVHD in the recipients, but formal demonstration in a clinical trial with third-party donor derived γδ-TCR T cells is currently lacking. γδ-TCR T cells meet many features required for universal donor T cell therapies (**Table 1**), and the interest in the field of exploiting this cell type is high.

## UNIVERSAL TCR

TCRs derived from both αβ- and γδ-TCR repertoires that allow the targeting of a broad range of tumors in an HLA independent manner have been identified. These TCRs can be considered "universal," as they redirect immune cells to broadly shared tumor-specific antigens. TCRs recognizing targets derived from altered cell metabolism are of particular interest, as these TCRs reliably distinguish between cancer and healthy cells. Three examples from the literature include Vγ9Vδ2 TCRs (174, 175), mLPA-specific CD1c-restricted αβ-TCRs (64), and an MR1-restricted αβ-TCR with unknown specificity (108). These TCRs have been successfully introduced into polyclonal αβ-TCR T cells and were able to redirect the engineered cells to a variety of cancers in an HLA-independent manner in preclinical studies. Autologous Vγ9Vδ2-TCR engineered αβ-TCR T cells are currently under phase I clinical evaluation in patients with hematologic malignancies (NTR6541, UMC Utrecht). More TCRs with similar features are likely to be identified in the future. TCRs targeting cancer-specific ligands in the context of non-polymorphic molecules are likely to become interesting candidates for engineered ACTs.

## CONCLUSIONS AND FUTURE OUTLOOK

VSTs and unconventional T cells possess several features that would enable their universal use without the need of genome editing to avoid unacceptable alloreactivity. Among these different T cell subsets, γδ-TCR T cells, especially Vγ9Vδ2 T cells, and iNKT cells show the highest universal potential (**Table 1**). However, whether or not host-mediated rejection of the infused cells will affect their engraftment and long-term persistence remains to be addressed in the upcoming clinical trials. The only data available to date on persistence, safety, and efficacy come from third-party banked VSTs where, despite excellent clinical activity, persistence seems to be reduced compared to other trials that used HLA-matched products. Characterized by a memory-like status, VSTs and unconventional T cells respond rapidly to antigen exposure, leading to strong cytolytic activity, and cytokine production (19). Several of these T cell types have successfully been redirected to tumors by genetic engineering with a CAR or a TCR and mostly retain their intrinsic characteristics. For example, $CAR^+$ VSTs maintain their anti-viral responses (48, 49), γδ-TCR T cells express both endogenous and introduced αβ-TCR (69), and $CAR^+$ iNKT cells continue to respond to α-GalCer (57, 59, 176). The retention of these cell-specific features can also promote their *in vivo* re-expansion after ACT through re-exposure to their natural cognate antigen (49). Another advantage is the relatively restricted pattern of target antigens recognized by their endogenous TCRs. CD1-restricted and γδ-TCR T cells, for example, recognize ligands derived from altered tumor metabolism, and thus spare the corresponding healthy cells (36, 43). Despite still limited understanding of the biology of certain unconventional T cell subsets, the developing clinical translational pipelines outlined in this review demonstrate that the future potential for some of these experimental therapies as off-the-shelf products is high (**Table 3**). We expect that some of these cell types or universal receptors will become important players in the field cancer immunotherapy.

## AUTHOR CONTRIBUTIONS

CP and CA: concept and writing of the manuscript. IG: preparation of the figure. All authors have read and agreed with the final version of the manuscript.

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# Rapid GMP-Compliant Expansion of SARS-CoV-2–Specific T Cells From Convalescent Donors for Use as an Allogeneic Cell Therapy for COVID-19

*Rachel S. Cooper[1†], Alasdair R. Fraser[1†], Linda Smith[1], Paul Burgoyne[1], Stuart N. Imlach[1], Lisa M. Jarvis[2], David M. Turner[3], Sharon Zahra[1], Marc L. Turner[1] and John D. M. Campbell[1*]*

[1] Tissues, Cells and Advanced Therapeutics, Scottish National Blood Transfusion Service, Edinburgh, United Kingdom, [2] National Microbiological Reference Unit, Scottish National Blood Transfusion Service, Edinburgh, United Kingdom, [3] Histocompatibility and Immunogenetics, Scottish National Blood Transfusion Service, Royal Infirmary of Edinburgh, Edinburgh, United Kingdom

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**Edited by:**
Peter Bader,
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Catherine Bollard,
Children's National Hospital, United States
Halvard Bonig,
Goethe University Medical School, Germany

***Correspondence:**
John D. M. Campbell
john.campbell4@nhs.scot

[†]These authors have contributed equally to this work

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COVID-19 disease caused by the SARS-CoV-2 virus is characterized by dysregulation of effector T cells and accumulation of exhausted T cells. T cell responses to viruses can be corrected by adoptive cellular therapy using donor-derived virus-specific T cells. One approach is the establishment of banks of HLA-typed virus-specific T cells for rapid deployment to patients. Here we show that SARS-CoV-2–exposed blood donations contain CD4 and CD8 memory T cells which recognize SARS-CoV-2 spike, nucleocapsid and membrane antigens. Peptides of these antigens can be used to isolate virus-specific T cells in a GMP-compliant process. The isolated T cells can be rapidly expanded using GMP-compliant reagents for use as an allogeneic therapy. Memory and effector phenotypes are present in the selected virus-specific T cells, but our method rapidly expands the desirable central memory phenotype. A manufacturing yield ranging from $10^{10}$ to $10^{11}$ T cells can be obtained within 21 days culture. Thus, multiple therapeutic doses of virus-specific T cells can be rapidly generated from convalescent donors for potential treatment of COVID-19 patients.

**Keywords: COVID-19, T cell, adoptive T cell immunotherapy, CD4, CD8, memory T cell**

## INTRODUCTION

Coronavirus disease 2019 (COVID-19), caused by the severe acute respiratory virus syndrome-coronavirus 2 (SARS-CoV-2) emerged in Wuhan, China in December 2019. In the majority of cases infection with SARS-CoV-2 is asymptomatic or leads to relatively mild self-limiting disease, but a proportion of patients progress to severe disease with about a 1% overall mortality rate (1, 2). Declared a pandemic by the WHO on March 11, 2020, the virus has spread rapidly to all parts of the world with >56 million infections and >1.35 million deaths reported by November 2020 (3).

Patients with progressive severe disease demonstrate a high neutrophil to lymphocyte ratio and a lymphopenia in the blood accompanied by a hyperinflammatory and prothrombotic diathesis leading to Acute Respiratory Distress Syndrome (ARDS) and multiorgan failure (4–6). Some

success in treating severe disease has recently been reported with therapeutic agents such as remdesivir (7), dexamethasone (8), and nebulized interferon-beta (9).

A particular feature of progressive COVID-19 disease is rapid exhaustion of the memory T cell compartment—characterized by overall lymphopenia and accumulation of naïve/exhausted T cell memory phenotypes (10, 11). This undesirable phenotype is associated with a systemic hyperinflammatory response and poor outcomes (reviewed in (12)). Conversely, protection in self-limiting disease is associated with strong CD4 and CD8 T cell responses to the spike, membrane and nucleocapsid proteins of the virus and development of virus-specific antibodies (13–15). Convalescent plasma (CP) is currently being trialed in a number of countries as a potential therapeutic option, although the level and duration of protection afforded by the antibody response against re-infection remains unclear at present (16).

New therapies to support the immune response to SARS-CoV-2, preventing the collapse of the lymphocyte compartment and supporting protective immunity would have significant impact on outcome for hospitalized patients. Anti-viral T cells specific for viruses such as cytomegalovirus (CMV), adenovirus (ADV) and Epstein Barr Virus (EBV) have been successfully used as adoptive cellular therapies to combat such infections in patients with immune deficiency (17–22). Following selection of antigen-specific T cells from a blood donation from an individual who has been infected with the relevant virus, T cells may be administered to the patient without further manipulation. An alternative strategy is to expand virus-specific T cells *in vitro* using donations from HLA-typed donors. These T cells can be cryopreserved from multiple donors as an allogeneic "off the shelf" therapy and are typically used as a "best-HLA match" to the recipient. We and others have adopted this approach in the treatment of EBV or CMV-driven disease (21, 22) with evidence of *in vivo* efficacy, disease remission and low incidence of Graft versus Host Disease (GvHD) (21, 22). Despite a number of *in silico* studies (23), more data are required on HLA restriction of SARS-CoV-2 peptides in different populations to understand which HLA alleles and loci should be preferentially matched between donors and patients to optimise the efficacy of SARS-CoV-2 T cell therapy.

In this study we present clear evidence to show that donations from individuals who have been infected with SARS-CoV-2 with mild symptoms and have recovered retain normal T cell compartment profiles, with CD4 and CD8 memory and effector T cells specific for SARS-CoV-2 spike, nucleocapsid and membrane antigens. These virus-specific T cells (VSTs) can be isolated using Good Manufacturing Practice (GMP)-compatible selection technology and rapidly expanded *in vitro* using closed culture vessels and GMP-compliant reagents and medium. The mononuclear cell fraction of a single whole blood donation from a COVID-19 convalescent donor (CCD) can be used to generate up to $10^{11}$ T cells within 21 days with the desired central memory phenotype as a potential new therapy for SARS-CoV-2. This offers the potential for the manufacture of a bank of HLA-matched donor T cell products for use in clinical trial and future treatment of COVID-19 patients.

# MATERIALS AND METHODS

## Study Design

The aim of this study was to characterize the SARS-CoV-2 peptide-specific T cell memory populations present in donations from CCD and to explore the feasibility of isolating and expanding these T cells to clinical scale. The expanded T cells could then form the basis of an HLA-typed allogeneic 'off the shelf' VST therapy for COVID-19. SNBTS is leading the Scottish COVID-19 convalescent plasma program, and COVID-19 Convalescent Donors (CCD) were also recruited from the local Scottish population to donate peripheral blood buffy coats for this study. CCD were eligible to donate if they had a confirmed positive SARS-CoV-2 PCR test and were a minimum of 28 days after resolution of infection symptoms, as well as fulfilling the current criteria for whole blood donation. Uninfected Donors (UD - adults confirmed as having no evidence of COVID-19 symptoms at time of donation) were used to compare initial phenotyping and SARS-CoV-2 antigen T cell responses with CCD. Buffy coats from CCD (n = 15) and UD (n = 17) were obtained under SNBTS Sample Governances 20~02 and 19~11 respectively. All donations were fully consented for research use.

## SARS-CoV-2 Antibody Detection

The Euroimmun anti-SARS-CoV-2 assay (Euroimmun US, NJ, USA) clinical diagnostic indirect ELISA was used to detect antibodies to SARS-CoV-2 spike protein from donor serum according to the manufacturer's instructions. The results were expressed as a ratio against a calibrator control, where values of <0.8 were considered negative and >1.1 were considered positive.

## Buffy Coat Peripheral Blood Mononuclear Cell (PBMC) Isolation

Buffy coats were diluted [1:3] with PBS and added to Leukosep tubes containing Ficoll-Paque (GE Healthcare). Tubes were centrifuged at 450*g* for 40 min and the resulting buffy layer extracted. Isolated PBMCs were then washed in PBS and counted on MACSQuant10 Analyzer (Miltenyi Biotec).

## Scale-Up to Representative Manufacturing Process

For full-scale clinical manufacturing the starting material was taken from leukapheresis collections with no requirement for Ficoll preparation. Two demonstration products were generated using commercially-acquired leukapheresis material (5L Optia process, supplied by Key Biologics Ltd/Cellero, TN, USA) from CCD. Alternatively, automated devices such as the Sepax device (GE Healthcare) to Ficoll buffy coat to make a mononuclear fraction could be used for manufacturing if leukapheresis is not available (24).

## HLA Typing

Following extraction of DNA from PBMC samples, HLA genotyping of donors was undertaken for HLA-A, B and DRB1 loci using Lifecodes HLA eRES SSO Typing kits (Immucor Inc, USA).

## Immunophenotyping

Freshly isolated PBMC and T cells from VST cultures (see below) were analyzed for surface immunophenotype. For this, $2 \times 10^6$ cell samples were taken and washed with PBS buffer supplemented with EDTA and human serum albumin (PEA buffer). Cell pellets were re-suspended in 100 µl PEA and incubated with 5 µl Fc Receptor blocking reagent to prevent non-specific antibody binding. Antibody surface marker multi-color panels detailed in **Figure S1** were then added for 20 min at 4°C. Samples were then washed and re-suspended in PEA, with dead cell dye DRAQ7 (eBioscience) added prior to acquisition on a MACSQuant10 Analyzer (Miltenyi Biotech) recording a minimum of 100,000 events.

## SARS-CoV-2 VST Detection Within PBMC Population

SARS-CoV-2 Peptivator peptide pools (Miltenyi Biotech) containing 15-mer sequences with 11 amino acids overlap for the immunodominant section of the spike protein, and the full sequence for nucleocapsid protein and membrane protein (**Table S1**) were reconstituted in DMSO/water according to manufacturers' guidelines.

PBMC were plated in TexMACS medium (Miltenyi Biotech) in a 24-well plate at $5 \times 10^6$ cells/ml per well with treatments: negative control, PMA/ionomycin positive control, individual spike, nucleocapsid, and membrane peptide pools, and combined pools (spike + nucleocapsid + membrane [SNM]). SARS-CoV-2 peptide pools were used at (0.3 nmol/ml), and cell activation cocktail (BioLegend) added to the positive control well at (1×). The negative control well contained DMSO/water at the same volume as the peptide wells. Cells were stimulated for a total of 5 h at 37°C, 5% CO2; with Brefeldin A (BioLegend) added at (5 µg/ml) for the final 3 h (Brefeldin A was only used in this analysis, not in any cell selection experiments).

## Intracellular Labeling

Plates were harvested into FACS tubes and washed with PEA. Samples were treated with Fc Receptor blocking reagent as above, and surface marker antibodies for multi-color panels (see **Figure S2** for details) were then added for 20 min at 4°C. Cells were then washed and stained with fixable viability dye (FVD) eFluor780 (eBioscience) for 30 min at 4°C. Cells were subsequently fixed and permeabilized using Cytofix/perm kit (BD Biosciences) for 20 min at 4°C, then washed and labeled with antibodies for intracellular cytokines (detailed in **Figure S2**) for 20 min at 4°C. Cells were washed and analyzed with a MACSQuant10 Analyzer recording a minimum of 150,000 events.

## SARS-CoV-2 VST Isolation

SARS-CoV-2 VSTs were isolated from PBMC whole population using a cytokine capture system (CCS) assay. Briefly, PBMC were plated at $5 \times 10^6$ cells/ml/cm$^2$ in standard Corning multi-well plates and incubated overnight at 37°C, 5% $CO_2$. The following morning, plates were stimulated with pooled SARS-CoV-2 peptide pools (spike + nucleocapsid + membrane) each at (0.3 nmol/ml) for 6 h at 37°C, 5% $CO_2$. The virus-specific IFN-γ secreting cells were then isolated using the IFN-γ CCS assay by either manual column or CliniMACS Prodigy isolation as described in (17). Following isolation, each fraction was counted and phenotyped using the lymphocyte panel as described above and illustrated in **Figure S1**.

## SARS-CoV-2 VST Culture Optimization

Non-target cells from the IFN-γ CCS assay were irradiated at 40 Gy and used as feeders for the IFN-γ+ target cells. Cultures were initially seeded at either $1 \times 10^7$ total cells per cm$^2$ (200–400 non targets: 1 target), or $3 \times 10^6$ total cells per cm$^2$ (100 non targets: 1 target) in G-Rex culture vessels (Wilson Wolf). Cells were cultured in GMP-grade TexMACS medium, and supplemented to determine culture optima using (200 U/ml) IL-2 (GE Healthcare), (155 U/ml), IL-7 (Miltenyi Biotech), (2%) human AB Serum (SNBTS) or (2%) nLiven (Sexton Biotechnologies). As nLiven is not fully European GMP-compliant it was replaced with pathogen-inactivated, xeno-protein-free GMP-compliant T-Liven (Sexton Biotech) for the scale up demonstrator VST products from leukapheresis. Cells were cultured for up to 28 days with feeds every 3 to 4 days and cultures split as necessary to maintain a density of 0.5 to $3 \times 10^6$ T cells/cm$^2$. At day 14, VST cultures from six donors were split to test feeder re-stimulation, where thawed irradiated non-target cells were added to cultured VSTs at (10 non targets: 1 VST) alongside a control culture with no feeder re-stimulation. Samples were taken every 3 to 4 days for immunophenotyping with the lymphocyte panel described above.

## Representative Scale Manufacture of VST

For scale manufacture, the 14-day procedure was chosen to maximize T cell yield without the requirement for a second feeder cell co-culture step. Leukapheresis material was obtained from SARS-CoV-2 convalescent USA-based donors (KBL/Cellero) and $2 \times 10^9$ MNC taken and processed for VST isolation using the Miltenyi Prodigy cell processor and the CCS isolation program. Combined peptide pools (6 ml starting volume) were prepared by passing through a 0.22-µm filter into a 20-ml transfer bag, which was attached to the prodigy tubing set TS500 *via* a sterile welder. The positive target fraction was assessed and the negative fraction irradiated as before, then the cells combined and cultured in G-Rex CS100M flasks at $3 \times 10^6$ cells/cm$^2$. Cells were counted and split at days 7 and 11, and flasks harvested at day 14 with closed processing used throughout (Gatherex, Wilson Wolf Ltd). The cells were then assessed for cell numbers, surface phenotype and functional response as before. The final product was frozen in 33% plasmalyte/67% CryoStor10 (CS10, Stem Cell Technologies).

## Generation of Monocyte-Derived Dendritic Cells (DC)

Monocyte-derived DC were generated from isolated PMBC CD14+ monocytes. Briefly, monocytes were isolated from purified PMBC using anti-CD14 microbeads (Miltenyi Biotech) as per manufacturer's instructions. Cells were cultured at 37°C, 5% $CO_2$ for 6 days in RPMI (Life Technologies)

supplemented with (5%) AB serum, (2 mM) Glutamax (Sigma-Aldrich), (20 ng/ml) GM-CSF, and (15 ng/ml) IL-4 (both Miltenyi Biotech). Media was replaced on days 2 and 4 of culture. After 6 days, cells were harvested using (1×) TrypLE (Life Technologies) and frozen in CS 10 (Stem Cell Technologies) until required for VST stimulation.

## SARS-CoV-2 VST Stimulation Assay

Expanded T cells form the 5 complete optimized expansion runs starting with PBMC, and the 2 expansion processes using Leukapheresis material were taken at day 14 to test in a stimulation assay with peptide-loaded DC. Briefly, frozen autologous immature DC were thawed, plated with RPMI medium and stimulated with individual SARS-CoV-2 peptide pools and combined pools at (0.3 nmol/ml) for 6 h at 37°C, 5% $CO_2$. Where possible, VSTs were tested for specificity to SARS-CoV-2 by testing against other virus-specific peptides (Adenovirus Hexon peptide, Epstein-Barr Virus consensus peptide pool, and GAD65 peptide, all Miltenyi Biotech). Unloaded DC were included as a negative control. DC were then washed and re-plated in RPMI supplemented with poly I:C (20 μg/ml) and $PGE_2$ (1 μg/ml) at $2.5 \times 10^5$ cells/$cm^2$ overnight to drive DC maturation. The next morning DCs and VSTs were co-cultured at $2.5 \times 10^6$/$cm^2$ (10 VST: 1 DC). Control wells were included to measure baseline stimulation of VSTs co-cultured with unloaded DCs. Plates were then stimulated for 5 h and labeled for the cytokine and activation panels as described above.

## Flow Cytometry Data Analysis

Analysis of all flow cytometry data was performed using either MACSQuantify (Miltenyi Biotec) for cell counts, or FlowJo version 7 (TreeStar Inc) for wider phenotyping analysis. All analyses were subject to a basic initial gating strategy in which debris was first excluded on the basis of forward and scatter properties, and sequentially gated for singlets using FSC Area versus Height, and finally sequentially gated on live cells (DRAQ7 or FVD negative cells). Populations and acquisition of activation markers/cytokines were then quantified using percentages corrected to negative controls (see **Figures S1** and **S2** for full gating strategies).

## Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 8.4.2 (GraphPad Software). Comparisons of population frequencies between healthy donors and COVID-19 convalescent donors were performed using unpaired two-tailed Student's t tests with Holm-Sidak correction for multiple comparisons. Tests comparing population frequencies intra-donor between time-points (i.e. day 0 versus day 14, and day 14 versus day 21) for lymphocyte phenotype markers or comparing acquisition of activation markers within the CD8 versus CD4 population were paired two-tailed Student's T t-tests, corrected for multiple comparisons using the Holm-Sidak method where relevant. Response comparisons between the three individual SARS-CoV-2 peptides (spike, nucleocapsid and membrane) were tested using repeated-measures one-way ANOVA with the Geisser-Greenhouse correction to assume equal variability of differences within VST cultures. Correlation between intra-donor frequency of populations compared to other baseline characteristics was done by computing linear correlation coefficients using Pearson's correction with confidence intervals of 95%. Unless otherwise stated, data are represented as mean values ± SEM.

# RESULTS

## Donor Characteristics and Leukocyte Phenotype

Buffy coats from CCD (n = 15, see **Table 1**) were collected from local Scottish donors between 34 and 56 days after resolution of symptoms (diagnosis and resolution of infection were confirmed by SARS-CoV-2 PCR). Donors were 23 to 58 years old (median, 49 years) and evenly split by gender (7 female, 8 male). In all cases, donors exhibited mild symptoms of COVID-19 infection and did not require hospital treatment. The HLA-A, B and DRB1 typing results are also shown in **Table 1** for 12/15 donors that gave specific consent for HLA typing to be carried out.

Immunophenotyping of buffy coat-isolated peripheral blood mononuclear cells (PBMC) from CCD compared to uninfected donors (UD, n = 17) is shown in **Figure 1**. PBMC were sequentially gated as per **Figure S1**. The mean percentages of T cells (CD3+/CD56-), NKT cells (CD56+/CD3+) and monocytes (CD14+) were comparable between UD and CCD (**Figure 1A**). The mean percentage of T cells with an activated phenotype (HLA-DR+/CD38+), reported as elevated in other studies with moderate to severe disease, were not found to be significantly different between UD and CCD in this study. NK cell levels were significantly elevated ($p = 0.0073$) in CCD compared to UD and the mean percentage of B cells in CCD was significantly lower than UD ($p = 0.0003$). In this study, age did not correlate with NK cell or B cell levels in CCD (**Figures 1B, C**), though a significant correlation (Pearson correlation $p = 0.04$, $r = 0.524$) was identified between percentage of B cells and SARS-CoV-2 antibody content (**Figure 1D**). Within the T cell compartment the percentage of CD4 and CD8 T cells, as well as CD3+/CD4-/CD8- (double negative) and CD3+/CD4+/CD8+ (double positive) remained unchanged between CCD and UD (**Figure 1E**). In addition, analysis of co-expression of T cell memory markers CD62L, CD45RO, and CD45RA reveals no difference in CD4 and CD8 memory subpopulations between UD and CCD for either CD4 T cells (**Figure 1F**) or CD8 T cells (**Figure 1G**).

## CCD T Cell Responses to Spike, Nucleocapsid, and Membrane SARS-CoV-2 Peptides

PBMC were stimulated with SARS-CoV-2 peptide pools for spike protein, nucleocapsid protein and membrane glycoprotein or combined pools of all three and subsequently labeled for T cell surface markers (CD3, CD4, CD8) and

**TABLE 1** | Baseline characteristics of COVID-19 convalescent donors and immune response at donation.

| Donor Code | Blood Group | HLA Type | | | | | | Days from symptoms onset to resolution | Days from symptoms resolution to donation | Antibody level | % SARS-CoV-2 VST |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Ax | Ay | Bx | By | DRB1x | DRB1y | | | | |
| C19BC1 | O pos | 31:01 | 32:01 | 35:01 | 51:01 | 01:01 | 13:01 | 8 | 38 | 10.5 | 0.22 |
| C19BC2 | O pos | 01:01 | 68:01 | 51:01 | 57:01 | 07:01 | 14:01 | 10 | 36 | 5.9 | 0.22 |
| C19BC3 | A pos | | | | * | | | – | 34 | 9.3 | 0.13 |
| C19BC4 | O pos | 03:01 | 26:01 | 07:02 | 38:01 | 15:01 | 16:01 | 13 | 45 | 3.1 | 0.12 |
| C19BC5 | O pos | 03:01 | 32:01 | 07:02 | 27:03/05 | 08:01 | 13:01 | 14 | 52 | 3.9 | 0.03 |
| C19BC6 | A neg | 01:01 | 01:01 | 08:01 | 08:01 | 03:01 | 07:01 | 14 | 55 | 2.3 | 0.05 |
| C19BC7 | O pos | | | | * | | | 14 | 56 | 6.2 | 0.07 |
| C19BC8 | B pos | 02:01 | 11:01 | 07:02 | 56:01 | 01:01 | 08:01 | 10 | 46 | 9.5 | 0.10 |
| C19BC9 | O neg | 02:01 | 11:01 | 18:01 | 44:02 | 01:01 | 15:01 | 18 | 37 | 7.4 | 0.16 |
| C19BC10 | O pos | | | | ** | | | – | – | 11 | 0.05 |
| C19BC11 | B neg | 01:01 | 26:01 | 08:01 | 44:02 | 03:01 | 04:01 | 7 | 49 | 11 | 0.26 |
| C19BC12 | A pos | 02:01 | 24:02 | 35:01 | 44:02 | 04:01 | 04:04 | 7 | 53 | 9.42 | 0.13 |
| C19BC13 | O pos | | | | * | | | – | – | 5 | 0.08 |
| C19BC14 | O pos | 02:01 | 02:01 | NT | NT | 04:04 | 15:01 | – | – | 4.51 | 0.07 |
| C19BC15 | O neg | 03:01 | 29:02 | 44:03 | 57:01 | 07:01 | 15:01 | 13 | 53 | 3.48 | 0.03 |

*Antibody level refers to Euroimmun assay values (>1.1 = positive). Percentage SARS-CoV-2 VST refers to percentage CD3+/IFN-γ+ cells responding to combined SARS-CoV-2 pooled peptides (see **Figure 2** for data analysis).*

**Not consented for HLA typing.*

***No sample taken for HLA typing.*

*NT, not tested as insufficient DNA to type locus.*

intracellular cytokines (IFN-γ, TNF-α, IL-2) or activation markers (CD38, CD154, CD137). Representative flow analysis for a UD and CCD stimulated with combined SARS-CoV-2 peptide pools, with gating applied from a no-peptide control, is shown in **Figure 2A**. The percentage of SARS-CoV-2 VSTs in CCD positively correlated (Pearson correlation $p = 0.0381$, $r = 0.5391$) with SARS-CoV-2 antibody level (**Figure 2B**). Interestingly, the percentage of SARS-CoV-2 VSTs in CCD was found to decline significantly over time (Pearson correlation $p = 0.0021$, $r = 0.6275$) (**Figure 2C**). The mean percentage of CD3+ cells expressing IFN-γ, TNF-α and CD154 (**Figure 2D**) was significantly higher in CCD compared to UD for stimulation with each individual peptide pool and also for the combined peptide pools. CCD T cell IFN-γ, TNF-α and CD154 responses to individual peptide pools ($n = 10$) was compared using repeated measures (RM) one-way ANOVA to determine whether there was a preferential response to specific SARS-CoV-2 antigens. While the mean percentage of CD3+/IFN-γ+ cells and CD3+/TNF-α+ cells was comparable between the three peptide pools, the CD4+/CD154+ response was significantly higher ($p = 0.042$) to membrane peptides than to nucleocapsid peptides. Altogether, these data indicate there is no consistent preferential T cell cytokine response to one particular SARS-CoV-2 antigen (**Figure S3A**).

Further dissection of the cytokine response to SARS-CoV-2 peptide pools within lymphocyte subsets CD4+ T cells, CD8+ T cells and NK cells (CD56+/CD3- PBMCs) from CCD indicates the IFN-γ response is primarily by CD4+ T cells (**Figure S3B**). The mean percentage of CD4+/IFN-γ+ PBMC was significantly higher than either CD8+/IFN-γ+ or CD56+/IFN-γ+ cells for each individual peptide pool. Stimulation with combined peptide pools drove induction of higher percentages of CD4+/IFN-γ+ T cells than either CD8+/IFN-γ+ or CD56+/IFN-γ+ cells. The percentage of CD8+/IFN-γ+ cells was significantly increased over CD56+/IFN-γ+ cells. Conversely the TNF-α response to pooled peptides demonstrated significantly higher CD56+/TNF-α+ cells than CD8+/TNF-α+ cells (**Figure S3C**).

## Isolation of SARS-CoV-2 VSTs Using Peptide-Driven IFN-γ Selection and Expansion in Culture

PBMC were stimulated with combined peptide pools and reactive VSTs were isolated with the CliniMACS IFN-γ Cytokine Capture System (CCS) kit. Analysis of the IFN-γ selected T cells (**Figure 3A**) revealed an equal ratio of monocytes to T cells with negligible levels of NK or NKT cells. CD3+ T cells in the isolated fraction were a mix of CD4+ (53.02 ± 3.94%) and CD8+ (35.73 ± 3.23%) cells; where CD4+ T cells were predominantly central memory (86.52 ± 3.44%), CD8+ T cells showed mostly effector memory and terminal effector RA (TEMRA) phenotype. The non-target cells from the CCS isolation were irradiated and co-cultured with the isolated IFN-γ+ target cells to act as feeders for VST culture expansion in G-Rex culture vessels. After 14 days expansion (**Figure 3B**), cultures were highly enriched for T cells (87.95 ± 2.99%) with minimal expansion of NK and NKT cells. T cells were predominantly CD4+ (77.86 ± 5.19%) with smaller proportion of CD8+ T cells (18.05 ± 4.4%). Both the CD4+ and CD8+ populations were heavily skewed towards central memory phenotype. Direct comparison of populations between isolation and day 14 expansion showed significant differences in monocyte and T cell content, CD4+, CD8+ and Double Negative (DN) T cells, and memory subpopulations in both the CD4 and CD8 compartment (**Figure S4A**) demonstrating an enrichment of central memory CD4 cells in our culture process. In addition, expanded VSTs showed negligible co-expression of T cell

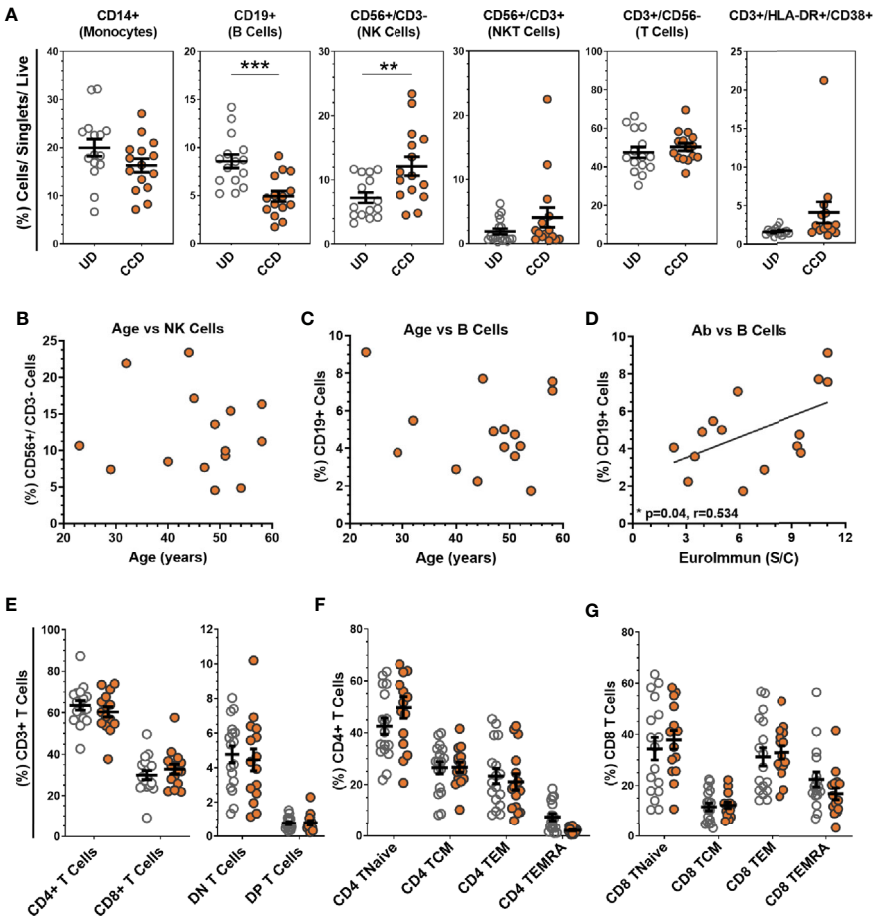


**FIGURE 1** | Analysis of COVID-19 convalescent donor buffy coat-derived PBMCs. **(A)** Buffy coat-derived PBMCs from COVID-19 convalescent donors (CCD, n = 15, orange circles) and healthy uninfected donors (HD, n = 17, clear circles) were assessed for leukocyte lineage by flow cytometry (see **Figure S1A** for gating strategy). No correlation was seen between age of COVID-19 convalescent donors with **(B)** NK cells or **(C)** B cells, but significant correlation between **(D)** SARS-CoV-2 serum antibody content and the percentage of B cells between donors ($p = 0.04$, $r = 0.534$, Pearson correlation coefficient). **(E)** Analysis of the T cell compartment (see **Figure S1B** for gating strategy) shows comparable mean levels of T cell subtypes, as well as **(F)** CD4+ and **(G)** CD8+ memory populations between HD and CCD. Data is represented as mean ± SEM. Significance determined by unpaired t-test with Holm-Sidak correction for multiple comparisons. DN double negative (CD4−/CD8−), DP double positive (CD4+/CD8+), TNaive (CD62L+/CD45RA+/CD45RO−), TCM central memory (CD62L+/CD45RA−/CD45RO+), TEM effector memory (CD62L−/CD45RA−/CD45RO+), TEMRA terminal effector memory CD45RA revertant (CD62L−/CD45RA+/CD45RO−). $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$.

exhaustion markers PD-1 and Tim-3 in both the CD4 and CD8 compartment (**Figure S4B**) indicating the culture expansion has not induced an exhausted T cell phenotype.

## Expanded SARS-CoV-2 VSTs Show Specific Response to All SARS-CoV-2 Peptides

SARS-CoV-2 peptide pool-loaded dendritic cells (DCs) and unloaded DC controls were then co-cultured with 14-day expanded VST at (1 DC: 10 VST) and analyzed for T cell activation and cytokine expression. Both CD4+ and CD8+ VSTs demonstrated specific anti-viral reactivity *via* expression of IFN-γ, TNF-α, CD154, CD107a, and CD137 when co-cultured with autologous DC loaded with SARS-CoV-2 pooled peptide (representative plots **Figure 4A**). There was a stronger response to peptide re-stimulation in CD4+ T cells than in the CD8+ T cells for IFN-γ, TNF-α, CD154 (**Figure 4B**), but equivalent CD107a and CD137 expression. The total T cell IFN-γ and TNF-α response to individual peptide pools for each donor VST are shown in **Figures 4C, D**, respectively, indicating donor-specific variation. When the data were collated, equivalent reactivity to all three peptide pools was observed (**Figure 4E**). Although the total T cell population did not show predominance for any of the SARS-CoV-2 antigen pools, the IFN-γ response was also assessed for the individual peptide pools, gated on CD8+ T cells and CD4+ T cells specifically (representative plot, **Figure 4F**). In CD4+ T cells there was a similar response to each peptide pool, but in CD8+ T cells, the nucleocapsid peptide pool was clearly immunodominant, inducing the strongest response (**Figures 4G, H**).

We assessed whether other non-SARS virus-specific T cells were coincidentally expanded in this process. Stimulation with

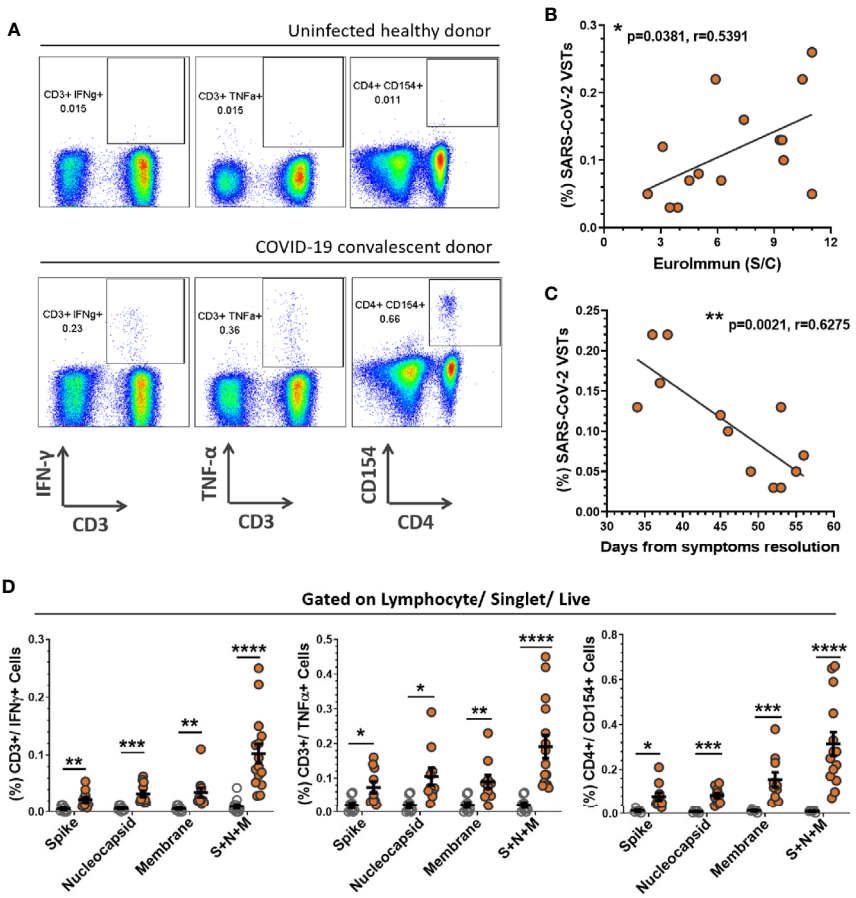


**FIGURE 2** | PBMC responses to SARS-CoV-2 peptides. PBMCs derived from buffy coats were incubated with SARS-CoV-2 peptides (Spike + Nucleocapsid + Membrane) for 5 h and corrected against a no antigen control well for positive expression of cytokines and activation markers. **(A)** Representation of flow cytometric analysis from a healthy uninfected donor (HD) and COVID-19 convalescent donor (CCD), note all flow analyses were gated on lymphocytes/single cells/live cells and subsequently quantified for percentage CD3+/IFN-γ+ cells, CD3+/TNF-α+ cells and CD4+/CD154+ cells (S2 for gating strategy). The percentage of SARS-CoV-2 VSTs in the CCD PBMC population (i.e. CD3+/IFN-γ+ cells reactive to pooled S+N+M peptides corrected to no antigen control) significantly correlated with **(B)** antibody titer at donation ($p = 0.0381$, $r = 0.5391$) and **(C)** days from resolution of symptoms to donation ($p = 0.0021$, $r = 0.6275$). Calculation was performed using Pearson correlation coefficient. **(D)** Mean percentages of CD3+/IFN-γ+ cells, CD3+/TNF-α+ cells and CD4+/CD154+ cells for individual and pooled peptides corrected to no antigen control were compared between HD (n = 12, clear circles) and CCD (n = 15, orange circles). Data is represented as mean ± SEM. Statistical significance was determined using unpaired t-tests corrected for multiple comparisons using the Holm-Sidak method where *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$.

EBV, adenovirus or irrelevant (GAD65) peptides did not demonstrate any significant levels of T cells directed to other viruses as measured by intracellular IFN-g response (**Figure S5**).

## Culture Optimization to Enhance SARS-CoV-2 VST Expansion for Clinical Manufacture

Establishing a bank of HLA-typed, donor-derived VSTs rely upon significant cell expansion in order to provide sufficient doses for clinical trial or therapeutic treatment. Combined growth curves of VST samples C19BC8 to C19BC14 (n = 6) cultured at optimized seeding density demonstrated a two- to three-log expansion from the initial isolated IFN-γ+ cells at day 0 to day 14, followed by a general plateau in expansion beyond this point (**Figure 5A**). Additionally, some VST cultures were expanded in different medium supplements for optimization of culture conditions, supplemented with IL-2, IL-7 or commercial pathogen-inactivated human platelet lysate (hPL). Addition of IL-7 had no effect on culture expansion between day 0 and day 8 (**Figure 5B**), whereas addition of hPL induced a markedly higher fold expansion between day 0 and day 14 compared to IL-2 alone in each donor culture tested (**Figure 5C**). After day 14 culture expansion plateaued, and an increased transition from central memory to effector memory phenotype by day 21 was observed (representative plot, **Figure 5D**). When cultures were administered a second feeder cell re-stimulation (FR) with autologous irradiated cells at day 14, central memory phenotype was retained at day 21. FR induced a subsequent two log expansion between days 14 and 21 (**Figure 5E**) in all VST cultures tested (n = 5). The final VST numbers harvested under

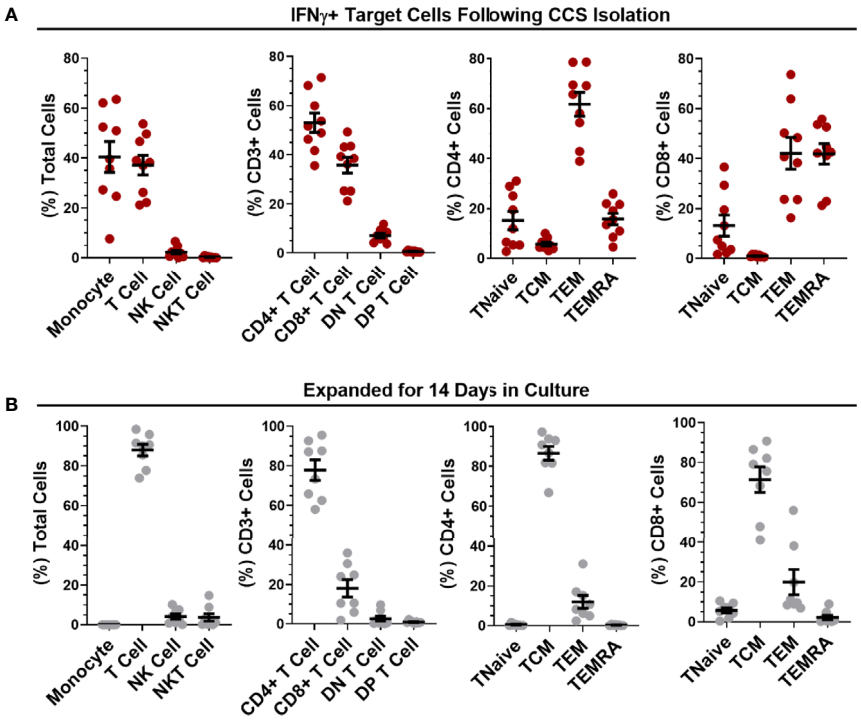


**FIGURE 3** | Phenotypic analysis of isolated and expanded SARS-CoV-2 VSTs. The percentages of leukocytes, T cell subpopulations and CD4/CD8 differentiation status were quantified for **(A)** IFNγ+ target cells directly after SARS-CoV-2 peptide-mediated cytokine capture system (CCS) isolation and **(B)** following expansion in culture for 14 days. All data is represented as mean ± SEM.

optimized conditions from a single CCD buffy coat ranged from 1 to 4.6 × $10^9$ at day 14, and 0.3 to 2 × $10^{11}$ at day 21 following FR (**Figure 5F**). Cultures were monitored throughout expansion to determine whether FR affected culture composition, but no significant differences in lymphocyte subsets (**Figures 5G, H**) or T cell memory status (**Figures 5I, J**) were observed between cells harvested at day 14 or at day 21.

## Scale Manufacture of Demonstrator VST Products

Successful isolation and expansion of two VST products was performed using at-scale manufacturing processes. VST from leukapheresis material was isolated using Prodigy and cultured for 14 days in closed-process flasks prior to harvest. This yielded at least 3 × $10^9$ VST and the VST demonstrated consistent phenotype and function to the developmental products (**Figure S6**).

# DISCUSSION

In this study we have characterized the virus-specific T cell compartment in SARS-CoV-2 convalescent donors, who volunteered to donate convalescent plasma (CCD). The immunophenotyping of donor PBMC demonstrated broadly similar percentages of different immune subpopulations compared to UD, though there was a significant decrease in CD19 B cells in CCD in common with many other reports (11, 25–27). However, we identified a significant increase in the NK cell compartment in CCD. Though increased innate lymphocyte levels have been correlated with increasing age (28), there was no correlation between donor age and frequency of NK cells or B cells. There was a significant correlation between B cell level and SARS-CoV-2–specific antibody levels. The T cell compartment showed no significant differences in CD4/8 ratios or differentiation status between CCD and UD, which indicates that mild COVID-19 does not significantly affect the overall T cell composition. There is however clear evidence of double-positive T cells, associated with recent viral infections (29).

Only recovered donors with confirmed infection and mild symptoms (non-hospitalized) were investigated in this study. Samples were collected between 34 and 56 days after resolution of symptoms, and in agreement with other studies we find that in these patients a robust T cell response is generated (30) against the spike, nucleocapsid and membrane glycoprotein peptide pools. A number of reports have indicated that protein or peptides from the C terminal of the spike protein can elicit T cell responses in donors known to be SARS-CoV-2 negative, indicating a cross-reaction with conserved motifs in other coronaviruses (31, 32) but this was not observed using this spike peptide pool. There was some indication that peptide pools from different proteins elicited a differential cytokine response, with IFN-γ and TNF-α responses stronger to nucleocapsid peptides, though CD154 was

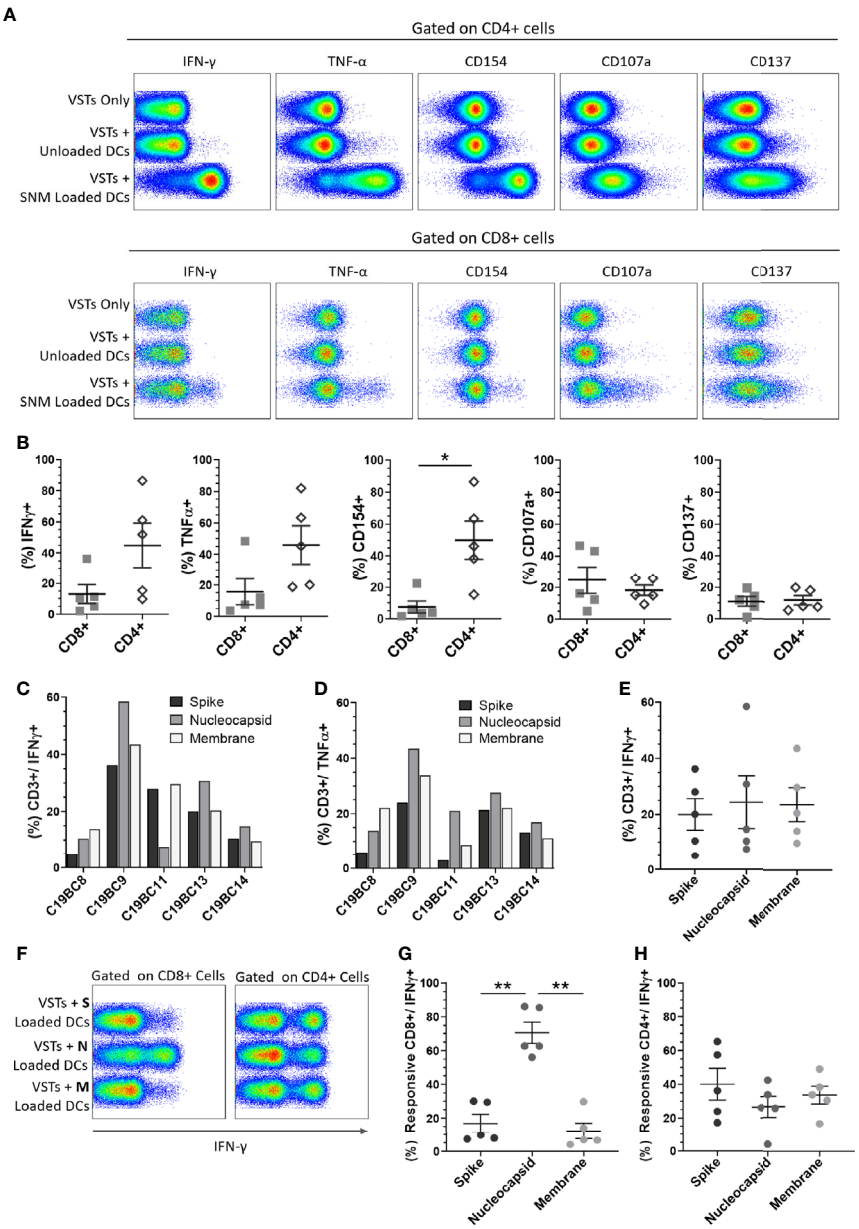


**FIGURE 4** | Cultured SARS-CoV-2 VST peptide specificity. Isolated and expanded SARS-CoV-2 VST (n = 5) at day 14 culture were co-cultured with peptide-loaded mature autologous DC. **(A)** Flow cytometric analysis on either CD3/CD4+ or CD3/CD8+ cells for IFN-γ, TNF-α, CD154, CD107a, and CD137 is shown for negative controls (VSTs Only, and VSTs + Unloaded DCs), and VST with pooled SARS-CoV-2 peptide loaded DCs (VST + SNM-loaded DCs). **(B)** The mean percentage of CD4+ and CD8+ positive for each marker of DC-stimulated VST was compared using paired t-test Holm-Sidak correction for multiple comparisons, *p ≤ 0.05. **(C, D)** Individual donor VST were assessed for T cell response (% CD3+/IFN-γ+ and % CD3+/TNF-α+ respectively) against DCs loaded with individual SARS-CoV-2 peptide pools: spike, nucleocapsid and membrane. **(E)** Collated responses to the individual peptides in the total CD3+ population indicated no significant difference. **(F, G)** A significantly higher CD8+/IFN-γ+ cells response was seen with nucleocapsid stimulation than with the other peptide pools (significance determined using RM one-way ANOVA with Geisser-Greenhouse correction **p ≤ 0.01). **(H)** CD4+/IFN-γ+ cells responded similarly to all the three peptide pools. All data represented as mean ± SEM.

preferentially increased in response to membrane peptides. This correlates well with findings in other cases of mild COVID-19 (33). The relatively low percentage of VSTs detectable has been reported in other studies on COVID-19 patients with mild disease (31). The key finding from this initial work was that we could successfully elicit IFN-γ responses in SARS-CoV-2 VSTs from CCD peripheral blood after peptide stimulation, which then confirmed that we could isolate and expand these T cells using an established clinical-grade cytokine capture assay. The principal confounding factor was identifying that SARS-CoV—2 VST levels

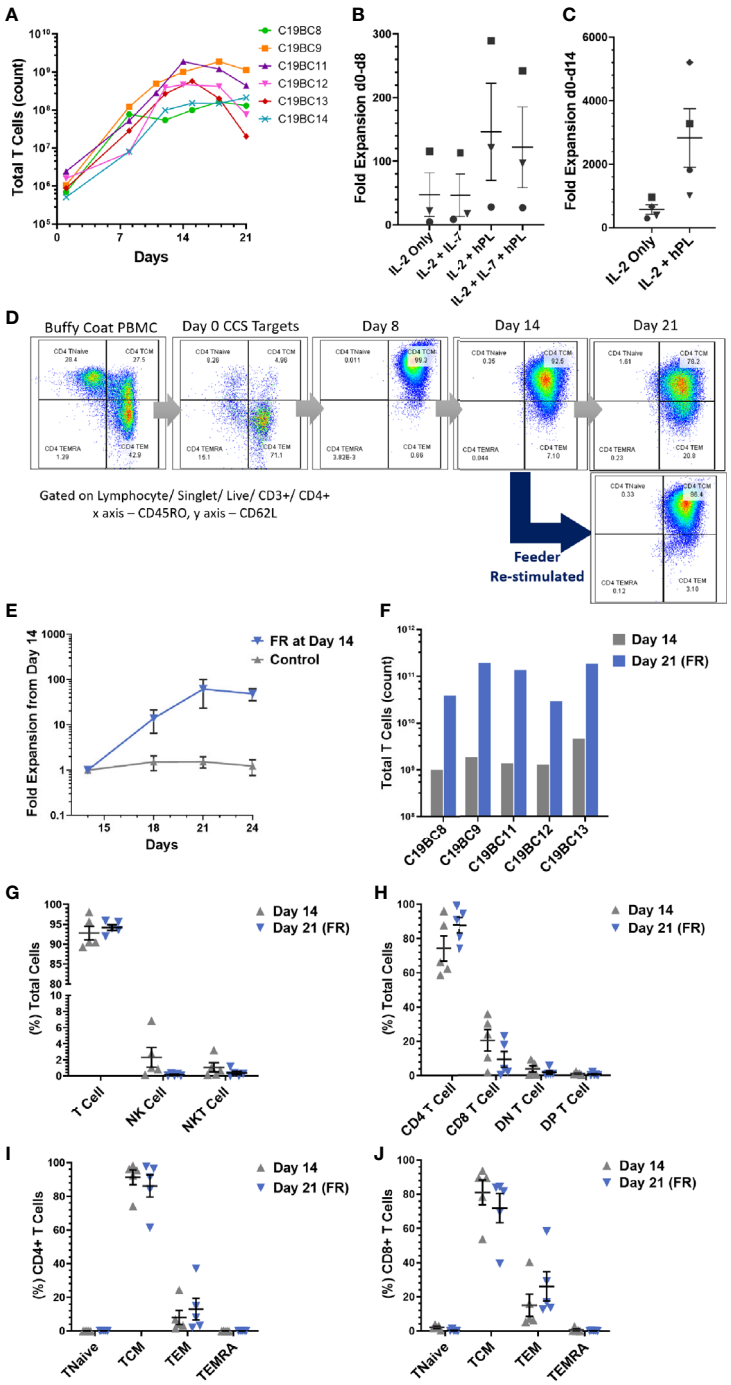


**FIGURE 5** | SARS-CoV-2 VST culture optimization. **(A)** Isolated SARS-CoV-2 VST from donors C19BC8-14 had a two- to three-log expansion over 21 day culture using an optimized culture expansion protocol. Variation in the start numbers of VST reflect donor variation in initial buffy coat PBMC numbers. Fold expansion between **(B)** day 0 and day 8 and **(C)** day 0 and day 14 was assessed in cultures after supplementation with IL-2, IL-7, and human platelet lysate (PL). Donors C19B9 (square), C19BC11 (triangle), C19BC12 (circle), and C19BC13 (diamond) were divided to compare medium supplementation condition. **(D)** Representative culture C19BC9 by day 21 without re-stimulation indicated some transition of CD4 TCM to CD4 TEM (Day 21 top panel). CD4 CM phenotype was retained when cultures re-stimulated at day 14 with autologous irradiated feeders (Day 21 bottom panel). **(E)** VST cultures were split at day 14 to directly compare standard continuation in culture (control) and re-stimulation with autologous irradiated feeders. Data is represented as mean T cell count ± SEM (n = 5). **(F)** VST from a single donor buffy coat were compared for optimal cell yields at day 14 (grey), and day 21 with feeder re-stimulation at day 14 (Day 21 FR, blue). **(G–J)** Final product phenotype and T cell memory status was compared at both harvest time-points: Day 14 (grey triangles) and Day 21 FR (blue triangles). Data is represented as mean ± SEM. No significant differences were observed using paired t-tests with Holm-Sidak correction for multiple comparisons.

were closely correlated with time from resolution of infection, with VSTs dropping to less than 0.03% by 60 days post-resolution of infection. Further work is ongoing to determine whether SARS-CoV-2 VST remain detectable later in convalescence, though preliminary investigations suggest residual T cell responses up to six months (34). However subsequent re-exposure to the virus may result in reinforcement and expansion of these residual cells.

Virus antigen-stimulated T cells were isolated using a GMP-compliant IFN-γ bead selection process and rapidly expanded *in vitro*. The isolated peptide-reactive T cells were predominantly differentiated effector T cells, with an equal CD4:CD8 split, but after 14-day culture, there was an overwhelming shift to central memory phenotype with a strong skew to CD4 T cells and negligible expression of T cell exhaustion markers seen in some donors (35). This correlates closely with the CD4-predominant expanded populations produced using the methods described by Keller et al. (36). This change may reflect a loss of effector T cells and a rapid expansion of the central memory (TCM) compartment. The cultured VSTs also retain strong specificity for viral peptides as co-culture with autologous peptide-loaded DC drives a pronounced CD4 activation and cytokine response, indicating that these expanded T cells retain proliferative capacity. Interestingly, the CD8 response was significantly lower than CD4 VSTs, with low expansion and weaker responses to the SARS-CoV-2 antigens. This reduced CD8 response may be an advantage for a therapeutic product, as in models there is a clear protective role for CD8 T cells against acute SARS infection (37), but there is evidence that hyper-activation of CD8 T cells can be linked to severity of COVID-19 disease (38). The expanded CD8 VST cell demonstrated differential responses to each protein peptide pool and there was clear indication that the nucleocapsid protein is the immunodominant antigen for the cytotoxic T cell population.

Adoptive anti-viral T cell therapy has been an important therapeutic approach for other infections such as EBV, CMV and adenovirus (20, 22, 39). Various manufacturing methods have been developed, but cytokine capture has proved effective for isolation of T cells for clinical therapy (17–19). Other methods for isolation and/or expansion of antigen-specific T cells have been developed, including isolation of tetramer-binding T cells (40), selection of activated T cells post antigen-exposure using CD137 selection (41), and expansion of virus-specific T cells through optimized *in vitro* stimulation with peptides and cytokines (36).

Direct "collect and select" methods such as tetramer selection or cytokine-based selection followed by infusion without expansion do have advantages in terms of rapidity of manufacture, but are very limited in the doses of T cells that they yield. Selection by surface marker such as CD137 has potential advantages in that it is not restricted to a subset of cells making one cytokine, but requires pre-depletion of interfering components such as CD25+ regulatory cells, and also selects activated NK cells, so may still require substantial manipulation and potential cell losses to achieve a memory T cell product (41).

The dose of cells required to treat SARS-CoV-2 infection is currently unknown, however treatment of patients where rising viremia is targeted such as cytomegalovirus (CMV) and adenovirus (AdV), have used total doses of up to $10^7$ cells and single or few repeat doses (19, 40). Whether interventions established for these DNA viruses will be applicable to an RNA virus remains to be determined. We are concentrating on manufacturing an HLA-typed bank of donated cells, as even a modest manufacturing process would yield 100 to 200 doses at a target dose of $10^7$ cells per treatment. This approach could also be achieved using the recently published methods (also based on previous work with other virus infections such as CMV/ADV) by Keller et al. in October 2020 (36).

A VST product based on this manufacturing method, or others currently in development (42), will require careful First in Human clinical trials to determine the best route for use of VST in SARS-CoV-2 infection. Patients treated with VSTs for CMV- or Epstein-Barr Virus (EBV)-mediated disease are commonly immune-suppressed as a result of the treatment pathway for stem cell or solid organ transplantation (19–21). A rapidly increasing CMV titer against a background of leukopenia, or proliferating EBV-infected lymphoma cells in an immune-suppressed solid organ graft patient will present differently to COVID-19 infection in terms of homeostasis for allogeneic T cell engraftment and antigen availability to stimulate T cell expansion. First clinical indications would be likely be in patients who are already immune-suppressed, capable of accepting a T cell graft, and at increased risk of severe COVID-19 disease. Recent evidence in the cancer field suggests that T cells up to $1 \times 10^9$ per dose (43) can be introduced with indicators of *in vivo* efficacy with no prior leukodepletion – this may also support the introduction of SARS-CoV-2 VSTs in "at risk" individuals in early COVID-19 infection without conditioning. SARS-CoV-2–specific VST may also be supportive in patients with current COVID-19 infections who are at high risk of increased disease severity due to pre-existing co-morbidities or who have susceptible immunotypes (44), and protect against exacerbation of infection.

The initial 14-day culture of SARS-CoV-2 VSTs in GMP-compliant reagents demonstrated that a suitable therapeutic T cell product could be manufactured from even small numbers of VSTs present in a single unit of blood, yielding up to $5 \times 10^9$ cells per manufacturing run with greater than 90% central memory T cells and a 3.3 log expansion. Further expansion was provided by a second round of stimulation with autologous feeder cells, resulting in an approximate 2-log further increase in cell numbers with a consolidation of central memory phenotype, plus reduction in NK cells. There was a further skew towards CD4 T cells during this second expansion. Thus, scaling up to a full manufacturing process using lymphocyte-optimized leukapheresis instead of buffy coats, with GMP-compliant isolation using CliniMACS Prodigy cell processing and expansion in closed-culture G-Rex culture flasks we could generate sufficient material to treat multiple patients from a single suitably HLA-matched donor, with or without a second expansion phase. In this study donors were not selected based on HLA type. However, HLA typing of consented donors revealed the presence of HLA class I and II alleles common in the UK population and donor genotypes contained alleles known to present multiple SARS-CoV-2 peptides *in silico* (23, 45). As

further information becomes available on HLA allele/peptide binding, donors could be selected for optimal VST efficacy on the basis of HLA type which would clarify which loci and alleles should be matched between a T cell donor and patient for best effect. HLA matching is important for effective function of adoptive T cell therapies, and it is clear that some HLA types are poorer at presenting SARS-CoV-2 peptides such as B*46:01 (45). However, the donor HLA range seen in **Table 1** indicates HLA subtypes with strong peptide-presenting capacity such as A*02:01 and DRB*15:01, so supplementation of immune response with donor T cells from a matched donor could be a therapeutic option where few others exist. Therefore, we demonstrate the feasibility of generating large quantities of virus-specific T cell products for clinical trials in support of severe SARS-CoV-2 infections where the endogenous T cell response is compromised, representing a potentially significant advance in therapy for COVID-19 (46).

## LIMITATIONS OF THIS STUDY

This study has used the three SARS-CoV-2 antigen peptide pools (Spike, Nucleocapsid and Membrane) available from April 2020 to generate the phenotyping and expansion data of antigen-specific T cells. Although quality tested, the peptide pools used in this study are research grade and require reconstitution and filtration. GMP-grade peptide pools designed for unmanipulated use with e.g. the prodigy system should be adopted once available. Additional antigens may reveal a fuller picture of the immune response in COVID-19, and their addition to the T cell expansion method described here could have a positive additive effect. However this could also increase the degree of cross-reaction with other non-SARS coronavirus-specific T cells. The demographics of the local donor pool used is necessarily limited to blood donors who have undergone mild COVID-19 disease. Different T cell responses and proliferation characteristics may be found in other donor populations recovering from a range of COVID-19 symptoms. Clinical use of a VST product based on this manufacturing strategy will require controlled clinical trials for safety and efficacy assessment.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by SNBTS Sample Governance Committee, SNBTS. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

RC, AF, MT, and JC conceived and designed the study. RC, AF, MT, and JC drafted and revised the manuscript. RC, AF, LS, PB, SI, and DT performed experimental work. RC, AF, LS, PB, SI, DT, and JC analyzed data. LJ, SZ, and MT performed clinical tasks including identification, consenting, and testing of donors. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.598402/full#supplementary-material

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REVIEW
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# Allogeneic CAR Cell Therapy—More Than a Pipe Dream

*Kenneth J. Caldwell, Stephen Gottschalk and Aimee C. Talleur**

*Department of Bone Marrow Transplantation and Cellular Therapy, St. Jude Children's Research Hospital, Memphis, TN, United States*

Adoptive cellular immunotherapy using immune cells expressing chimeric antigen receptors (CARs) has shown promise, particularly for the treatment of hematological malignancies. To date, the majority of clinically evaluated CAR cell products have been derived from autologous immune cells. While this strategy can be effective it also imposes several constraints regarding logistics. This includes i) availability of center to perform leukapheresis, ii) necessity for shipment to and from processing centers, and iii) time requirements for product manufacture and clinical release testing. In addition, previous cytotoxic therapies can negatively impact the effector function of autologous immune cells, which may then affect efficacy and/or durability of resultant CAR products. The use of allogeneic CAR cell products generated using cells from healthy donors has the potential to overcome many of these limitations, including through generation of "off the shelf" products. However, allogeneic CAR cell products come with their own challenges, including potential to induce graft-versus-host-disease, as well as risk of immune-mediated rejection by the host. Here we will review promises and challenges of allogeneic CAR immunotherapies, including those being investigated in preclinical models and/or early phase clinical studies.

**Keywords: allogeneic, CAR, cell therapy, immunotherapy, cancer**

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*Banc de Sang i Teixits, Spain*

***Correspondence:**
*Aimee C. Talleur*
*aimee.talleur@stjude.org*

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## INTRODUCTION

Adoptive cellular therapy refers to the isolation of immune cells, followed by *ex vivo* manipulation and subsequent delivery into patients as a therapeutic intervention. An area of interest is the exploration of cellular or immunotherapeutic approaches for the treatment of oncologic diseases, including using chimeric antigen receptors (CARs) (1–3). CARs combine the specificity of an antibody with signaling domains of effector cells and costimulatory molecules (1–3). When constitutively expressed on the surface of an immune cell through non-viral or viral transduction, CARs enable an effector cell to recognize targets in an antigen-specific manner. CARs designed to target a specific tumor-associated-antigen (TAA) can then be used for anticancer therapy (1–3).

Cell therapy with T cells expressing CARs (CAR T cells) represent a significant advance in the field of cancer immunotherapy and is fueling the development of CAR-based immunotherapies using other immune cells. The most successful CAR cell therapy approach thus far has been the treatment of patients with highly relapsed/refractory CD19-positive hematological malignancies using CD19-CAR T cells derived from autologous T cells. Across numerous institutions, using a variety of CAR constructs and manufacturing strategies, CD19-CAR T cell therapy has been extremely efficacious (4, 5). This success

led to the FDA approval of three such products: tisagenlecleucel (Kymriah, Novartis), axicabtagene ciloleucel (Yescarta, Kite Pharmaceuticals), and brexucabtagene autoleucel (Tecartus, Kite Pharmaceuticals) (6–9). Additionally, autologous CAR T cells have shown robust anti-tumor activity for hematological malignancies targeting BCMA, CD20, CD22, and CD30 (10–13).

The autologous (patient-derived) CAR T cell paradigm has also highlighted the limitations of such therapies, including the challenges of leukapheresis, manufacturing and efficacy in an often heavily pre-treated patient population (14). Seeking to overcome these barriers, allogeneic CAR strategies are actively being developed. Significant challenges of using allogeneic cells exist and center upon the inherent immunologic mismatch between donor and recipient. However, despite these challenges, allogeneic CAR strategies hold the potential to offer quicker, more efficacious and more accessible CAR therapies.

In this review, we will discuss a variety of allogeneic CAR cell therapy platforms that are being developed, including the use of different immune cells and/or subtypes, as well as gene-editing techniques (**Figure 1**). Additionally, we will highlight clinical experiences with allogeneic CAR cell therapies and on-going clinical trials to treat malignancies.

## THE NEED FOR ALLOGENEIC CAR THERAPIES

Most CAR cell therapies to date, including the FDA approved products, are generated using autologous T cells. This has several important advantages, including infusion of CAR-engineered cell products without immunologic mismatch between donor and recipient. However, the use of autologous immune cells also has clinical and economical disadvantages. Autologous CAR cell production can be long and complicated. The process includes navigating the logistics of performing successful leukapheresis for a patient with relapsed/refractory malignant disease, accessing a manufacturing/treatment facility, and shipping and manufacturing times that commonly take several weeks. This time delay can be significant, particularly in a group of patients with aggressive relapsed/resistant cancers, who are at risk of clinical deterioration which could preclude proceeding with CAR cell therapy. Furthermore, generation of a cell product is not guaranteed and for those whom a product can be successfully generated, a proportion have limited short- or long-term efficacy. This is likely in part due to poor autologous immune cell fitness in cancer patients, particularly following aggressive cancer-directed therapies (15). Earlier collection of T cells may ameliorate some issues related to autologous T cell fitness. However, this strategy would only benefit the subset of patients determined to be at high-risk of needing CAR therapy early on in their disease process. Lastly, autologous cell therapy is performed for individual patients and is associated with significant costs, limiting broader applications of this therapy (16, 17).

The use of immune cells from donors, or allogeneic cell therapies, offers many advantages over autologous cells including the potential to be cost effective, readily available, and provide a higher quality product (**Figure 2**). Healthy donor cells confer a more uniform starting material, allowing for more predictable manufacturing and performance of generated cell product. Following the single donor to single recipient model, use of a family member would provide an easily accessible and highly motivated allogeneic donor. Furthermore, allogeneic therapies have the potential to provide a ready to use "off the shelf" immunotherapeutic, such that a single manufacturing run would allow dosing for several patients and/or multiple dosing for individual patients. Likewise, by increasing the scale of production and creating an inventory or bank of manufactured CAR immune cells from healthy donors, the cost per patient would decrease while access to product would increase.

## CHALLENGES WITH ALLOGENEIC THERAPIES

Despite the recognized potential benefits of allogeneic CAR therapies, they are not without risk and significant challenges

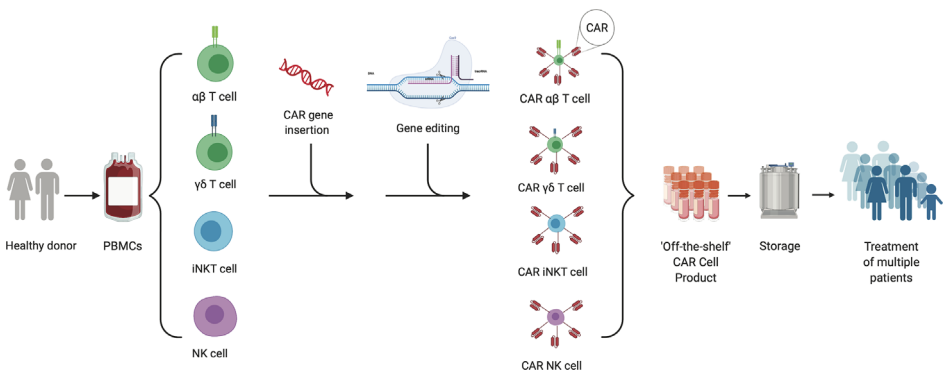


**FIGURE 1** | "Off the Shelf" allogeneic cellular therapy production. The production process for an allogeneic cellular therapy starts with collection of peripheral blood mononuclear cells (PBMCs) *via* leukapheresis from a health donor. Cells can then be sorted and selected for depending on the desired starting cellular material. CAR-encoding genes can be either inserted by non-viral or viral transduction or gene editing into immune cells. Additional gene editing can be performed to knock out genes of interest to mitigate risks such as immunogenicity and/or graft-versus-host-disease. The final product created from a single donor can be expanded, stored and used to treat multiple patients.

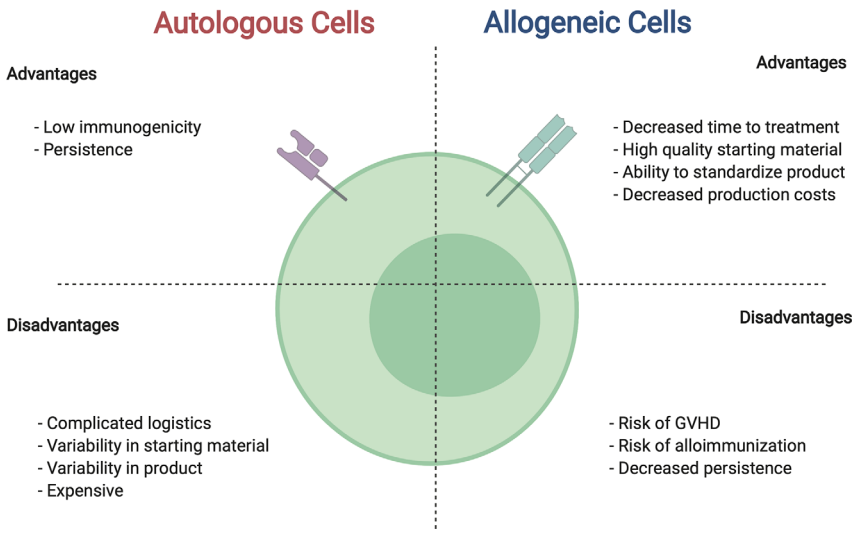


**FIGURE 2** | Comparison between autologous and allogeneic cells for use in CAR cell therapies. For details see text.

must be overcome in order to successfully implement this approach. These challenges stem from the immunologic mismatch between donor and recipient, and the resultant bidirectional risk to the cellular product and to the recipient *in vivo*. If the administered allogeneic cells recognize and attack healthy recipient tissues, the cellular therapy may cause unwanted graft-versus-host-disease (GVHD). Conversely, if the recipient's immune system recognizes and reacts against the allogeneic product the cell therapy may be rejected, limiting the therapeutic effect.

## Graft-Versus-Host-Disease

Allogeneic hematopoietic cell transplant (HCT) highlights the significant risk of GVHD by adoptive transfer of allogeneic T cells (18). Human leukocyte antigen (HLA) mismatch between donor and recipient leads to donor immune recognition of, and subsequent alloreactivity against, recipient tissues (19–21). Clinically, this is manifested as GVHD. T cells are primarily responsible for causing acute GVHD, triggering tissue cell death *via* FAS ligand, perforin, granzyme and other signaling pathways (22, 23). The risk of GVHD correlates with increasing donor/recipient HLA-disparity. Most commonly affecting the skin, gastrointestinal tract and liver, GVHD carries a significant risk of post-HCT morbidity and mortality (24, 25). In HCT, the risk of GVHD may be mitigated through donor selection, T cell depletion/selection and/or use of immunosuppressive pharmacologic therapies (26–30). However, some of these strategies are in direct opposition with the goals of allogeneic CAR therapies which depend on highly immunocompetent cells. Therefore, decreasing the risk of GVHD from allogeneic CAR immune cells must balance with the need to retain high levels of immune activity of the effector cell. Such strategies may include T cell-subset selection or gene editing approaches, as well as continued exploration of cell products such as γδ T cells, invariant (i)NKT cells, or allogeneic NK cells that do not induce GVHD.

## Immunogenicity

Expansion and persistence of CAR immune cells are important to achieve both short- and long-term efficacy. While desired duration of persistence may vary based on the malignancy being treated, it has been shown that prolonged remission of acute leukemia correlates with duration of persistence of autologous CAR T cells (31). In the autologous setting, CAR cell longevity can be compromised through immunological rejection of the CARs "foreign" proteins. The use of allogeneic immune cells carries further increased risk of immunogenicity as both the CAR and effector cells are "foreign." Acutely, this would result in impaired short-term responses as cells are rejected before exerting the intended therapeutic effect. The use of lymphodepleting chemotherapy prior to infusion of allogeneic CAR cellular products should mitigate the risk of acute rejection, augmenting CAR cell persistence (32). However, subsequent recipient immune reconstitution may result in delayed rejection of the adoptively transferred cells, providing an opportunity for malignant relapse. Furthermore, the use of allogeneic cells confers risk of alloimmunization, where the recipient develops donor-specific anti-HLA antibodies (DSAs). Alloimmunization is a well-recognized cause of graft failure and rejection in HCT (33). While desensitization strategies exist, the development of DSAs may preclude a patient from proceeding with HCT in the future or limit re-dosing of the allogeneic CAR product. Genetic modification to remove donor major histocompatibility complex (MHC) molecules or expansion of donor pools to allow for increased HLA-matching may mitigate these risks.

# ALLOGENEIC CAR STRATEGIES BY EFFECTOR CELL TYPE

The most widely used CAR platform currently in clinical practice are CAR T cells. These products are largely manufactured using a

batched pool of autologous donor T cells collected *via* peripheral blood leukapheresis and the CAR-T product administered without selection of specific cell types. While this strategy may work in the autologous setting, to mitigate potential risk of using allogeneic immune cells strategies using various T cell subgroups or different immune cell types are being tested in both preclinical and clinical settings. Here we review strategies being explored to make allogeneic CAR immune therapy possible, as well as ongoing clinical trials evaluating these strategies (**Table 1**).

## T Cells

T cells are a powerful component of the human immune system, providing surveillance for, and protection against, foreign antigen. Antigen recognition occurs *via* the T cell receptor (TCR), a heterodimer complex composed of two subunits and located on the surface of T cells. In a healthy donor, the majority (> 90%) of circulating T cells have a TCR consisting of an alpha (α) and beta (β) chain, referred to as an αβ T cell (34). The remaining T cells contain a TCR composed of a gamma (γ) and delta (δ) subunit, γδ T cells (35). The interaction between the TCR and antigen triggers a signaling cascade through the TCR which activates the T cell. αβ T cells recognize foreign or non-self-antigen presented through the MHC on antigen presenting cells, while γδ T cells are MHC-independent (36). Furthermore, αβ T cells can be subdivided based on function (i.e., CD4-positive and CD8-positive T cells) and/or degree of differentiation (i.e., naïve and memory T cells) (37, 38). When a T cell is transduced with a CAR, the CAR adds an additional receptor to the T cell without interruption to the native TCR. CAR T cells can expand and contract in response to antigen stimuli *via* the CAR, allowing for robust responses in the setting of active target recognition, but also potential for memory-surveillance state when an intended target is not currently present. Below, we review different types of T cells used to generate allogeneic CAR T cells.

### T Cells From Prior Allogeneic Transplant Donor

The use of donor lymphocyte infusion (DLI) after allogeneic HCT is a standard clinical practice. The therapeutic intent of unmodified DLI centers on the properties of donor T cells, such that they can

**TABLE 1** | Selected clinical studies with allogeneic CAR immune cells.

| Target | Diagnosis | Strategy to reduce GVHD and/or rejection | Other genetic modification | NCT # |
|---|---|---|---|---|
| **T cells** | | | | |
| BCMA | MM | TRAC KO | CAR (LV), CD52KO | NCT04093596 |
| | MM | TRAC and B2M KO | CAR (knock in) | NCT04244656 |
| CD7 | T-cell leuk or lymph | TRAC KO | CAR (*), CD7 KO | NCT04264078 |
| CD19 | NHL | TRAC KO | CAR (LV), CD52KO | NCT03939026 |
| | NHL | TRAC KO | CAR (LV), CD52KO | NCT04416984 |
| | B-ALL | TRAC KO | CAR (LV), CD52KO | NCT02808442 |
| | ALL | * | CAR (*) | NCT04173988 |
| | B-cell leuk or lymph | TRAC and B2M KO | CAR (LV) | NCT03166878 |
| | B-cell leuk or lymph | TRAC and B2M KO | CAR (knock in) | NCT04035434 |
| | B-cell leuk or lymph | * | CAR (*) | NCT04384393 |
| | B-cell leuk or lymph | * | CAR (*) | NCT04264039 |
| | Leuk or lymph | * | CAR (*) | NCT04227015 |
| | B-ALL or lymph | TRAC KO | CAR (knock in) | NCT03666000 |
| | B-cell leuk or lymph | * | CAR (*) | NCT03229876 |
| | B-ALL | * | CAR (*) | NCT04166838 |
| CD19/20/22 | Leuk or lymph | TRAC KO | CAR (*) | NCT03398967 |
| CD20 | Lymph | * | CAR (*) | NCT04176913 |
| | B-cell lymph or CLL | * | CAR (knock in) | NCT04030195 |
| CD22 | B-ALL | TRAC KO | CAR (LV), CD52KO | NCT04150497 |
| CD70 | Leuk or lymph | TRAC and B2M KO | CAR (knock in) | NCT04502446 |
| | RCC | TRAC and B2M KO | CAR (knock in) | NCT04438083 |
| CD123 | AML | TRAC KO | CAR (LV) | NCT03190278 |
| CS1 | MM | TRAC KO | CAR (LV) | NCT04142619** |
| NKG2DL | CRC | * | CAR (RV) | NCT03692429 |
| Mesothelin | Mesothelin+ ST | TRAC KO | CAR, PD1 KO | NCT03545815 |
| **EBV-specific T cells** | | | | |
| CD19 | Leuk or lymph | Cell product | CAR (RV) | NCT01430390 |
| CD30 | Lymph | Cell product | CAR (RV) | NCT04288726** |
| **γδ T cells** | | | | |
| NKG2DL | ST | Cell product | CAR (RV) | NCT04107142** |
| **iNKT cells** | | | | |
| CD19 | B-cell leuk or lymph | Cell product | CAR (RV), IL15 | NCT03774654 |
| **NK cells** | | | | |
| CD19 | B-cell leuk or lymph | Cell product (cord blood) | CAR (RV), IL15 | NCT03056339 |
| CD19 | B-cell lymph or CLL | Cell product (iPSC) | CAR, IL15, CD16 | NCT04245722 |

*ALL, acute lymphoblastic leukemia; CLL, chronic lymphatic leukemia; CRC, colorectal cancer; NHL, non-Hodgkin lymphoma; MM, multiple myeloma; Leuk, Leukemia; Lymph, lymphoma; RCC, renal cell carcinoma; ST, solid tumor.*

*LV, lentivirus; RV, retrovirus; *not disclosed; **not yet recruiting or currently closed for recruitment (as of 10'2020).*

correct mixed donor/recipient chimerism and combat viral infections (39). However, DLIs are not specific for TAAs and therefore have minimal anti-cancer benefit, especially outside the setting of minimal residual disease (39–41). As CAR T cells began to be explored clinically for the treatment of active disease, an initial venture into allogeneic CAR T cell products focused on the post-HCT population using T cells from the HCT donor. Brudno et al. evaluated the use of allogeneic CD19-directed CAR T cells derived from an individual patient's HCT donor to treat patients with progressive disease after transplant, who had a median donor chimerism of 100%, demonstrating anti-tumor benefits and safety of this approach, including no reports of new-onset GVHD (42). This study exemplified the possibility of increasing the potential for graft-versus-tumor effect of donor-derived T cells, without significantly increasing risk of GVHD.

The use of HCT-donor-derived CAR T cells is limited to post-HCT patients with an available and willing donor, whom are largely treated at facilities with the capability to manufacture clinical grade CAR T cell products. Therefore, this method is innately lacking some of the benefits of an "off the shelf product" and is not widely accessible. However, several benefits of using an allogeneic product are retained, including the use of healthy donor cells, ease of leukapheresis timing and minimal risk of diminished persistence *in vivo* due to lack of HLA-mismatch. Additionally, this approach allows for exploration into the use of CAR T cells in different disease settings such as prophylaxis post-HCT to reduce relapse in high-risk populations. Data suggest that this strategy is feasible without added toxicity and, in addition to providing leukemic control, may help with control of viral-reactivations post-HCT *via* native TCR recognition (43, 44).

## Virus-Specific T Cells

The adoptive transfer of allogeneic virus-specific T cells (VSTs) has emerged as a safe and effective means of providing antiviral benefit in multiple patient populations (45–48). This has led to the generation of partially HLA-matched banks comprised of libraries of "off the shelf," purified allogeneic VSTs. Importantly, across numerous clinical studies including in allogeneic HCT populations, the incidence of GVHD has been very minimal. Although the complete mechanism is not fully understood, decreased TCR diversity in VSTs (i.e., memory T cells) is felt to decrease the risk of alloreactivity (i.e., GVHD).

The safety profile seen using allogeneic VSTs created interest in the development of an allogeneic platform using CAR-transduced VSTs. Demonstrating feasibility of VSTs as effector cells, autologous CAR transduced VSTs targeting TAAs (CD30 [Hodgkin lymphoma], HER2 [glioblastoma] and GD2 [neuroblastoma, osteosarcoma]) have successfully been manufactured and infused into patients, with encouraging safety and efficacy profiles (49–51). This strategy has also been explored in the post-HCT patient population using primarily donor-derived VSTs and thus far results are promising. In one study, CD19-redirected VSTs were generated using peripheral blood mononuclear cells (PBMCs) collected from the HCT donor and then infused into patients with B-cell malignancy at escalating doses (52). Manufacturing time for this product was significant, requiring culture for 5 – 6 weeks. Treatment was well tolerated with no GVHD and there was evidence of anti-leukemia activity, as well as retained recognition of viral stimuli. Similar preliminary results have been reported in an on-going trial evaluating allogeneic EBV-specific T cells transduced with a CD19-CAR (NCT 01430390) (53). Notably, donor sources in this trial include the HCT donor or 3$^{rd}$ party donors when the HCT donor is not available, with recipients of the CAR-transduced 3$^{rd}$ party cells also showing encouraging response rates.

Clinical experience to date with allogeneic CAR-transduced VSTs has shown intended anti-tumor effects with minimal GVHD risk. Additional benefit includes the finding that viral-specificity is retained and can trigger CAR T cell expansion *in vivo*, thereby potentially providing on-going, intermittent stimulus and promoting persistence. A limitation of studies thus far centers on the fact that data are largely confined to CAR-transduced VSTs derived from a patients' HCT donor, thereby drastically minimizing the challenges of rejection and alloimmunization. Drawing from clinical experiences with unmodified VSTs from 3$^{rd}$ party banks, persistence of VSTs is typically limited to a few months (48); therefore, we would hypothesize that the issue of rejection and limited persistence of CAR-transduced VSTs remains.

## Memory T Cells

When devising strategies for allogeneic cellular therapies the use of memory T cell subsets as effector cells may confer a decreased risk of GVHD. T cell maturation and differentiation inversely correlates with alloreactivity, such that memory T cell subsets are less alloreactive than naïve T cells. Therefore, memory T cells are less likely to cause GVHD in the HLA-mismatched setting (54). Functionally it has also been noted that the effectiveness and persistence of CAR T cells is influenced by the degree of differentiation of the T cell subsets in autologous CAR T cell platforms (55–58). While the use of memory T cells to generate autologous CAR T cells is actively being studied using a variety of CAR constructs (NCT03389230, NCT02146924, NCT02051257, NCT03288493), their use in the allogeneic setting has not yet been evaluated.

T cell subsets can be distinguished through identification of extracellular surface markers, including CD45RO, CD45RA, CD62L, CCR7 and CD27 (37, 38). Several studies have highlighted that generating CAR T cells from central memory (CD45RO+/CD62L+ or CCR7+) T cells or memory stem cells (Tscm) T cell populations is associated with improved CAR T-cell effector function (59–61). Other groups have just focused on utilizing CD45RA-negative T cells, which includes the central memory and effector memory T cell subsets, since these subsets have decreased alloreactive potential (62). After showing promise in animal models, CD45RA-depletion began to be studied in human allogeneic HCT. Clinical studies demonstrated that this approach is feasible and carried a decreased risk of GVHD, both when utilized in primary graft manipulation and post-transplant DLI (63–67). Building upon this clinical experience, the role of memory T cell subsets as effector cells in CAR therapy has been studied pre-clinically. Investigators have shown that CD45RA-negative T cells expressing either a NKG2DL-specific or CD19-CAR have anti-cancer effects and decreased *in vivo* and *in vitro* alloreactivity (68–70). Using a similar approach, CD19-CAR-engineered CD27-negative T cells (effector and terminal effector memory subsets)

have also shown promise in preclinical models (71). These data suggest that the approach of using allogeneic memory T cells as effector cells in CAR therapy may have merit in the clinical arena; however, additional studies are needed to define the optimal memory T cell subset, which should be used as a source to generate memory CAR T cells with reduced alloreactivity.

## Genetically Modified αβ T Cells

Several strategies are being explored to improve allogeneic αβ T cells, which are summarized in **Figure 3**. Gene editing of T cells to reduce the risk of GVHD and rejection is perhaps the most promising and widespread approach, particularly for the development of an "off the shelf" product. Given that GVHD is driven in large part by TCR recognition of host tissue, gene-editing approaches focused on the native αβ TCR of the effector cell are under investigation. Many groups have explored disrupting the T cell receptor constant alpha chain (TRAC) or beta chain (TRBC). Torikai et al. showed that knocking out the αβ TCR from CD19-CAR T cells did not significantly alter the cells ability to kill CD19-positive targets (72). This initial report in 2012 used zinc finger nuclease mediated knockout of TRAC or TRBC. In recent years, with advancement in gene editing techniques, numerous groups have demonstrated knockout of TRAC using transcription activator like effector nucleases (TALENs) as well as CRISPR/Cas9 (73, 74). Another technique, targeting the CAR to the TRAC locus, was associated with improved anti-tumor activity in one preclinical study (74). In addition to gene-editing approaches, protein-based strategies are being developed to retain the TCR within the Golgi apparatus using an anti-TCR linked to the KDEL motive (75). While these techniques have become increasingly efficient, any remaining T cells that continue to express αβ TCR can be removed magnetically *ex vivo* using anti- αβTCR antibodies. *Stenger et al.* showed that TCR knockout of CD19-CAR T cells had high anti-leukemic functionality in the absence of alloreactivity. However, the gene-edited CAR T cells did not persist as long *in vivo* compared to CAR T cells with endogenous TCR, demonstrating a possible concern with this technique (76). Furthermore, modification to the endogenous TCR does not address the issue of immunogenicity.

To decrease immunogenicity, investigators have targeted β-2 microglobulin (B2M), a component of HLA class I molecules that is present on all T cells (77). As recipient T cell recognition of allogeneic CAR T cells can occur *via* interaction of HLA/MHC, knockout of the B2M gene in CAR T cells may prevent alloantigen presentation by infused the T cells. This thereby offers another strategy to limit host recognition and clearance of infused allogeneic CAR T cells. Ren et al. showed that CAR T cells including knockout of B2M had reduced alloreactivity *in vivo* (78). Kagoya et al. showed that B2M knockout to eliminate HLA class I as well as knockout of class II major histocompatibility complex transactivator (CIITA) to eliminate HLA class II improved CAR T cells persistence *in vitro* (79). Other strategies to mitigate immunogenicity rely on depleting resident immune cells. Lymphodepleting chemotherapy prior to cellular infusion by itself should reduce immune-mediated clearance of CAR T cells, however immune responses to

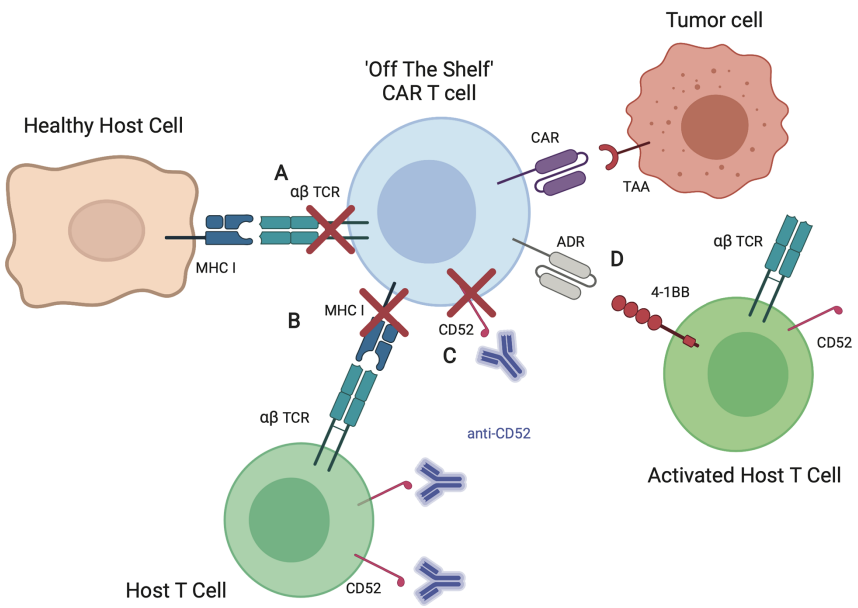


**FIGURE 3** | Strategies to improve allogeneic CAR T cells. **(A)** Elimination of the αβ TCR by knockout of TRAC or retaining TCRs within the Golgi apparatus reduces the risk of allogeneic T cell recognition of healthy host tissues, thereby decreasing risk of graft-versus-host-disease (GVHD). **(B)** Elimination of MHC I on allogeneic T cells by knockout of β-2 microglobulin (B2M) reduces the risk of host T cell recognition and elimination CAR T cells, increasing likelihood of allogeneic CAR T cell persistence. **(C)** Knockout of CD52, a T cell marker, allows for the use of CD52 antibody (anti-CD52) for enhanced lymphodepletion of host T cells without affecting infused allogeneic CAR T cells. **(D)** Expression of alloimmune defense receptor (ADR) destroys alloreactive host T cells targeting 4-1BB, decreasing the risk of rejection.

components of the CAR have been reported (80–82). To increase immunosuppression post-CAR T cell infusion, investigators have explored the use of the monoclonal antibody, alemtuzumab, which targets the pan-lymphocyte antigen CD52 (83). Since the mean half-life of alemtuzumab is 6.1 days (84), this approach would require the knockout of CD52 on allogeneic CAR T cells to prevent their depletion post infusion (85). Since the use of alemtuzumab is associated with an increased risk of viral reactivation/infection (e.g. cytomegalovirus, adenovirus) after allogeneic HCT (86, 87), close monitoring of recipients of alemtuzumab and CAR T cells is warranted for now since the risk of viral reactivation/infection in the setting of CAR T cell therapy is currently unknown. Lastly, active depletion of alloreactive host T cells is being explored including the expression of so called alloimmune defense receptors (ADRs) on infused allogeneic T cells that selectively recognizes 4-1BB, a cell surface receptor that is temporarily upregulated by activated host T cells (88).

Several clinical trials using gene-edited allogeneic CAR T cells are actively enrolling with some early results presented in abstract format. Many of these products include knockout of both CD52 and TRAC, to address both risk of GVHD and rejection. Pooled data from the CALM (NCT 02746952) and PALL (NCT02808442) studies (cellular product: UCART19) were presented by Allogene Therapeutics and Servier. UCART19 features an anti-CD19 scFv, as well as TRAC and CD52 knockout and was first shown to have clinical activity in two infants with B-ALL who achieved molecular remission after treatment (89). Data presented included 17 patients with relapsed/refractory B-ALL treated with a lymphodepleting regimen including alemtuzamab, 14 of whom had a CR or CRi. Additionally, UCART19 showed overall an acceptable safety profile (90). Neelapu et al. presented early results from the ALLO-501 trial (NCT03939026), which evaluates an allogeneic CAR T cell product targeting CD19 with knock out of TRAC and CD52, and includes a novel CD52 antibody for lymphodepletion prior to CAR T cell infusion. Twenty-two patients had been enrolled, with response seen in 12 of 19 evaluable patients (7 complete responses) (91). Wang et al. presented the first 5 patients treated with TruUCART GC027, an allogeneic anti-CD7 CAR T cell product with knock out of TRAC and CD7 by CRISP/Cas9 gene editing technology to avoid both GVHD and fratricide. Four of the initial 5 treated patients showed a complete response with an acceptable safety profile (92).

Gene editing has emerged as the leading strategy being tested in the clinic as investigators seek to develop an "off the shelf" allogeneic CAR therapy platform. As gene editing techniques advance, this method offers great potential to mitigate potential risks and downsides associated with the use of allogeneic cells. By increasing the number of genetic edits made to a single cell the possibilities increase; however, it will take time to thoroughly investigate the short- and long-term safety of these gene edited products in patients.

### γδ T Cells

Animal models show that γδ T cells play an important role in tissue homeostasis and cancer immunosurveillance (93). Allogeneic γδ T cells have been given to patients with cancer after lymphodepleting chemotherapy and were shown to expand *in vivo* without causing GVHD (94). γδ T cells recognize cancer through a broad spectrum of receptors, rather than in a single clonally expanded fashion, which may mitigate tumor escape *via* single antigen loss (95). These T cells are also typically abundant in tissues, which may provide an advantage over αβ T cells when developing therapeutics to treat solid tumors (93). Their ability to recognize targets in an MHC independent manner confers a low risk of alloreactivity and GVHD, thereby increasing the potential of using allogeneic γδ T cells in CAR T cell therapies.

Polyclonal γδT cells transduced with a CD19-CAR have been shown to expand and demonstrated anti-tumor effects *in vitro* and in *in vivo* murine models (96). Capsomidis et al. demonstrated CAR dependent antigen specific killing *in vitro* using GD2-CAR γδ T cells (97). Several companies are now moving forward with clinical trial development using allogeneic CAR-transduced γδ T cells including, Adicet Bio, Cytomed Therapeutics, GammaDelta Therapeutics and TC BioPharm (98). Furthermore, a clinical trial evaluating allogeneic CAR γδ T cells targeting NKG2DL for the treatment of solid tumors has been registered on clinicaltrials.gov, but is not yet recruiting (NCT04107142).

## iNKT Cells

Invariant natural killer T (iNKT) cells are a rare subclass of immune cells that are restricted by CD1d, a glycolipid presenting HLA I like molecule expressed on B cells, antigen presenting cells and some epithelial tissues (99–101). Thus, since iNKT express an invariant TCR, they do not cause GVHD. iNKT cells have been shown to be decreased in number and defective in cancer patients (102–104). iNKT cells also protect from GVHD after allogeneic HCT (105–107). Preclinical studies of iNKT engineered with CARs targeting CD19 and GD2 have been effective in murine models against lymphoma and neuroblastoma, respectively (108–110). CAR-engineered iNKT cells also appear to be safe in humans and are promising for "off the shelf" use given the lack of GVHD. Preliminary results from an ongoing trial (NCT03294954) using autologous GD2-CAR iNKT cells with co-expression of IL15 for the treatment of pediatric patients with neuroblastoma have shown this approach to be feasible and safe (111). Clinical experience of allogeneic CAR iNKT cells is yet to be published so possible adverse events cannot be predicted, however an ongoing clinical trial evaluating allogeneic CAR19-iNKT cells for the treatment of hematological malignancy (NCT03774654) aims to test safety and efficacy.

## NK Cells

NK cells, are of great interest in the treatment of cancer as they contribute to graft-versus-tumor effect and do not cause GVHD (112, 113). Endogenous NK cells are part of the innate immune system and can target cancer cells that downregulate HLA class I molecules (112). Tumor cells often downregulate their HLA molecules as an escape mechanism against T cells, making them susceptible to NK cells (114–116). Clinically, adoptively transferred non-CAR engineered allogeneic NK cells have shown to be safe in patients with cancer (117–120). NK cells are a promising alternative to T cells for CAR engineering given the low risk of GVHD and their innate anti-cancer properties. Numerous preclinical studies have shown CAR engineered NK cells to be effective against hematologic malignancy targets (CD19 and CD20), as well as solid tumor targets

(WT1 and GD2) (121–126). Notably, Liu et al. published results on 11 patients with lymphoid tumors, treated on an early phase clinical trial using allogeneic (cord blood derived) NK cells transduced with a gene containing a CD19-CAR, IL-15, and an inducible caspase 9 safety switch. In this study, 73% of patients demonstrated anti-tumor response. Inclusion of IL-15 in the CAR construct may have contributed to the persistence of the NK cells, which were shown to expand and persist for at least 12 months. There were no major toxic effects of the therapy (127).

Allogeneic NK cells can be prepared form numerous sources, including peripheral blood mononuclear cells (PBMCs), cord blood, and pluripotent stem cells (iPSCs) (128). In addition, a NK-cell line, NK-92, genetically modified to express a CAR, is actively being explored in early phase clinical testing (NCT03383978) (129). Current strategies to generate clinical grade NK cells from PBMCs or cord blood rely on the use of irradiated feeder cells, most commonly K562 cells genetically modified to express i) 4-1BBL and membrane-bound (mb) IL15 or ii) mbIL21 and exogenous IL2 (130–132). More recently, exosomes or plasma membrane particles derived from mbIL21 expressing K562 cells have also been successfully used for the *ex vivo* expansion of NK cells (133). iPSCs present an attractive source for generating NK cells without feeder cells (134, 135), and an early phase clinical study with unmodified iPSC-derived NK cells is in progress (NCT03841110). In addition, iPSC cells can be genetically modified and/or gene edited prior to NK cell differentiation, enabling the provision of an unlimited supply of modified NK cells (136–138).

## DISCUSSION

Autologous CAR T cell therapy has revolutionized the treatment of hematological malignancies, highlighting the therapeutic potential of cellular therapies, as well as opportunities for continued improvement. Subsequently, the number of CAR therapy trials has increased dramatically in recent years, exploring new targets, manufacturing strategies, CAR constructs, patient populations and effector cells. While many of these trials continue to use autologous immune cells, the number using allogeneic CAR products are rapidly increasing. The apparent benefits of allogeneic therapies have spurred a robust interest in developing techniques that counter the predicted limitations, including exploration of various effector cell types and gene editing techniques. One hurdle that will definitively need to be addressed is immunogenicity, which already has emerged as a potential roadblock of autologous CAR T cell therapies, especially when no lymphodepleting chemotherapy is given prior to T cell infusion (139). Thus, recipients of "off the shelf" cell products might require immune-modulation post cell infusion to enable their long-term persistence. However, given the extensive experience with allogeneic HCT and solid organ transplantation, we believe that immunogenicity will not present an unsurmountable barrier. While production of "off the shelf" therapeutic products require increased resources during the development and manufacturing process, the ultimate goal is to develop cell products that have a favorable safety and efficacy profile and are widely accessible and affordable. However, at present it is too early to estimate the cost of an allogeneic cell product; this will depend on the required genetic modifications, which might include not only viral transduction but also gene-editing. Another driving factor of cost will be how many cell doses can be prepared from one lot of "off the shelf" cell products since release testing of genetically-modified cell products is cost intense. Nevertheless, we believe that continued investment in the optimization of these allogeneic strategies is warranted based on the current data and that allogeneic cell products will usher in a new era of cell therapy.

## AUTHOR'S NOTE

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## AUTHOR CONTRIBUTIONS

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# HLA Class I Knockout Converts Allogeneic Primary NK Cells Into Suitable Effectors for "Off-the-Shelf" Immunotherapy

*Keven Hoerster[1], Markus Uhrberg[2], Constanze Wiek[3], Peter A. Horn[1,4], Helmut Hanenberg[3,5] and Stefan Heinrichs[1,4]**

[1] Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Essen, Germany, [2] Institute for Transplantation Diagnostics and Cell Therapeutics, Heinrich-Heine University, Düsseldorf, Germany, [3] Department of Otorhinolaryngology & Head/Neck Surgery, University Hospital Düsseldorf, Heinrich Heine University, Düsseldorf, Germany, [4] German Cancer Consortium (DKTK), partner site Essen/Düsseldorf, Essen, Germany, [5] Department of Pediatrics III, University Children's Hospital of Essen, University Duisburg-Essen, Essen, Germany

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**Edited by:**
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***Correspondence:**
Stefan Heinrichs
stefan.heinrichs@uk-essen.de

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Cellular immunotherapy using chimeric antigen receptors (CARs) so far has almost exclusively used autologous peripheral blood-derived T cells as immune effector cells. However, harvesting sufficient numbers of T cells is often challenging in heavily pre-treated patients with malignancies and perturbed hematopoiesis and perturbed hematopoiesis. Also, such a CAR product will always be specific for the individual patient. In contrast, NK cell infusions can be performed in non-HLA-matched settings due to the absence of alloreactivity of these innate immune cells. Still, the infused NK cells are subject to recognition and rejection by the patient's immune system, thereby limiting their life-span *in vivo* and undermining the possibility for multiple infusions. Here, we designed genome editing and advanced lentiviral transduction protocols to render primary human NK cells unsusceptible/resistant to an allogeneic response by the recipient's $CD8^+$ T cells. After knocking-out surface expression of HLA class I molecules by targeting the B2M gene *via* CRISPR/Cas9, we also co-expressed a single-chain HLA-E molecule, thereby preventing NK cell fratricide of B2M-knockout (KO) cells *via* "missing self"-induced lysis. Importantly, these genetically engineered NK cells were functionally indistinguishable from their unmodified counterparts with regard to their phenotype and their natural cytotoxicity towards different AML cell lines. In co-culture assays, B2M-KO NK cells neither induced immune responses of allogeneic T cells nor re-activated allogeneic T cells which had been expanded/primed using irradiated PBMNCs of the respective NK cell donor. Our study demonstrates the feasibility of genome editing in primary allogeneic NK cells to diminish their recognition and killing by mismatched T cells and is an important prerequisite for using non-HLA-matched primary human NK cells as readily available, "off-the-shelf" immune effectors for a variety of immunotherapy indications in human cancer.

**Keywords: NK cells, B2M knockout, HLA class I, off-the-shelf, allogeneic, genome editing, immunotherapy, adoptive cell transfer**

## INTRODUCTION

Adoptive cell transfer (ACT) of autologous genetically modified immune cells has emerged as an attractive treatment option for various malignancies of hematologic origin. Yet, the highly personalized nature of these cell products generates extreme costs and patient-specific factors can still impede the manufacturing process due to large variabilities. For a significant number of patients, an autologous final product cannot be generated in time for treatment [reviewed in (1)].

To alleviate these problems, research in the field is moving towards "off-the-shelf" products, making use of immune effector cells from healthy donors. However, this endeavor is complicated by problems of alloreactivity and immune tolerance for mismatched HLA constellations. A severe side effect of allogeneic cellular therapy is Graft-versus-Host-Disease (GvHD), a life-threatening complication caused by the transplanted alloreactive T cells and known since the early days of hematopoietic stem cell transplantation (HSCT) (2–5). To circumvent this complication, several approaches have been developed. Virus-specific cytotoxic T (VST) cells, for example, have successfully been used to control latent infections post HSCT without causing GvHD (6, 7). Accordingly, they have been proposed as a potential T cell population to create "off-the-shelf" therapeutic products (8, 9). Another option is the selective depletion of T cell subsets alloreactive towards specific HLA (10, 11). However, this bias towards certain T cell subsets is again limiting the application potential of the products. An interesting approach to abrogate unwanted or alloreactive signaling from the endogenous T cell receptors (TCRs) in chimeric antigen receptor (CAR) T cells uses genome editing on common TCR domains (12, 13), however this genomic editing will require additional gene transfer systems and therefore will add several layers of complexities to CAR T cell clinical trials.

Thus, an obvious solution is to simply use another type of immune effector cells: natural killer (NK) cells. Importantly, even when infused at large quantities into immunocompromised patients, NK cells do not cause GvHD in the first place and can even prevent it (14, 15). In contrast to T and B cells, NK cells express germline-encoded activating and inhibitory receptors and integrate signals to distinguish between healthy and transformed or stressed cells (16). This *innate* recognition of transformed cells and absence of GvHD have proven to be of great potential for the treatment of malignancies in animal models and clinical trials (17–24). While the infusion of autologous NK cells is ineffective in various cancers, donor NK cell infusions after or through haploidentical HSCT demonstrated that NK cells with killer cell immunoglobulin-like receptor (KIR) mismatches with the recipient do not cause any damage to normal tissue, but still can eliminate residual malignant cells (25–32). Importantly, mature NK cells contained within the stem cell graft were shown to be responsible for the anti-tumor effects observed early after transplantation and therefore were unlikely to originate from the reconstituted NK cell compartment (33–35). The efficacy and apparent safety of NK cells in allogeneic adoptive cell therapies has made them an attractive cell type for the manufacturing of "off-the-shelf" cell-based products. However, two major aspects of human NK cells, the marked resistance to standard genetic modifications with lentiviral vectors and the limited *ex vivo* expansion capacities, have hampered their use for both allogeneic and also autologous CAR-redirected immunotherapies. Hence, researchers have resorted to stable NK cell lines, such as NK-92 (36–42), or to using NK cells differentiated from CD34+ hematopoietic stem and progenitor cells (HSPCs) or pluripotent stem cells (PSCs) (43–46) with subsequent expansion using feeder cells (47–50).

While alloreactivity and GvHD are activities initiated by the graft, graft rejection by the host's immune system is another factor to consider in allogeneic non-myeloablative therapies. To prevent rejection of the graft by the host immune system, a wide variety of concepts have been used, including the expression of the immune checkpoint inhibitors CTLA4-Ig and PD-L1 (51) or engagement of the "don't eat me"-signal CD47 (52). Rather than equipping cells with means to fend off attacking immune cells, some studies arm the therapeutic cells with receptors to fight back and lyse the approaching alloreactive host T cells (9, 53). Others have set out to disrupt the HLA barrier/antigens entirely, hiding the infused cells from recognition by alloreactive host T cells. The latter aim has been achieved either by targeting genes essential for the HLA processing machinery such as the class II transcriptional activator (CIITA) and beta-2-microglobulin (B2M) or by disrupting individual HLA genes (54–67). In most of these preclinical studies however, the starting material was either a transformed cell line or PSC cells that subsequently had to be differentiated into the tissue of choice in elaborated and time-consuming protocols.

In this study, we used recent advances in genome editing and HLA biology to generate NK cells ideally suited for adoptive cellular therapy. Based on the recent breakthrough for genetically modifying human NK cells (68), we constructed a chimeric envelope with the surface and transmembrane domains of the baboon endogenous retrovirus and the cytoplasmic tail of the amphotropic murine retrovirus for efficient gene transfer, similarly to constructs described before (69–71). We then disrupted HLA class I expression in human NK cells by targeting B2M *via* a CRISPR/Cas9 lentiviral vector (72) and finally equipped the HLA class I knockout NK cells with a modified single-chain HLA-E molecule (58, 73). Consequently, these double-modified NK cells neither activated nor expanded allogeneic T cells and were also protected from autolysis/fracticide by NK cells. Combined with novel NK cell culture expansion protocols for GMP settings (74, 75), highly cytotoxic, primary "off-the-shelf" human NK cells were generated in relevant amounts without the need of lengthy differentiation protocols, PSCs or feeder cells.

## MATERIALS AND METHODS

### Cells and Cell Lines

NK cells were purified from PBMC using a negative selection protocol with the NK cell isolation kit (#130-092-657, Miltenyi Biotec) and MACS LS columns (Miltenyi Biotec) according to

the manufacturer's protocol. NK cells were cultured in NK MACS medium (Miltenyi Biotec), supplemented with 1% of the enclosed NK MACS Supplement, 5% heat-inactivated human AB serum (Sigma-Aldrich, H4522), 1% Penicillin/Streptomycin (Sigma-Aldrich), 500 U/ml rhIL-2 and 140 U/ml rhIL-15 (both from Miltenyi Biotec) and termed NK MACS complete medium. T cells were purified from whole blood using the RosettaSep HLA T cell kit (Stemcell Technologies) according to the manufacturer's protocol and cultured in DMEM (Thermo Fisher Scientific) supplemented with 5% heat-inactivated human AB serum, 1% Penicillin/Streptomycin and 50 U/ml rhIL-2 if not stated otherwise, in the following termed T cell medium. Whole blood was obtained from healthy donors at the University of Düsseldorf after informed consent. PBMNCs were isolated by Ficoll density gradient centrifugation. SKM1, K562 and Kasumi-1 cell lines where maintained in RPMI medium (Thermo Fisher Scientific) supplemented with 10% heat-inactivated FCS, termed R10 medium.

## Lentiviral Vectors

The CRISPR/Cas9 vector pLE38-Cas9-sgB2M/gNKG2A is a third-generation self-inactivating (SIN) lentiviral vector based on the pRRL SIN backbone (76). Expression cassettes for the U6-promoter/gRNA and the EFSns-promoter/Cas9 were derived from pLCv2 (72). The targeting sections of the gB2M sequence (5′-GAGTAGCGCGAGCACAGCTA-3′) and gNKG2A sequences (5′-TGAACAGGAAATAACCTATG-3′) were designed using the GPP sgRNA designer tool (Broad Institute, Cambridge, MA, USA) and cloned into the Esp3I sites of the pLE38-Cas9-stuffer vector using annealed oligonucleotides. The sc-HLA-E coding sequence was designed after Gornalusse et al. (58). Briefly, the fragment encoding the HLA-E*03:01 heavy chain was cloned from HEK293T cells with the forward-primer incorporating the last repeat of the $(G_4S)_4$ linker for the final sc-HLA-E sequence and a BamHI restriction site and the reverse-primer harboring a *MluI* restriction site for assembly into the vector. The fragment encoding the sequence for B2M-leader/HLA-G-leader/$(G_4S)_3$-linker/mutatedB2M-chain/$(G_4S)_4$-linker was synthesized by LGC genomics. The gB2M targeting site and the protospacer adjacent motif (PAM) site were mutated from 5′-CCTTAGCTGTGCTCGCGCTACTC-3′ to 5′-CACTGGCC GTGCTGGCCCTGCTG-3′ to avoid editing of the sc-HLA-E by gB2M. The HLA-G-leader-B2M-sequence was PCR amplified using primers harboring restriction sites for *Xba*I and *BamHI* and assembled together with the amplified HLA-E*03:01 heavy chain encoding fragment into a lentiviral transfer vector *via* the *Xba*I and the *Mlu*I restriction sites. Expression was driven from the SFFV promoter (77).

For construction of the pcoBaEVTM chimeric baboon envelope vector, the surface and transmembrane subunits of the wild-type sequence of the M7 strain of the Baboon endogenous virus (NC_022517) was fused to the cytoplasmic sequence of the amphotropic murine leukemia virus (AF411814), synthesized as a codon-optimized cDNA by GeneArt (ThermoFisher) according to our design and then cloned in our envelope expression plasmid using *Eco*RI and *Not*I (78).

Lentiviral particles were produced in HEK293T cells by cotransfection of pcoBaEVTM, pCMV-ΔR8.91 (76) and the lentiviral transfer vector. Supernatants were harvested 48 and 72 h after transfection, concentrated by high-speed centrifugation, resuspended in non-supplemented NK MACS medium supplemented with 20 mM HEPES and titered on K562 and SKM1.

## NK Cell Transduction

Transductions were performed 7 days after the preparation of CD56+ CD3- cells and start of the NK cell expansion protocol. Briefly, lentiviral particles corresponding to an MOI of 1 (titered on K562/SKM1) were adjusted to a volume of 100 µl using plain NK MACS medium without additives, mixed with the equal volume of plain NK MACS supplemented with 5 µg/ml Vectofusin-1 (Miltenyi Biotec), incubated at room temperature for 8 min and mixed with 50 µl cell suspension containing $1 \times 10^6$ NK cells in NK MACS complete medium. For simultaneous double-transductions, both particle populations were used at MOIs of 1 and pooled prior to mixing with NK MACS and Vectofusin-1. Subsequently, the 250-µl cell/particle mix was transferred into 48-well plates and centrifuged for 90 min at 400*g*, 32°C. After spinoculation, cells were incubated at 37°C for additional 4 h before 500 µl NK MACS complete medium where carefully added to the cells.

## Flow Cytometry and Phenotyping

Flow cytometric data where acquired using a CytoFLEX (Beckman Coulter). Antibodies from Thermo Fisher Scientific were anti-HLA-E (clone 3D12; Thermo Fisher). Antibodies from BioLegend were: anti-pan-HLA class I (clone W6/32), CD3 (clone HIT3a), CD4 (clone RPA-T4), CD8 (clone SK1), CD56 (clone 5.1H11), CD16 (clone 3G8), CD57 (clone HNK-1), KIR2DL2/3 (CD158b, clone DX27), KIR2DL1/S1/3/5 (CD158a,h, clone HP-MA4), CD107a (clone H4A3), CD137 (clone 4B4-1), anti-NKG2D (CD314, clone 1D11), anti-NKp30 (clone P30-15), anti-NKp44 (clone P44-8), anti-NKp46 (clone 9E2). Antibodies from Miltenyi Biotec were anti-NKG2A (CD159a, clone REA110), anti-NKG2C (CD159c, clone REA205). Antibody stainings were performed in PBS (Thermo Fisher Scientific) supplemented with 0.5% BSA (Sigma-Aldrich) and 2 mM EDTA (Sigma-Aldrich), termed MACS buffer. Of note, the clone W6/32 does not recognize the sc-HLA-E due to the covalently linked N-terminus of the incorporated B2M (79), enabling discrimination between endogenous HLA class I and the sc-HLA-E. Analysis was performed using the CytExpert software (Beckman Coulter) and FlowJo V10.6.2 (Becton Dickinson).

## NK Cell Cytotoxicity Testing

NK cells were co-cultured for 6 h with K562 and Kasumi-1 cells in 100 µl R10 medium supplemented with 500 U/ml rhIL-2 and 140 U/ml rhIL-15 at the effector target ratios 4:1, 1:1 and 0.25:1. To allow for discrimination between NK cells and targets while avoiding to gate out dead target cells, a "no-wash" protocol was applied. Briefly, CD56-antibody was added to the wells at the end of the incubation period, mixed and stained at 4°C for 20 min.

Subsequently, the cell mixture was diluted in 300 µl of MACS buffer supplemented with 7AAD and incubated for 2 min at room temperature before data acquisition. The specific lysis of targets was determined by the percentage of $7AAD^+$ cells within the $CD56^-$ singlets.

## Fratricide Assay

NKG2A-KO NK cells were generated using gNKG2A, which targets the *KLRC1* gene encoding for NKG2A, and the transduction protocol as described above. The mixture of sc-HLA-E only and sc-HLA-E/B2M-KO NK cells, obtained after double transduction, was co-cultured for 24 and 48 h with either untransduced, parental NK cells from the same donor or NK cells after knockout of NKG2A. Cells were stained with 7AAD and antibodies for pan-HLA class I, HLA-E and NKG2A before acquisition. Selective depletion of B2M-KO cells was evaluated by gating on all sc-HLA-$E^+$ target cells and then discriminating between HLA class $I^+$ and HLA class $I^-$ cells.

## T Cell Proliferation Assay

Proliferation of allogeneic T cells was evaluated by CFSE dilution. Briefly, $5 \times 10^6$ freshly isolated T cells were resuspended in 5 ml prewarmed PBS/0.1% BSA. 10 µl of a 5-mM CFSE solution were added and cells were incubated at 37°C, 5% $CO_2$ for 7 min, subsequently topped up with 16 ml cold DMEM/10% FCS, incubated at 4°C in the dark for 5 min and washed twice. For the assay, 200,000 T cells where co-cultured with 50,000 NK cells in 200 µl T cell medium supplemented with 2 U/ml rhIL-2 for 6 days. Cells stimulated with PMA/Ionomycin served as a qualitative positive control and medium controls were used as negative controls. On day 6, cells were stained for CD56, CD4, CD8 and 7AAD. CFSE dilution was analyzed in $CD4^+$ and $CD8^+$ T cells after gating on $7AAD^-/CD56^-$ singlets.

## T Cell Reactivation and Degranulation Assay

Alloreactive T cells were expanded from isolated T cells by incubation for 14 days with 30-Gy irradiated PBMC of the respective NK cell donors. To test reactivation and degranulation of alloreactive T cells, 160,000 to 200,000 expanded T cells where incubated with 80,000 to 100,000 either parental or modified NK cells (Effector-Target ratio of 2:1) for 24 and 48 h in T cell medium. For degranulation assays, monensin and anti-CD107a antibody were added to the cultures 4 h before acquisition. For analysis, samples were stained with 7AAD and antibodies for CD3, CD4, CD8. For the reactivation assay, cells were additionally stained for CD137. Degranulation and reactivation were analyzed in $CD4^+$ and $CD8^+$ T cells after gating on $7AAD^-/CD3^+$ singlets. A baseline measurement was performed on the day the assay was set up and medium, as well as autologous and 3rd party NK cells, served as negative and specificity controls at the time points of analysis.

## T Cell Cytotoxicity Assay

For T vs NK cell cytotoxicity assays, CFSE-stained T cells were cultured for 20 h with the NK cell lines at the effector target ratios 4:1, 2:1 and 1:1 (calculated on $CD8^+$ T cells) in 200 µl T cell medium. A "no-wash" protocol was applied to prevent loss of dead target cells: Before acquisition, the cell mixture was diluted with the same volume of MACS buffer supplemented with 7AAD and incubated at room temperature for 2 min before acquisition. Autologous NK cells served as negative controls and for gating purposes. NK cell lysis by T cells was determined as the percentage of $7AAD^+$ cells within the $CFSE^-$ singlets.

## Statistics

Statistical analysis was performed using GraphPad Prism with the tests given in the figure legends. The level of statistical significance was set to $p < 0.05$. Statistically significant differences are reported in the figure legends.

# RESULTS

## Concomitant Single-Chain HLA-E Expression on Primary NK Cells Allows for a Functional Knockout of HLA Class I Surface Expression Without Leading to Fratricide

In primary NK cells, the efficient knockout of the classical HLA class I genes A, B and C by CRISPR/Cas9-based genome editing is challenging due to the extensive polymorphism and the presence of six genomic target sites. The functional elimination of all HLA class I proteins with a single hit can be achieved by using a single CRISPR/Cas9 lentiviral vector targeting the beta-2-microglobulin gene (B2M), the shared invariant light chain of all HLA class I molecules (**Figure 1A**). We first tested two distinct gRNAs targeting B2M (gB2M) in the pLE38-Cas9 vector for their knockout efficiencies by transducing the human diploid AML cell line SKM1. Analyzing the transduced cells after staining with the pan-HLA class I monoclonal antibody W6/32 revealed a decrease in surface expression of classical HLA class I molecules by flow cytometry, starting four days after transduction and generating stable knockouts with both guide sequences when analyzed 10 days later (**Figure 1B**). We decided to use gB2M#1 for further experiments as it yielded a higher gene editing rate.

Despite the high gene transfer efficiencies that can be achieved in NK cells with baboon envelope-pseudotyped lentiviral vectors (68), we observed only approximately 16% HLA class I-negative cells (**Figure 1C**) four days after transduction of primary NK cells with the CRISPR/Cas9 HLA class I targeting vector. This percentage of HLA class I-negative cells steadily decreased over time and dropped below 1% on day 14 post transduction (**Figure 1D**, red line). We hypothesized that this progressive loss of successfully targeted NK cells was most likely a consequence of the "missing self"-induced killing by neighboring NK cells, a phenomenon also called "fratricide". In parallel cultures, we therefore co-expressed a modified single-chain (sc-)HLA-E molecule (**Figure 1A**) on the surface of NK cells, as this chimeric protein can efficiently protect the HLA class I-negative NK cells from fratricide by engaging the inhibitory receptor dimer CD94/NKG2A (58). By itself, lentiviral overexpression of sc-HLA-E yielded a distinct positive

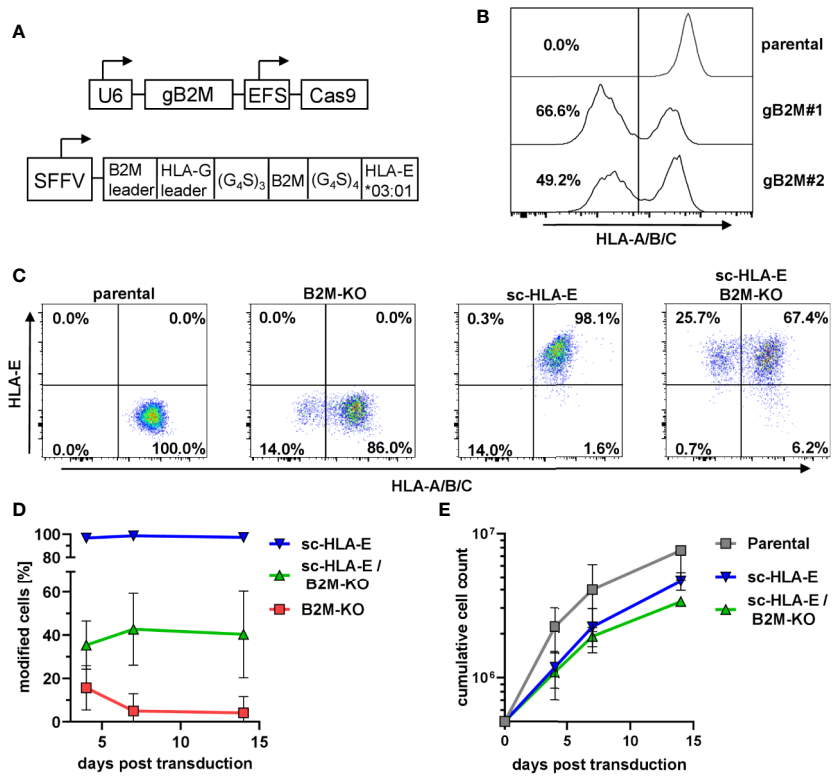


**FIGURE 1** | Knockout of B2M and simultaneous overexpression of sc-HLA-E in primary NK cells **(A)** Outline of the lentiviral expression cassettes used for CRISPR/Cas9 mediated B2M-knockout and expression of the sc-HLA-E molecule. Cas9 and gB2M are expressed from the same lentiviral vector, driven by an EFS and an U6 promoter, respectively. The HLA-E*03:01 heavy chain is linked to B2M and the HLA-G leader peptide by $G_4S$ linkers and expression is driven from an SFFV promoter. **(B)** Knockout of B2M in SKM1 cells abrogates surface expression of HLA class I detected *via* flow cytometry on day 10 after transduction. **(C)** Frequencies of HLA class I and sc-HLA-E expressing primary NK cells 4 days after transduction with lentiviral particles encoding gB2M and Cas9, sc-HLA-E or a combination of both compared to an untransduced control. **(D)** Frequencies of modified cells measured on day 4, 7 and 14 after transduction (mean ± SD; n = 6 for sc-HLA-E, n = 5 for sc-HLA-E/B2M-KO and B2M-KO). **(E)** Growth of NK cell cultures measured 4, 7 and 14 days after transduction. Shown is the cumulative cell count of all NK cells within the culture. The NK cells showed a fold-expansion from the day of transduction of 15.2 ± 5.72 for parental, 9.36 ± 1.29 for sc-HLA-E and 6.74 ± 0.31 for sc-HLA-E/B2M-KO NK cells (mean ± SD; n = 3).

population that was stable over time (**Figures 1C, D**, blue line). By pooling the two lentiviral supernatants encoding sc-HLA-E and sgB2M/Cas9, we achieved a mean gene editing/transduction frequency of 35.4% that remained stable around 40.4% on days 7 and 14 post transduction (**Figures 1C, D**, green line). NK cell expansion was documented for 14 days after transduction (3 weeks after isolation), demonstrating that sc-HLA-E and sc-HLA-E/B2M-KO modified NK cells indeed grew slower compared to the mock-transduced controls ("parental"), but still achieved an almost seven-fold expansion from the day of transduction (**Figure 1E**). NK cells were used for downstream experiments within 4 weeks from the day of preparation.

## Protection From Fratricide Is Dependent on NKG2A

In order to prove that the HLA-E-mediated protection of HLA class I-negative NK cells in mixed cultures was due to the engagement of the inhibitory receptor CD94/NKG2A (73), we designed the corresponding guide RNAs against *KLRC1*, the gene encoding NKG2A, and used the same lentiviral expression system as above (**Figure 2A**). Flow cytometry analysis demonstrated that transduction of primary human NK cells with the pLE38-Cas9 vector successfully abrogated NKG2A expression seven days post transduction (**Figure 2B**). To test whether *NKG2A*-KO NK cells lead to the relative reduction of sc-HLA-E/B2M-KO NK cells, as they would no longer be tolerated by NKG2A-deficient NK cells, the bulk-transduced NKG2A-KO NK cell cultures were co-cultured with a mixture of sc-HLA-E/B2M-KO and sc-HLA-E NK cells. Importantly, addition of NKG2A expressing parental NK cells did not have any effect on the frequency of sc-HLA-E/B2M-KO NK cells in a mix with B2M competent sc-HLA-E-expressing NK cells when compared to the baseline controls (**Figure 2C**, upper panel and **Figure 2D**). In contrast, a co-culture containing NKG2A deficient NK cells showed a strong depletion of the sc-HLA-E/B2M-KO NK cells after 24 h, leading to elimination of more than 85% of B2M-KO cells (**Figure 2C**, lower panel and **Figure 2D**). In support of these observations, we also noted that B2M-KO NK cells did not persist in NK cell cultures with low NKG2A-expression levels despite the presence of sc-HLA-E (data not shown). These results

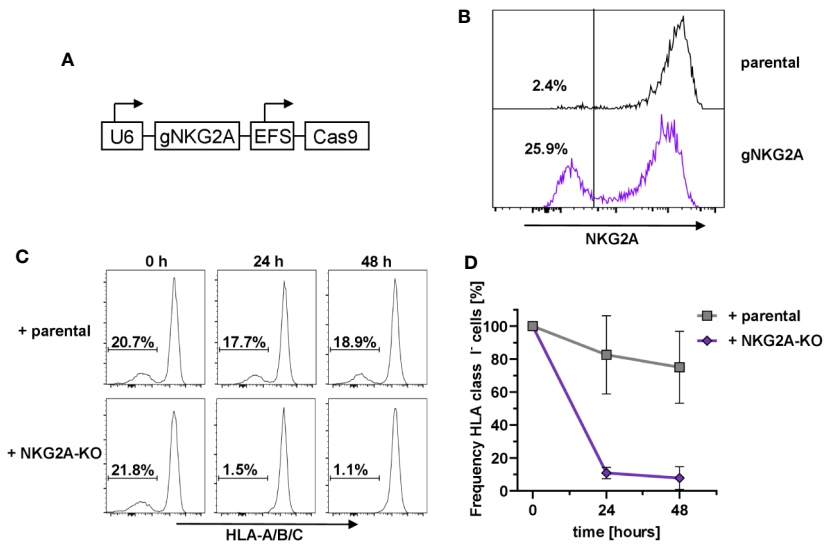


**FIGURE 2** | Dependency on NKG2A expression for sc-HLA-E-mediated protection from NK cell fratricide **(A)** Outline of the lentiviral expression cassettes used for CRISPR/Cas9 mediated NKG2A-knockout. **(B)** Transduction with Cas9 and gNKG2A led to abrogation of NKG2A surface expression in primary NK cells 7 days post transduction. **(C)** Representative histograms showing the frequency of sc-HLA-E/B2M-KO NK cells in presence of B2M-competent sc-HLA-E NK cells upon addition of parental cells (upper panel) or NKG2A-KO NK cells (lower panel) at different time points. **(D)** Quantification of changes in sc-HLA-E/B2M-KO NK cell frequencies relative to the time point (0 h) when parental or NKG2A-KO NK cells were added. Statistical analysis was performed by two-way ANOVA with Geisser-Greenhouse correction (ε = 0.6972) for repeated measures and Holm-Sidak testing for multiple comparisons between each time point within both groups. For the samples containing NKG2A-KO NK cells, the decrease in frequency of HLA class $I^-$ was statistically significant at both time points (mean ± SD; n = 4).

demonstrated that the prevention of fratricide is strongly dependent on the HLA-E/NKG2A signaling.

## B2M-KO NK Cells Are Phenotypically Similar to Unmodified Cells and Retain Uncompromised Effector Functions

HLA molecules serve a vital role in the education of NK cells *via* KIRs and NKG2A (80, 81). As the forced expression of HLA-E on neighboring cells might lead to tonic engagement of CD94/NKG2A, which can signal *via* downstream targets (82, 83), we next investigated if the genetic modifications in HLA expression impacted the NK cell phenotype and functions. To this end, we performed multi-color flow cytometric analysis using a panel comprising various maturation markers as well as activating and inhibitory receptors. The results in **Figures 3A, B** demonstrated that the modified NK cells exhibited an immunophenotypic profile similar to their unmodified counterparts: The majority of cells was $CD56^{bright}$ and CD16 was expressed on almost all cells with a slight bias towards $CD16^{bright}$ cells. Only a minor fraction of NK cells expressed CD57 usually associated with terminal maturation and replicative senescence (84), while almost all cultured NK cells were positive for the activating receptors NKG2D and NKp30. NKp44 and NKp46 was present on about 60 – 80% of NK cells from three donors, but little to no $NKG2C^+$ cells were detected. KIR2DL/S1/2/3 expression was detectable on roughly 50% to 60% of NK cells while NKG2A expression was present in over 90% of cells. Interestingly, the expression levels of NKG2A were diminished in both sc-HLA-E-expressing NK cells, rendering these cells $NKG2A^{dim}$. In addition, the frequencies of CD16, NKG2C and KIR expressing NK cells were slightly lower in the sc-HLA-E-expressing NK cell cultures compared to parental NK cells while the frequency of NKp44 expressing NK cells in cultures expressing sc-HLA-E was slightly higher.

To test the cytotoxic effector cell functions, the genetically modified NK cells were co-incubated with the AML cell lines K562 (HLA class $I^-$) and Kasumi-1 (HLA class $I^+$). Flow cytometric analysis after 6 h of co-incubation revealed uncompromised natural cytotoxicity towards both AML cell lines in a dose-dependent fashion with no statistically significant differences detectable (**Figure 3C**). Therefore, the high cytotoxicity towards the HLA class $I^+$ Kasumi-1 cells highlighted that the remarkable cytotoxicity of the NK cells against AML blasts is not inhibited by the genetic modifications using either CRISPR/Cas9 technology or lentiviral overexpression of HLA molecules.

## Expression of sc-HLA-E Suppresses Proliferation of Allogeneic T Cells

In the next set of experiments, we wanted to explore whether the modifications of HLA class I surface expression also conferred escape of immune recognition by allogeneic T cells. An allogeneic T cell response can be initiated *via* two different pathways, either a direct recognition by binding of the TCR to the foreign HLA proteins themselves or indirectly by donor peptides presented on self-HLA molecules by antigen-presenting cells (85). Through both pathways, T cells become activated, exert effector functions and undergo clonogenic expansion.

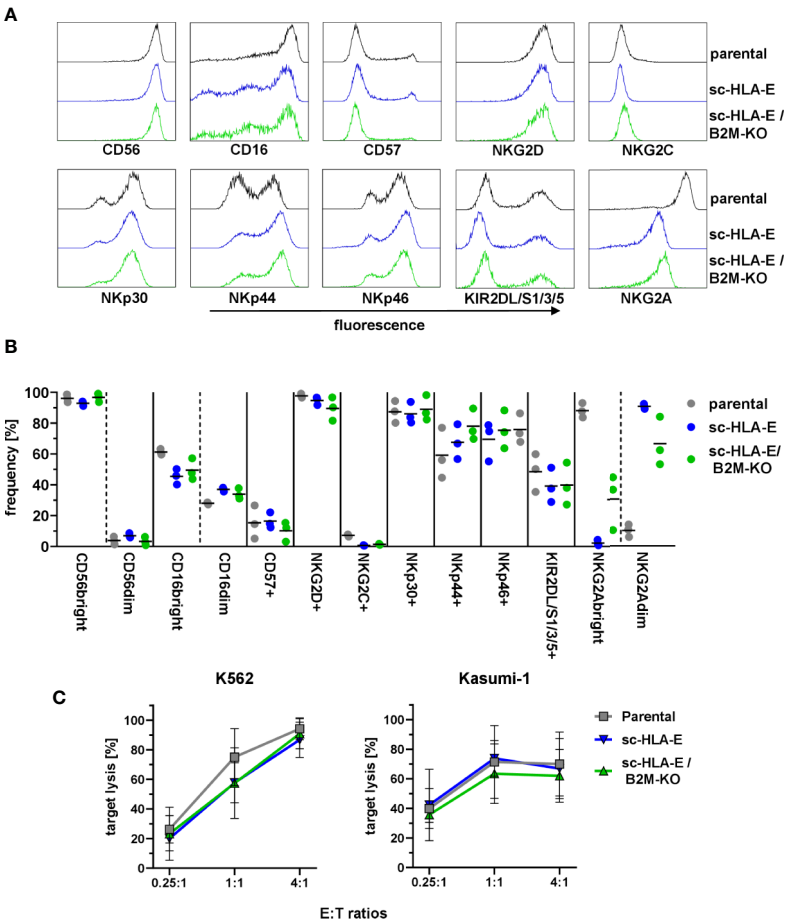


**FIGURE 3** | Functional and phenotypical analysis of the gene modified NK cells. **(A)** Representative flow cytometry plots of the phenotypical analysis of the NK cell cultures. **(B)** Quantification of NK cell frequencies for phenotypic markers in parental, sc-HLA-E and sc-HLA-E/B2M-KO NK cell cultures from left to right for each marker (all data points shown, n = 3). **(C)** Quantification of natural NK cell cytotoxicity by lysis of the AML cell lines K562 and Kasumi-1 after co-incubation for 6 h. Statistical analysis was performed by two-way ANOVA with Holm-Sidak testing for multiple comparisons. No statistically significant differences could be detected (mean ± SD; K562: n = 6 for parental and sc-HLA-E/B2M-KO and n = 5 for sc-HLA-E NK cells; Kasumi: n = 4 for parental and sc-HLA-E/B2M-KO and n = 3 for sc-HLA-E NK cells).

We therefore measured the expansion of allogeneic T cells from healthy unrelated donors as a surrogate for immune recognition in a mixed lymphocyte culture (MLC) with the modified NK cells in comparison to parental NK cells and a medium control. To this end, purified T cells were labeled with the dye CFSE and co-cultured with NK cells. After six days, the proliferation of the T cells was assessed by flow cytometry as the frequency of $CFSE^{dim}$ cells. The data in **Figure 4** demonstrated that the $CD4^+$ T cells did not proliferate in response to HLA mismatched NK cells irrespectable whether these cells overexpressed sc-HLA-E or not and also independent of the B2M status. In contrast, co-culture with parental unmodified NK cells activated alloreactive $CD8^+$ T cells and induced their proliferation, visible as the increased percentage of $CFSE^{dim}$ cells (**Figure 4A** second panel top row and **Figure 4B** first panel). Surprisingly, sc-HLA-E only NK cells also did not induce allogeneic $CD8^+$ T cell proliferation, despite intact HLA class I expression (**Figure 4A**, lower panel). Quantification showed that, while allogeneic responses vary greatly between the individual pairs in the mixed MLCs, a significant allogeneic stimulus was only generated by unmodified NK cells and only for $CD8^+$ T cells (**Figure 4B**).

## Only B2M-KO NK Cells Are Protected From Allogeneic $CD8^+$ T Cell Responses

As only unmodified allogeneic NK cells elicited a proliferative response in $CD8^+$ T cells, we hypothesized that the overexpression of sc-HLA-E can actively suppress T cell activation/proliferation and consequently cytotoxicity even after direct TCR-mediated recognition of the foreign HLA on the target NK cells. Therefore, in order to investigate whether mere overexpression of sc-HLA-E in NK cells is sufficient to protect them from alloreactive T cell cytotoxicty, we evaluated T cell degranulation and subsequent lysis of parental, sc-HLA-E or scHLA-E/B2M-KO NK cells by HLA-mismatched T cells. As only a fraction of T cells is capable of directly recognizing foreign HLA molecules for any given donor-recipient pair, expansion of the alloreactive T cells occured prior to

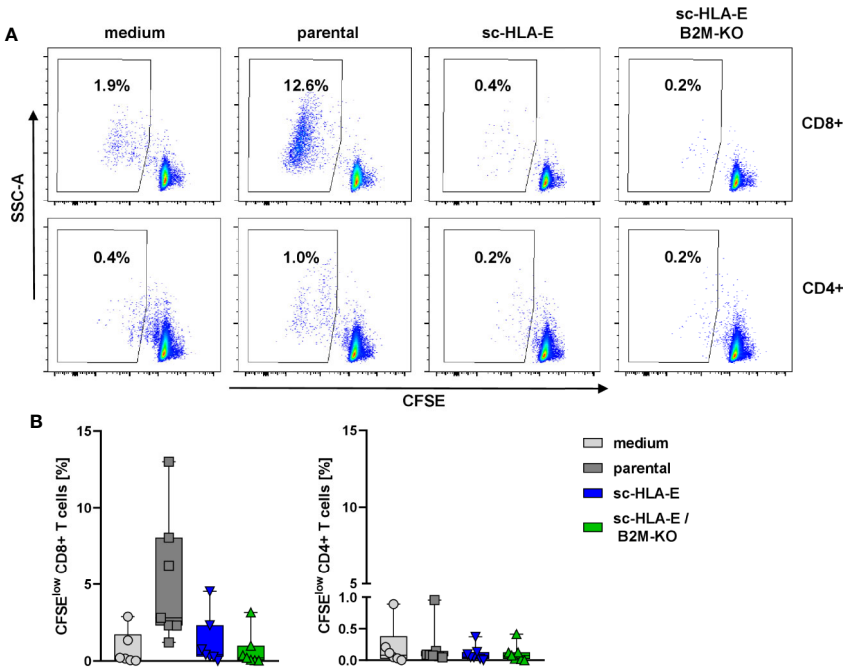


**FIGURE 4** | CFSE proliferation assay to measure the immune response of allogeneic T cells towards the modified NK cells. **(A)** Representative flow plots showing the frequency of $CFSE^{low}$ $CD8^+$ (upper panel) and $CD4^+$ (lower panel) T cells after 6 days of co-incubation with NK cells carrying the modifications depicted above the plots. **(B)** Quantification of activated $CFSE^{low}$ $CD8^+$ (left) and $CD4^+$ (right) T cells after co-incubation with the modified NK cells. Statistical analysis was performed using one-way ANOVA with Holm-Sidak testing for multiple comparisons. The higher frequency of $CFSE^{low}$ $CD8^+$ T cells was statistically significant compared to sc-HLA-E and sc-HLA-E/B2M-KO NK cells (Box plots including median, quartiles and all data points, n = 7).

the experiments by co-culture ("priming") with 30 Gy-irradiated PBMCs of the specific NK cell donor for 14 days. Subsequently, these T cells were co-cultured with NK cells and then analyzed for expression of CD137 or CD107a as markers for activation and degranulation, respectively. Autologous and also HLA-disparate "3rd party" NK cells served as important controls.

These co-culture experiments with primed T cells revealed a specific activation of $CD8^+$ but not $CD4^+$ T cells in presence of parental as well as sc-HLA-E-expressing NK cells for up to 48 h as measured by CD137 expression (**Figures 5A, B**). In contrast, sc-HLA-E/B2M-KO NK cells did not induce expression of CD137 in a significant fraction of $CD8^+$ T cells, similarly to co-cultures with autologous and also 3rd party NK cells, thus confirming the donor specificity of the assay. Analysis of degranulation by CD107a staining at 24 h of co-culture revealed a similar pattern (**Figure 5C**), with degranulation in the presence of parental and sc-HLA-E-expressing NK cells, while the CD107a levels of T cells challenged with sc-HLA-E/B2M-KO NK cells was comparable to those using autologous and 3rd party controls. Finally, the specific cytotoxicity towards the different genetically modified NK cells was assessed with purified populations at effector to target ratios of 4:1, 2:1 and 1:1 (calculated on $CD8^+$ T cells). After 20 h of co-culture with primed T cells (**Figure 5D**), between 10 and 40% of parental and sc-HLA-E expressing NK cells were killed in a dose-dependent manner. In contrast, sc-HLA-E/B2M-KO NK cells were not lysed at E:T ratios of 1:1 and 2:1, and even E:T ratios of 4:1 resulted in only <10% lysis.

## DISCUSSION

In this study, we have established a robust methodology to generate primary NK cells that are devoid of classical HLA class I molecule surface expression. Compared to their unmodified counterparts, the genome-edited NK cells escaped immune recognition by mismatched $CD8^+$ and $CD4^+$ T cells, thus making them suitable tools for "off-the-shelf" allogeneic immunotherapy. To achieve this, we first had to overcome the obstacle that NK cells are "hard-to-transduce" cells. This *relative* resistance of primary human NK cells to lentiviral and also alpha-retroviral vectors using VSV-G or RD114 pseudotypes was just recently documented again (86) and is simply due to the low expression levels of the cellular proteins that serve as surface receptors for entry of such pseudotyped vector particles (68). Based on the pioneering work of Els Verhoeyen and her colleagues establishing the envelope of the baboon endogenous virus (BaEV) as novel pseudotype for human primary cells (69), two recent studies demonstrated efficient NK cell transduction with the BaEVRless envelope using either CH296/retronectin-coated plates (87) or Vectofusin-1 as enhancers of viral uptake (68, 69, 88). However, as the BaEVRless envelope with the deletion of the

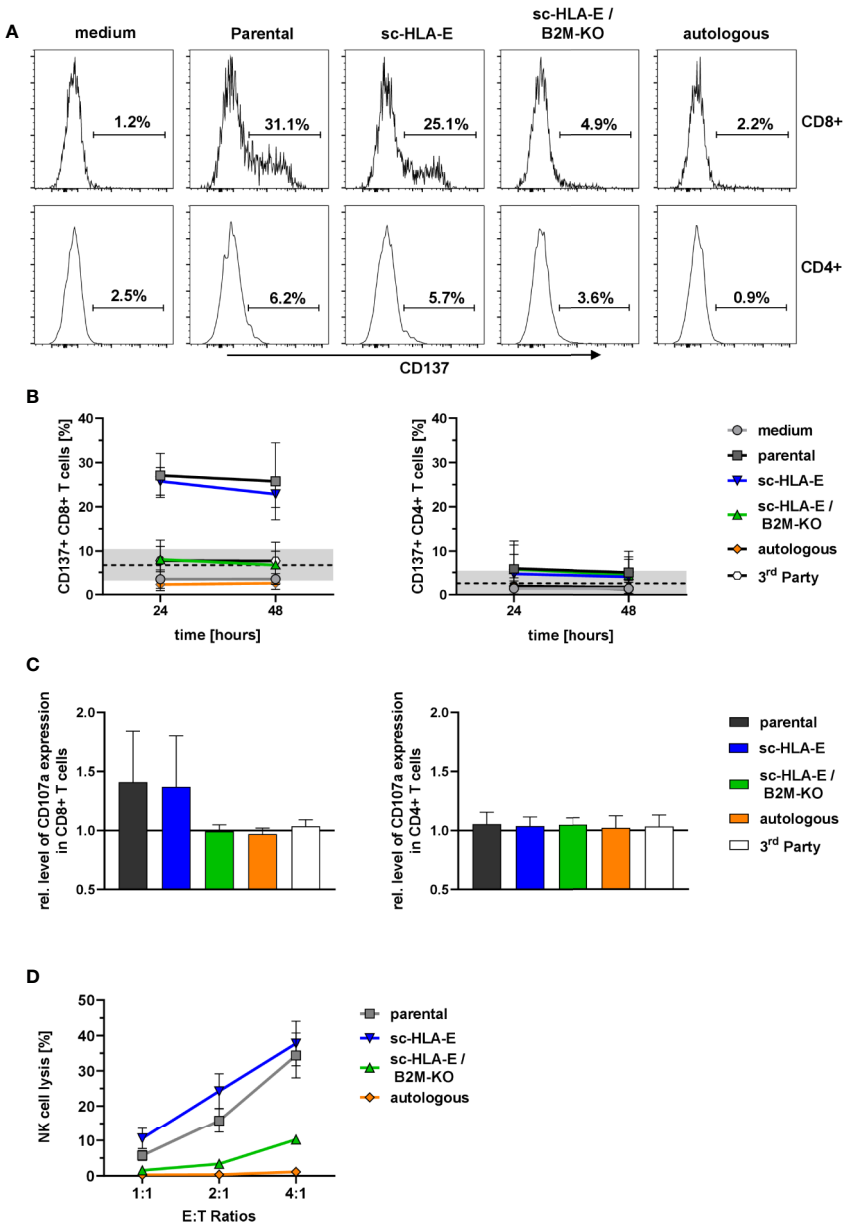


**FIGURE 5** | Activation and degranulation of primed T cells in presence of modified NK cells. **(A)** Representative flow plots showing the frequency of CD137$^+$ cells within CD8$^+$ (upper panel) and CD4$^+$ (lower panel) T cell subsets after 24 h of co-incubation with the different NK cell cultures depicted above the plots. **(B)** Quantification of CD137$^+$ frequencies among CD8$^+$ (left) and CD4$^+$ (right) T cells at two time points. Statistical analysis was performed by two-way ANOVA with matching by time points and Holm-Sidak testing. At both time points, the frequency of CD137$^+$ cells within the CD8$^+$ T cells was significantly lower when challenged with sc-HLA-E/B2M-KO NK compared to parental and sc-HLA-E NK cell containing cultures (mean ± SD; n = 7 for medium control, parental, sc-HLA-E, sc-HLA-E/B2M-KO NK cells, n = 4 for autologous and 3$^{rd}$ party controls; black line and grey box indicate the mean of the baseline measurements ± 95% confidence interval). **(C)** Levels of CD107a normalized to the medium controls in CD8$^+$ (left) and CD4$^+$ (right) T cells 24 h after co-incubation with NK cells. Statistical analysis was performed using Friedman test with Dunn's correction for multiple comparisons. Levels of CD107a were significantly lower for CD8$^+$ T cells incubated with sc-HLA-E/B2M-KO NK cells compared to T cells incubated with parental or sc-HLA-E NK cells (mean ± SD; n = 7 for medium control, parental, sc-HLA-E, sc-HLA-E/B2M-KO NK cells, n = 4 for autologous and 3$^{rd}$ party controls). **(D)** Lysis of NK cells by primed T cells at the effector targets ratios 4:1, 2:1 and 1:1 (calculated on CD8$^+$ T cells) after 20 h of co-incubation. Statistical analysis was performed by two-way ANOVA with Holm-Sidak testing for multiple comparisons. The reduced lysis of sc-HLA-E/B2M-KO NK cells was statistically significant at E:T ratio 4:1 compared to parental and sc-HLA-E NK cells and at ratio 1:1 compared to sc-HLA-E NK cells but not to parental NK cells, yet the p value almost met the criterion with p = 0.0516 (mean ± SD; n = 5).

R protein is highly fusogenic already in the packaging cells, we constructed another version of the BaEV envelope featuring a fusion of the surface and transmembrane regions with the cytoplasmic tail of the amphotropic endogenous murine retrovirus, as described (69). This pseudotype for lentiviral vectors enabled us to reproducibly and efficiently perform genome editing of primary NK cells.

In past clinical trials, mainly genetically non-manipulated allogeneic NK cells were used for immunotherapy of malignancies including AML, myeloma and solid tumors (17, 20, 22, 23, 89). The clinical response rates were highly variable, ranging from 26 to 50% and often with only transient improvements (17, 20, 22, 23). Remarkably, no GvHD was observed in these trials despite the various HLA mismatch constellations, except for one study with higher T cell contaminations (89). All NK cell trials had two things in common: (i) preconditioning therapy using fludarabine and cyclophosphamide to deplete recipient lymphocytes in order to avert immunological rejection, and (ii) subcutaneous injections of IL-2 to facilitate NK cell engraftment and maintenance. The study from Miller et al. (20) showed that only high-intensity conditioning using fludarabine and high-dose cyclophosphamide was able to facilitate engraftment of NK cells beyond day 5 post infusion, compared to regimens that administered only fludarabine or low-dose cyclophosphamide and prednisolone, arguing that rigorous lymphodepletion is indispensable for successful engraftment and post-injection expansion. Importantly, the lymphodepleting conditioning was accompanied by a rise of endogenous IL-15 levels which roughly correlated with NK cell *in vivo* expansion. In all trials with high-dose conditioning (17, 20, 22, 23), donor-derived NK cells were detected in the patients by PCR for up to 28 days post infusion. During these four weeks, a decline in numbers was usually evident between 8 and 17 days (17, 20, 22, 23), which coincided with the patients' with hematopoietic recovery and rise in endogenous T cell counts. Additionally, Shi et al. (23) reported that T cells from patients treated with NK cells showed reactivity towards donor-derived PBMNCs in an *in vitro* MLR. This finding is bolstered by the observation by Curti et al. (17) that a second infusion of NK cells is rejected even quicker than the first one: 5 days vs. 17 days, respectively. Taken together, these results strongly suggest that the mounting of an alloreactive immunological T cell memory response is a major contributing factor for the short-term NK cell persistence. Shi and colleagues even argued that the regular IL-2 injections might have facilitated the quick establishment of an allogeneic T cell response (23).

Thus, these clinical studies highlight the potential benefit of a knockout of HLA class I for allogeneic NK cell therapy to avoid donor-specific alloreactions of the patient's T cells and extend the persistence of the transfused NK cells. In addition, the evasion from a pool of alloreactive patient T cells, whose numbers would inevitably build up due to indirect allorecognition after infusion, should readily enable multiple infusions and even has the potential to make lymphotoxic conditioning obsolete.

We achieved the functional deficiency of HLA class I molecules by a lentiviral CRISPR/Cas9-mediated knockout of B2M. However, given that HLA class I expression protects against NK cell recognition, it is not surprising that B2M-KO NK cells did not persist in culture, but were lysed by their neighboring NK cells based on the "missing self" activation. The phenomenon of NK cells killing each other, called fratricide, has been observed before, yet in other contexts. In murine NK cells, for example, trogocytosis of NKG2D ligands from tumor cells can trigger fratricide, which has been proposed as a negative feedback loop to control NK cell activation (90). Patients with multiple myeloma, who were treated with the monoclonal antibody daratumumab targeting CD38, clinically benefitted from the antibody treatment. However, an unexpected side effect was the loss of CD38+ autologous NK cells in the peripheral blood and even in the bone marrow of the patients *via* an antibody-dependent cellular cytotoxicity (ADCC) (91, 92). In an experimental setting, this fratricide of autologous CD38+ NK cells was overcome by a CRISPR/Cas9-mediated knockout of CD38 in *in vitro* expanded NK cells (91), thus providing a potential therapeutic strategy to enhance the efficacy of the antibody infusions further.

To avoid fratricide, we co-expressed a sc-HLA-E molecule as described by Gornalusse and colleagues (58) as an efficient approach to protect HLA-deficient PSC-derived cells from NK cell lysis. Despite the necessity to introduce two genetic modifications in the NK cells, the knockout of B2M and the overexpression of sc-HLA-E, we noted only a minor reduction in the expansion kinetics/characteristics of our NK cell cultures, when we transduced the cells simultaneously with the mixture of both concentrated supernatants. It seems likely that this reduction can be attributed to the higher vector doses used to achieve efficient transduction and editing frequencies. For clinical purposes however, the NK cells will be expanded for at least 21 days in a closed system such as the Prodigy (74, 75), thus sufficient opportunities for sequential genetic manipulations can be established in an optimal cell expansion protocol. Additionally, there is no need to purify the edited cells, as they would simply persist due to their immune evasive properties, thus facilitating a simple manufacturing process. To further validate the fratricide hypothesis and exclude that the loss of B2M directly led to NK cell death, we performed the fratricide assays using NK cells in which the *KLRC1* gene, coding for the inhibitory receptor NKG2A that recognizes sc-HLA-E, had been knocked out by genome editing. In these experiments, NKG2A-deficient NK cells eliminated the B2M-KO cells, regardless of whether sc-HLA-E was expressed or not.

The phenotypical and functional analyses revealed robust concordances between the parental and the genetically modified NK cells. While the killing of established target cells for NK cells such as K562 was comparable, the only notable difference between the parental NK cells and those expressing sc-HLA-E, regardless of the B2M-KO, was the lower expression level of NKG2A. One obvious explanation of the diminished NKG2A surface expression here is that the overexpressed sc-HLA-E already binds to NGK2A within the cells, thus leading to retention of the complex. This idea is clearly reminiscent of the approach developed by Kamiya and colleagues, in which they engineered NKG2A$^{dim/-}$ NK cells for immunotherapy by cytoplasmically targeting NKG2A with a scFv fused to an

endoplasmatic reticulum (ER) retention peptide, thereby retaining NKG2A in the ER (93).

Curiously, sc-HLA-E-expressing NK cells with intact HLA class I expression did not evoke allogeneic T cell proliferation, while the sc-HLA-E positive NK cells were still efficiently lysed when the same T cells were pre-activated in an MLR with irradiated feeder cells for 14 days. One obvious explanation is that the frequency of alloreactive T cells against a specific HLA type is relatively low and that these few T cells upon activation upregulate NKG2A. The newly expressed NKG2A is rapidly engaged by a sc-HLA-E molecule on neighboring NK cells, thus hampering the activation and the proliferation of the alloreactive T cells. Although this situation can readily occur in an *in vitro* setting in which the activated T cell is surrounded by sc-HLA-E expressing NK cells, *in vivo* the likelihood of such interactions is very low and one can expect numerous events of indirect immune recognition that will inevitably generate a large pool of alloreactive T cells capable of eliminating all HLA divergent cells.

In summary, we think that the universal "off-the-shelf" effector cell product for adoptive cellular therapies should be B2M-deficient NK cells overexpressing sc-HLA-E. These cells will be completely invisible for allogeneic T cell responses and will be protected from NKG2A+ recipient NK cells. Whether these modifications are sufficient for such modified NK cells to evade recognition and destruction of the patient's immune system needs to be explored in clinical trials. Nevertheless, our modifications appear to be highly valuable to enhance efficacy of CAR-modified NK cells. Indeed, a recently published seminal NK cell study for CD19-positive lymphoid tumors by Liu and colleagues used a single dose of partly matched (mostly 4/6 with regard to A, B and DRβ1) allogeneic NK cells that had been transduced with a retroviral vector encoding three different transgenes: a CD19 CAR, soluble IL-15 and the iCASP9 suicide gene (19). In eleven treated patients, neither GvHD nor a cytokine release syndrome occurred. Thus, the suicide gene was never employed (19). Independent of the cell doses infused, eight patients (75%) had a clear immune response against the CD19+ malignant cells, which was complete and lasting in seven out of the eight patients. Remarkably, the additionally expressed IL-15 appeared to promote the long-term expansion of the donor NK cells *in vivo* for up to 12 months (19). Although cellular alloreactions by the recipients' T cell systems subsequent to the infusions were not tested and probably strongly influenced by the lymphodepleting conditioning in these heavily pretreated patients, it cannot be ruled out that the high degree of HLA matching, the expression of IL-15 and the variety of additional treatments and substances that the patients received after the NK cell infusions all played major roles.

Our study adds the knockout of B2M in combination with sc-HLA-E expression as another building block to the development of "off-the-shelf" cellular NK cell therapies to enable manufacturing of safer and more efficient cell products to benefit a larger group of patients.

## DATA AVAILABILITY STATEMENT

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

## AUTHOR CONTRIBUTIONS

KH and SH conceived the experiments. KH and CW performed experiments and analyzed data. KH, MU, HH, PH and SH wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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# Allogeneic CAR T Cells: An Alternative to Overcome Challenges of CAR T Cell Therapy in Glioblastoma

*Darel Martínez Bedoya*[1,2,3], *Valérie Dutoit*[1,2,3] *and Denis Migliorini*[1,2,3,4]*

[1] Center for Translational Research in Onco-Hematology, University of Geneva, Geneva, Switzerland, [2] Swiss Cancer Center Léman, Lausanne, Switzerland, [3] Brain Tumor and Immune Cell Engineering Group, Faculty of Medicine, University of Geneva, Geneva, Switzerland, [4] Department of Oncology, Geneva University Hospitals (HUG), Geneva, Switzerland

Chimeric antigen receptor (CAR) T cell therapy has emerged as one of the major breakthroughs in cancer immunotherapy in the last decade. Outstanding results in hematological malignancies and encouraging pre-clinical anti-tumor activity against a wide range of solid tumors have made CAR T cells one of the most promising fields for cancer therapies. CAR T cell therapy is currently being investigated in solid tumors including glioblastoma (GBM), a tumor for which survival has only modestly improved over the past decades. CAR T cells targeting EGFRvIII, Her2, or IL-13Rα2 have been tested in GBM, but the first clinical trials have shown modest results, potentially due to GBM heterogeneity and to the presence of an immunosuppressive microenvironment. Until now, the use of autologous T cells to manufacture CAR products has been the norm, but this approach has several disadvantages regarding production time, cost, manufacturing delay and dependence on functional fitness of patient T cells, often reduced by the disease or previous therapies. Universal "off-the-shelf," or allogeneic, CAR T cells is an alternative that can potentially overcome these issues, and allow for multiple modifications and CAR combinations to target multiple tumor antigens and avoid tumor escape. Advances in genome editing tools, especially *via* CRISPR/Cas9, might allow overcoming the two main limitations of allogeneic CAR T cells product, i.e., graft-vs.-host disease and host allorejection. Here, we will discuss how allogeneic CAR T cells could allow for multivalent approaches and alteration of the tumor microenvironment, potentially allowing the development of next generation therapies for the treatment of patients with GBM.

**Keywords: CAR T cells, glioblastoma, allogeneic, graft-vs.-host disease, allorejection, tumor microenvironment**

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*Lucas Coelho Marlière Arruda,*
*Karolinska Institutet (KI), Sweden*

***Correspondence:**
*Denis Migliorini*
*Denis.Migliorini@unige.ch*

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## INTRODUCTION

Chimeric antigen receptors (CAR) are synthetic receptors comprising an extracellular domain, most frequently derived from an antibody single-chain variable fragment (scFv), and an intracellular signaling and costimulatory domain derived from T cells. Genetic insertion of CARs, most frequently into the T cell genome but also in other immune cells, allows redirecting them to a desired antigen (1). Anti-CD19 CAR T cells, mainly generated from autologous peripheral

blood lymphocytes, have shown remarkable clinical responses in patients with B cell-derived hematologic malignancies (2). Clinical trials using anti-CD19 CAR T cells led to a paradigm change in cancer therapy, based on their unprecedented response rates in adult patients with recurrent/refractory diffuse large B cell lymphoma (DLBCL) or pediatric refractory B cell acute lymphoblastic leukemia (B-ALL) (3–6). Two CAR T cell products specific for the B-cell marker CD19, Kymriah (Novartis) and Yescarta (Kite Pharma), became the first therapeutic products registered by the FDA comprising a genetic engineering element for the treatment of B-ALL and DLBCL (7, 8).

However, occurrence of severe side effects is associated with the use of CAR T cell therapy. The best-characterized toxicity associated with CAR T cells is cytokine-release syndrome (CRS). It consists of a systemic inflammatory response derived from immune cell activation, with common symptoms being the presence of hypotension, capillary leak, high fever and multiorgan failure (9, 10). CRS is produced by a supra-physiological activation of CAR T cells that leads to exacerbated secretion of pro-inflammatory cytokines, including IFN-γ, TNF-α, IL-6, and IL-2, and chemokines such as MCP1, allowing the recruitment and activation of other immune and non-immune cells (11). Other toxicities associated with CAR T cell treatment are the immune effector cell–associated neurotoxicity syndrome (ICANS), hematological toxicity due to the lymphodepleting chemotherapy, increased risk of infection due to lymphodepletion or B cell aplasia (following anti-CD19 CAR T cell administration) and macrophage activation syndrome (12). To avoid CRS and other toxicities, CAR T cells with adaptive expression systems have been developed: (i) passive control using mRNA-encoded CARs, allowing for transient CAR expression, (ii) inducible control using inducible suicide systems (13), and (iii) autonomous control *via* logic-gated CAR T cells (14).

Up to now, most of published clinical trials testing CAR T cells have used autologous T cells, i.e., cells derived from the patient for whom the product is being made. However, therapies based on autologous T cells are endowed with limitations, mainly related to the fact that the product has to be generated from each patient's cells, in a time-consuming and costly process, and with the risk of manufacturing failure (15). Indeed, delay in treatment availability can be particularly problematic in patients with highly proliferative diseases (16). An additional hurdle lies in the quantity and quality of the starting autologous T cells as patients usually receive lymphodepleting chemotherapy and/or radiotherapy (17). In addition, heterogeneity of tumor antigen expression and immune evasion mechanisms developed by tumor cells require using CAR T cell products able to target multiple antigen specificities (18). As mentioned, the amount of functional autologous T cells available in heavily pre-treated patients are often limited. In contrast, using T cells obtained from healthy donors (allogeneic T cells), provides high amounts of fully functional cells and allows to generate multiple "off-the-shelf" CAR T cell products (15, 16).

Despite many desirable traits, allogeneic CAR T cells also come with challenges. Indeed, allogeneic T cells might cause severe graft-vs.-host disease (GVHD) and the host immune system might in turn induce allorejection, which will impede anti-tumor activity. There are different ways to avoid GVHD when designing allogeneic CAR T cells, the most widely used strategy being the generation of TCR-deficient T cells using genome editing tools such as Zinc finger nucleases (ZFN) (19–21), transcription activator-like effector nucleases (TALEN) (22–24) and CRISPR/Cas9 (25–27). Strategies to reduce allorejection are being evaluated as well, testing repeated rounds of administration (28), using chemotherapy-resistant CAR T cells allowing for prolonged or deeper lymphopenia (22, 29) or genetically eliminating key molecules governing CAR T cell immunogenicity. For the latter, an attractive method uses gene editing of MHC class I molecules by disrupting the β2-microglobulin locus (30). Creating an allogeneic T cell bank is an alternative as well and this has been used mainly with virus-specific and non-modified T cells (31–33), but also with anti-CD123 retrovirally transduced CAR for the treatment of acute myeloid leukemia (34).

Glioblastoma (GBM) is the most frequent and aggressive type of primary malignant tumors originating in the central nervous system (CNS) (35). Incidence increases with age, but GBM also occurs in younger patients, with a different genetic profile (36). Despite aggressive therapies including surgery followed by concomitant chemo-radiotherapy with temozolomide (TMZ) and adjuvant TMZ (37), survival of GBM patients has only discreetly improved over the past decades. A recent systematic review showed a median overall survival of 20.7 months in clinical trials using tumor-treating fields and a 5-year survival only reaching 5.8% (38). During the last 20 years, identification of tumor-associated and tumor-specific GBM antigens led to the implementation of immunotherapy for GBM patients (39, 40). Outcome of clinical studies in primary and recurrent GBM using vaccines have largely been disappointing, and early clinical trials with immune checkpoint inhibitors didn't show positive results (41, 42). However, some promising results were recently obtained in phase I/II studies using multipeptide vaccines (43–46) or neoadjuvant immune checkpoint blockers (47–49). Given their success in other tumor indications, CAR T cells have been considered promising for GBM (2). Early phase clinical studies using CAR T cells to treat GBM patients showed that these were safe, but did not generate sufficient anti-tumor activity (50, 51). However, some monovalent CAR T cells showed tumor control (52–54) and a complete response was even reported (52). In this review, we will discuss the potential of using allogeneic CAR T cells for the treatment of patients with GBM.

## ALLOGENEIC CAR T CELLS

As mentioned before, allogeneic T cells present many advantages over autologous T cells. However, they also come with specific challenges that need to be overcome to reach clinical success. These include (i) an appropriate selection of the T-cell source, (ii) avoiding GVHD and (iii) abrogating host immune rejection to obtain robust *in vivo* activation and expansion (16).

## Sources of T Cells

Patient-derived non-mobilized peripheral blood leukapheresis collection is the primary and most frequently used starting material for autologous CAR T cell manufacturing. In contrast, apheresis is performed from healthy adult volunteers in the allogeneic setting (55) (**Figure 1**). Using healthy donors provides high numbers of cells from a single volunteer, and peripheral blood mononuclear cells are fit, as donors, in contrast to cancer patients, do not receive chemo- or radiotherapy (16). Other cell sources can be considered for allogeneic CAR T cell development, such as umbilical cord blood (UCB)-derived T cells. GVHD frequency and intensity can be decreased when using T cells obtained from UCB, as these have reduced reactivity due to lower activation of the NF-κB pathway, resulting in decreased production of several pro-inflammatory cytokines (56, 57). In the context of hematopoietic stem cell transplantation (SCT) for treatment of hematological malignancies, UCB transplantation has indeed shown better results than matched unrelated donors and similar results as compared to matched related donor transplantation with regard to GVHD incidence, late complications and overall survival (58–60). CAR T cells derived from UCB have already been used, showing the feasibility of the approach, as well as efficacy, as UCB-derived CAR T cells were able to recognize and kill target cells (61). Another promising option is induced pluripotent stem cells (iPSCs). This allows generating pluripotent stem cells starting from adult somatic cell by introduction of specific transcription factors (62). iPSC-derived T cells have longer telomeres than mature T cells and show higher proliferation capacity. Until now, one study showed that anti-CD19 CARs can be obtained from iPSC-derived T cells, these CAR T cells being able to specifically eliminate target cells (63). However, not much progress has been reported with the use of iPSC for CAR T cell generation lately.

## Avoiding GVHD

Avoiding GVHD concentrates most efforts in allogeneic CAR T cell development, considering that GVHD is one of the main causes of death after allogeneic SCT (64). In lasts years, many groups have been working at refining the diagnostics and classification of GVHD. The current consensus defines two main categories of GVHD, acute and chronic, each divided in 2 subcategories (65). However, the literature related to CAR T cells, and especially allogeneic CAR T cells, does not address the differential impact of these therapies for each GVHD categories, particularly in chronic GVHD. As allogeneic CAR T cells advance in clinical settings, more research will be needed to understand their impact in both categories of GVHD.

Many groups consider that the principal responsible of GVHD are αβ T cells, the T cell type mostly used to generate CAR T cells (66). Two main strategies designed to reduce the risk of GVHD have been proposed, based on either selection of virus-specific T cells or genetic ablation of the TCR locus. As the risk of alloreactivity increases with donor TCR repertoire diversity and amount of T cells transferred (16), there is a rationale to use purified T cells with a low-diversity TCR repertoire. Indeed, the use of virus-specific memory T cells during hematopoietic SCT was able to control viral infections without occurrence of GVHD (67–69). However, even if repeated stimulations of donor T cells can increase virus-specific memory cells frequency, and in turn reduce the risk of GVHD, it is still not trivial to predict *a priori* the degree of alloreactivity of these cells (70). A small clinical trial using allogeneic virus-specific T cells expressing the anti-CD19 CAR construct demonstrated that these were safe and capable of anti-tumor activity without clinical manifestation of GVHD (71). New clinical trials are ongoing using anti-CD19 and anti-CD30 CAR T cells engineered with Epstein–Barr Virus-specific allogeneic T cells (72). The use of virus-specific T cells as a source of allogeneic CAR T cells remain an interesting option that needs to be fully validated for the next generation of clinical trials.

In recent years, rapid development of gene editing technologies has provided the necessary tools to abrogate expression of endogenous TCRs in order to minimize the risk of GVHD (**Figure 2**). Different groups are eliminating the expression of αβ TCRs on the T cell surface through genetic knockout of exons of the TCRα constant (TRAC) and/or TCRβ constant 1 (TRBC1) or 2 (TRBC2) loci, using small interfering RNA (73), ZFN (19, 20), TALEN (24, 74), megaTAL nucleases (75), engineered homing endonucleases (76), or CRISPR/Cas9 (26, 27). In a direct comparison between TALENs, CRISPR/Cas9 and megaTAL nucleases, the latter 2 were best at TCR disruption (75). Since there is only one gene for the α-chain constant region, this seems to be the most direct and efficient approach to disrupt the αβ TCR, and is consequently the most frequently used (75, 77). Additionally, multiplex editing is possible to further modify CAR T cells. Indeed, CRISPR/Cas9 has been used to generate TCR and MHC class I deficient allogeneic CAR T cells with additional PD1 (25), Fas or PD1/CTLA4 (78) knockout. The edition of multiple genes can contribute to reduction of CAR T cell alloreactivity while improving resistance to apoptosis and immunosuppression. However, it also increases the risk of off-target cleavage that could potentially lead to an excessive proliferation of CAR T cells due to disruption of tumor suppressor genes (79, 80). One of the most interesting alternatives to gain functional advantages and avoid GVHD in a more controlled way is to introduce the CAR transgene directly into the TRAC locus. Indeed, in addition to reducing GVHD, this manipulation allows for an homogenous and regulated expression of the CAR under the control of the TCR promoter, a feature which was shown to lead to decreased CAR T cell differentiation and exhaustion (26, 76, 77, 81). This variant is also explored in field of TCR-engineered T cells with similar benefits (82, 83).

Other strategies that have been considered to avoid GVHD are the use of non-αβ T cells (84, 85) or T cells derived from a hematopoietic SCT donor. The first include a population of innate-like lymphocytes such as NK (86, 87), invariant NKT (iNKT) (88, 89), or γδ T (90, 91) cells. In the case of γδ T cells, these rare cells (5% of T lymphocytes), are able to expand *ex vivo*, show strong cytotoxic anti-tumor activity and recognize their targets independently of MHC restriction, and there are unlikely to trigger GVHD (92). Some encouraging results have been showed in pre-clinical experiments with CAR γδ T cells, including targets associated with gliomas as the disialoganglioside GD2 (90). We will discuss more on NK and

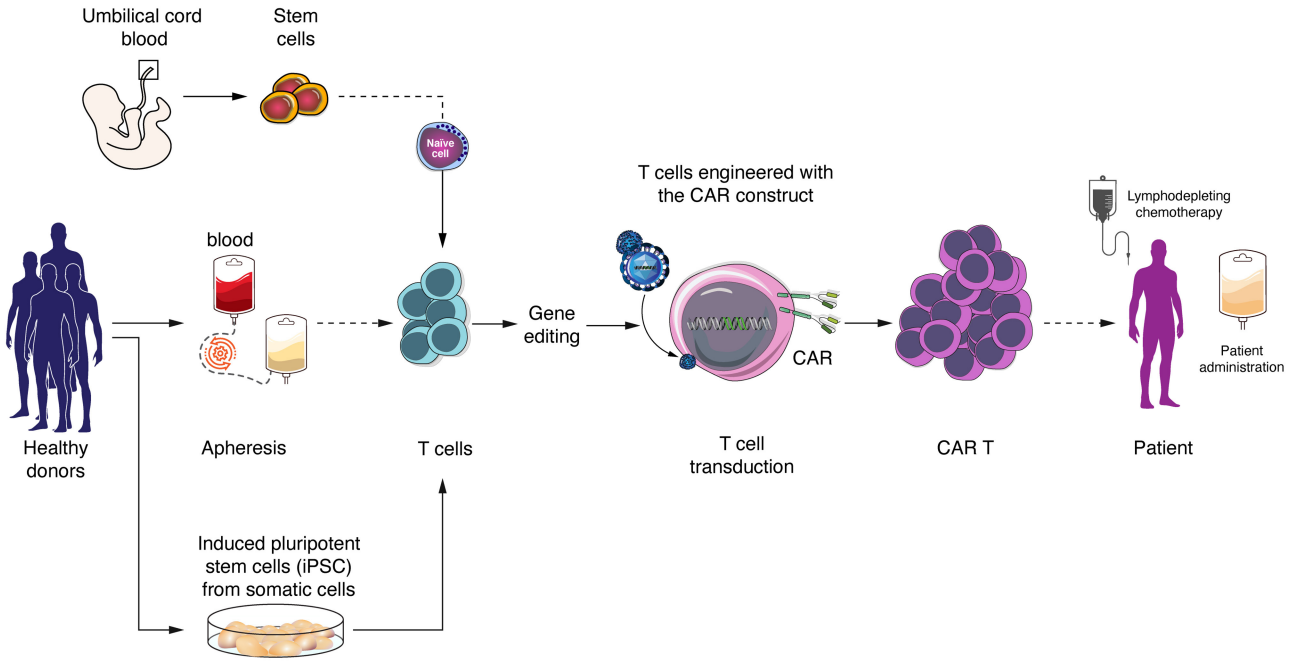


**FIGURE 1 |** Allogeneic ("off-the-shelf") CAR T cells generation and sources of T cells. Allogeneic T cells can be obtained from peripheral blood mononuclear cells from healthy donors, umbilical cord blood or derived from induced pluripotent stem cells (iPSCs). CAR T cells are generated by virus transduction and *in vitro* expansion before patient administration.

iNKT cells later. Using T cells derived from an SCT donor is limited to patients who have relapsed after an allogeneic hematopoietic SCT. Here, it is possible to use the same donor-derived CAR T cells at relapse, a procedure that showed GVHD only in 6.9% of patients from a meta-analysis of seven studies (93).

## Limiting Allorejection

A second major challenge of allogeneic CAR T cell therapy is that allogeneic CAR T cells have to persist and expand *in vivo*, a feature that has been associated with response to treatment in autologous CAR T cell trials in hematologic malignancies (94, 95) and neuroblastoma (96). As commented above, allogeneic CAR T cells do not share the limitation of autologous products in T cell functionality, and the main concern to increase *in vivo* persistence is thus to reduce their immunogenicity.

The fact that allogeneic CAR T cells can be produced in greater numbers as compared to autologous CAR T cells allows for repeated administrations. Some early results using this approach in an attempt to circumvent *in vivo* rejection showed that it was feasible (97). However, repeated administration requires repeated patient immunosuppression, and repeated encounters with host immune cells increase the risk of alloreaction, at least by the antibodies produced upon previous transfusions. Aiming at a more prolonged lymphopenia is an alternative, but will require generating CAR T cells that can resist lymphodepleting agents. To do this, αβ TCR-deficient CAR T cells were made resistant to multiple purine nucleotide analogs *via* deletion of the deoxycytidine kinase gene and were shown to be capable of efficient tumor cell killing in the presence of lymphodepleting agents (29). Alternatively, CAR T cells were made resistant to depletion *via* the anti-CD52 monoclonal antibody (alemtuzumab) used as pre-conditioning regimen by knocking out CD52 (22).

Independently of the number of doses infused and of the intensity of lymphodepletion, reducing the immunogenicity of allogeneic CAR T cells is always desired, and one direct approach is the genetic abrogation of MHC class I molecules (**Figure 2**). Despite being highly polymorphic molecules, all share the β2-microglobulin protein, and disrupting this subunit allows the elimination of all MHC class I molecules at the T cell surface (98, 99). A second level of allorejection could be mediated *via* the presence of HLA class II molecules on CAR T cells. Indeed, activated human T cells express the MHC class II molecules DR, DQ, and DP at the cell surface, which is regulated by the class II MHC transactivator (CIITA). The function of MHC class II molecules on T cells remains controversial (100), but it is conceivable that it can induce allorejection *via* $CD4^+$ T cell recognition. This issue probably could be avoided by genetic editing of the transcription factors regulatory factor X and CIITA (101, 102). Allogeneic anti-CD19 CAR T cells triple-knockout for HLA class I (β2-microglobulin KO), class II (CIITA KO) and TCR (α-chain KO) showed better persistence than double-knockout (β2-microglobulin and TCR) cells in a mouse tumor model, with anti-tumor activity, but without GVHD (103). Other cells potentially mediating an allogeneic response are NK cells (104), even if NK were shown to be functionally impaired in some tumors, particularly from

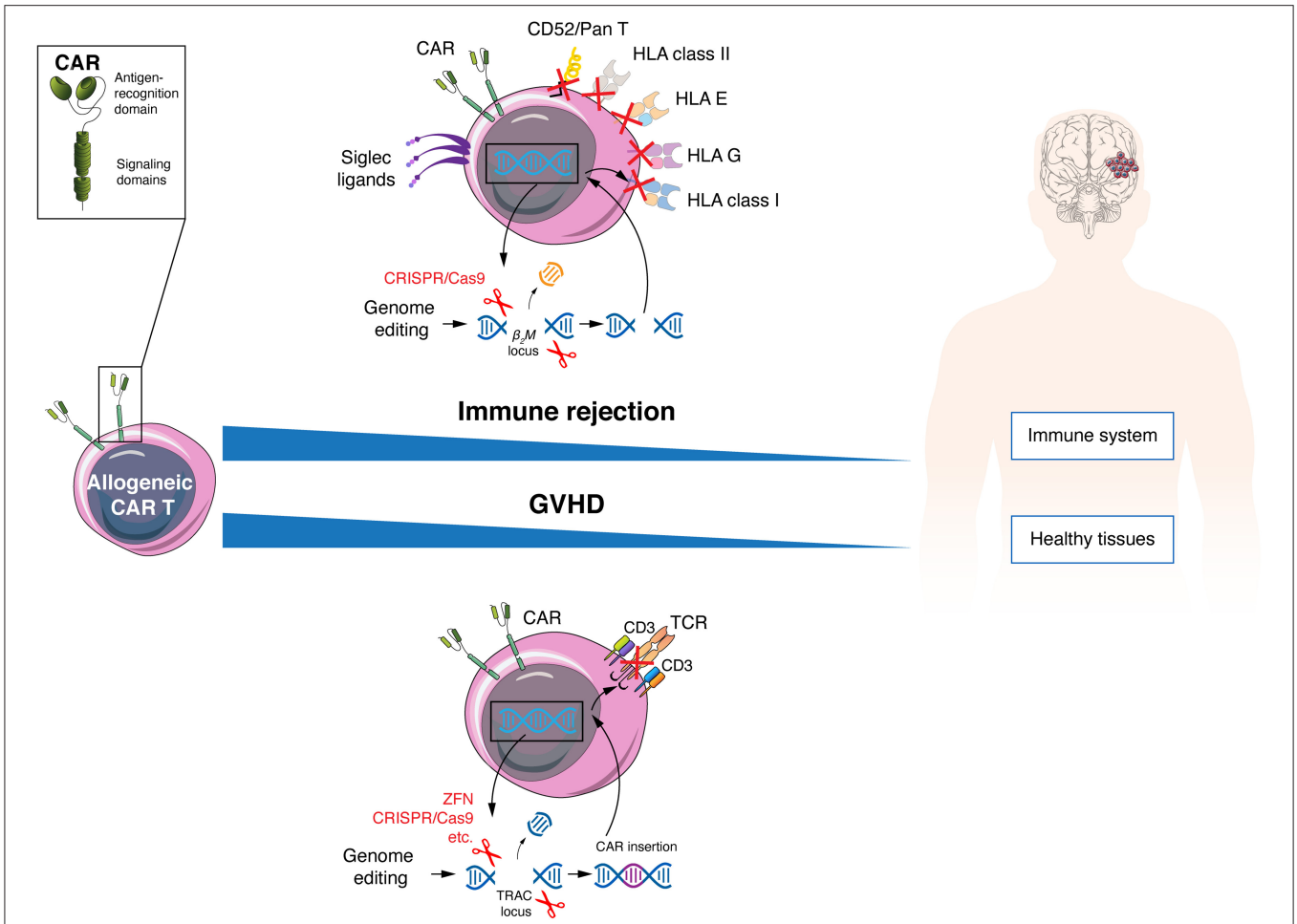


**FIGURE 2 |** Allogeneic CAR T cells must avoid host immune rejection and GVHD. Allogeneic CAR T cells can evade the patient immune response by genetic disruption of HLA class I and II molecules, resist lymphodepleting regimens using anti-CD52 antibodies by elimination of the CD52 molecule and inhibit NK elimination by increasing expression of Siglec ligands of HLA-E and G variants. To protect patients from GVHD, allogeneic CAR T cells can be engineered to lose TCR expression.

hematological origin (105, 106). Expression or overexpression of inhibitory ligands could be a possible solution to prevent NK cell-mediated allorejection, with HLA-E or G (107–109) or Siglec 7/9 ligands (110, 111) being among most promising options. Finally, new alternatives are being developed to avoid CAR T cell rejection, one promising strategy being the recent generation of a CAR that mediates deletion of activated host T and NK cells through expression of an extracellular 4-1BB ligand combined with the intracellular CD3ζ signaling molecule (112).

## Addressing Tumor Heterogeneity With Modular CAR T Cells

Antigen loss is a common tumor resistance mechanism to CAR T cell therapy (113) and has been reported as one of main causes of relapse in hematological malignancies (114) and GBM (52) as well as in pre-clinical models of solid cancers (115). An interesting approach to overcome antigen escape is the use of "universal" modular CAR designs. In these, the scFv targeting the antigen of interest is fused to an intermediate soluble molecule (or adaptor) which can be bound by the construct containing the activation signals expressed by the T cell (**Figure 3**). These CARs are based on antibody Fc receptors, streptavidin-biotin interaction, scFvs directed against a specific tag or other combinations (116, 117). Two of the most famous universal modular CARs are split, universal, and programmable (SUPRA) CARs and universal CARs (UniCAR). SUPRA CARs consist of a receptor with a leucine zipper on T cells and a separate scFv with a leucine zipper adaptor molecule targeting specific antigens (118). The design of SUPRA CARs confers some advantages, potentially significant in a clinical setting, over a classical CAR design, such as ability to change targets at will, adjusted control of activity and toxicity, and flexibility to change and combine signaling domains and immune cell types (15). The UniCAR system consists of two components as well, one being the CAR T cell that expresses a CAR directed to the nuclear antigen La-SS/B-derived peptide E5B9 and the second, termed target module, consisting of the E5B9 peptide fused to a tumor-specific antigen binding domain, typically an scFv (119). The UniCAR system can also target more than one

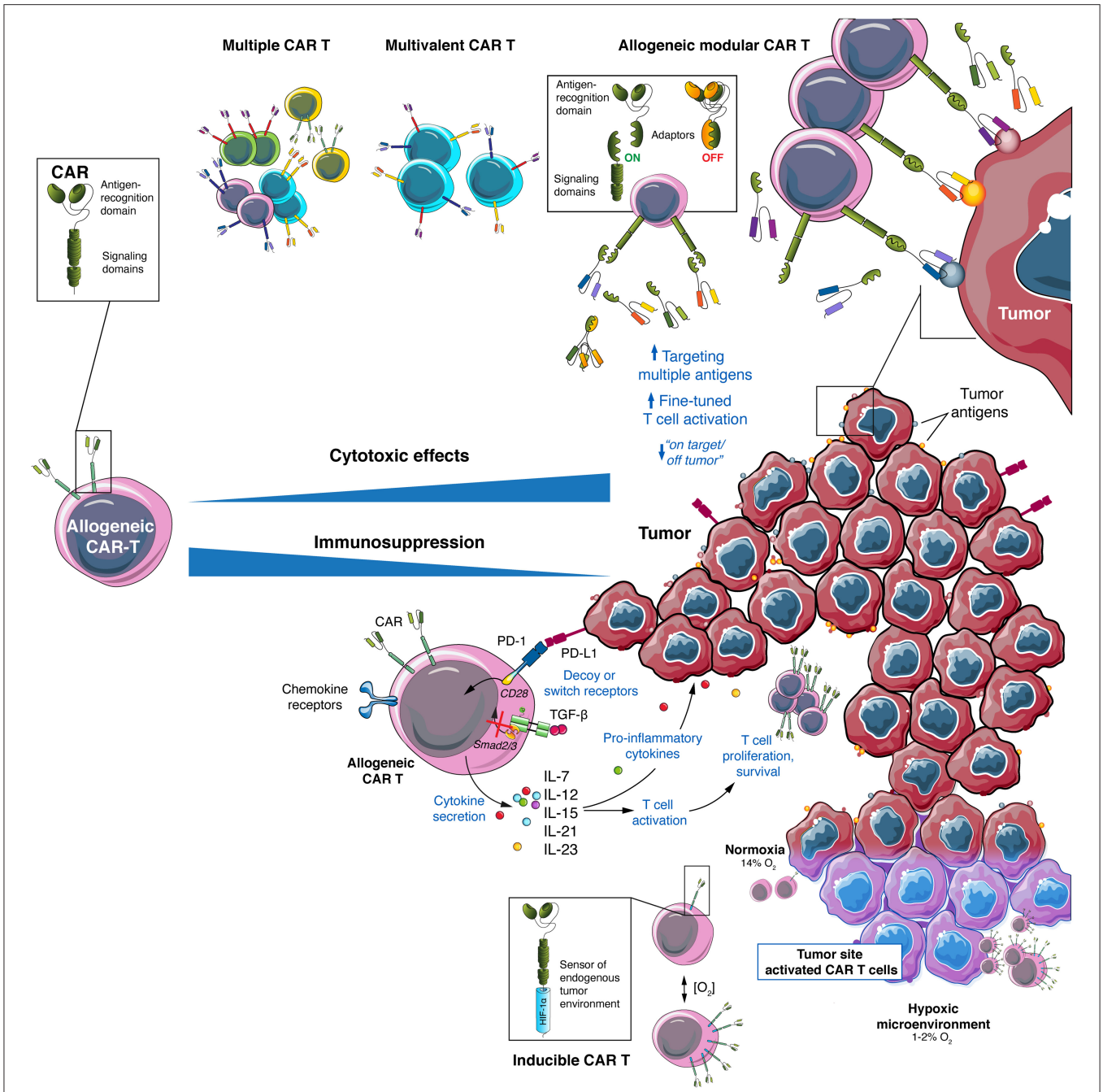


**FIGURE 3 |** Allogeneic CAR T cells provide a versatile platform to attack GBM and its environment. GBM heterogeneity require a multitarget approach that can be achieved using allogeneic CAR T cells using multiple CAR T cell mixes, multivalent CAR T cells or by modular CAR T combined with several adaptors. To overcome the immunosuppressive TME of GBM several strategies to engineer allogeneic CAR T cells can be used: secretion of pro-inflammatory cytokines (such as IL-7, IL-12, IL-15, IL-21, or IL-23), expression of decoy or switch receptors (to change immunosuppressive signals into activating ones), expression of chemokine receptors (to direct CAR T cells to the tumor site) and generation of locally activated CAR T cells (such as hypoxia-inducible CAR T cells).

antigen, combine different signaling domains and provide an on/off switch system that allows for a better control of CAR T cells activation (119–121).

In general, using adaptor molecules allows regulating CAR T cell activity through target selection, one or more of these targets being tackled simultaneously or sequentially. In addition, the effector activity can be turned on or off against each target separately by adding or removing the soluble adaptor, without the need to deplete CAR T cells. Thus, universal modular CAR T cells offer the opportunity to target multiple tumor

antigens, with reduced toxicity. In addition to these desirable features, potential side effects of modular CAR T cells could be mitigated by a customization of the adaptor dose (122, 123). However, modular CAR T cells still bear some disadvantages related to the exogenous nature of the adaptor molecules that can generate neutralizing antibodies in the host. Additionally, each new adaptor may require its own manufacturing development, clinical validation and regulatory approval related to safety and effectiveness (124). Universal off-the-shelf and universal modular CAR could be conjugated to obtain a "fully universal" CAR that would be readily available to switch target specificity and allow fine-tuned control at the same time. This approach could be particularly suited for GBM, a highly heterogeneous solid tumor.

### Alternative Sources of Allogeneic CAR Cells: NK and NKT Cells

Besides T cells, other cells can be used to generate CARs. NK cells are the most explored alternative to T cells, due to their potent cytotoxic anti-tumor activity combined with a favorable safety profile. A smaller risk of inducing GVHD has been historically associated with adoptive transfer of allogeneic NK cells because, as opposed to T cells, NK cells kill independently of MHC expression (125). In addition, allogeneic CAR NK cells do not require genetic modifications such as TCR deletion, which makes it easier to obtain cellular product devoid of any risk of GVHD (126). Even more interesting is the differences in the cytokine profile of NK cells compared to T cells, as NK cells do not produce IL-1 and IL-6, the main cytokines involved in CRS (127). Despite the fact that macrophages are usually the main source of cytokines such as IL-6, there is a debate about the role of activated T cells in IL-6 secretion during CAR T cell therapy, some studies pointing to T cells as the main source of IL-6 (128, 129), while others showing no IL-6 production by CAR T cells (130). However, generating CAR NK cells faces several practical issues, from the lower amount of these cells in blood of adult donors compared to T cells, to a limited ability to expand *ex vivo*, although progress is being made in the latter through the use of improved protocols with different cytokines such as IL-15 and IL-18 (131, 132). In addition, genetic engineering of NK cells is less efficient than with T cells, with low transduction rate and loss of cytotoxic activity (126, 133). However, CD19 CAR-transduced NK cells from cord blood were recently shown to induce 64% of complete responses in patients with hematological malignancies, with favorable cell product attributes, i.e., CAR expression, engraftment and expansion *in vivo*, and without showing serious toxicity (134). In addition, given the existence of various NK cell subsets, important differences in phenotype and functional activity can be observed depending on the cells used (135, 136). NK cell lines are an alternative with potential to overcome most primary NK cells handicaps. These cells lines are rare but at least one of them, NK-92, is endowed with a high cytotoxic activity due to expression of many NK activating receptors and loss of some of the NK inhibitory receptors (137, 138). Advantages of NK-92 cells are their unlimited expansion *in vitro* and easy maintenance in culture (requiring only IL-2 supplementation). In addition, they are an homogeneous source of NK cells with an invariant phenotype and cytotoxic activity, a safe profile, and are easily modifiable through genetic modifications (133, 137, 139). Additionally, using CRISPR/Cas9 editing, NK-92 cells have been modified to re-express CD16 or DNAM-1, allowing to increase their anti-tumor cytotoxic activity to levels close to primary NK cells (138). As NK-92 cells originate from a human NK cell lymphoma, one limitation as cell therapy is the mandatory irradiation of these cells before infusion in patients to avoid any risk of malignant expansion. Irradiation does not affect functionality of NK-92 cells, including cytotoxic activity, but impairs proliferation *in vivo*, reducing NK-92 engraftment to a few days (126, 139). In order to overcome this limitation, NK-92 CARs must be infused several times in patients, a concept that has already been proven as feasible in an anti-Her2 therapy (140–142). Furthermore, despite the fact that CAR NK-92 cells showed pre-clinical activity against several tumor targets such as CD19, EGFR, Her2, and PSMA, they still need to be validated in a clinical setting (137, 143). Finally, among other population that have been explored to generate allogeneic CARs are iNKT cells (144). iNKTs combine a strong cytotoxic capacity with an activation restricted to the monomorphic CD1d molecule (145), reducing the risks of off-target toxicity, since CD1d is expressed mainly by antigen presenting cells such as dendritic cells and B cells (146). Interestingly, allogeneic iNKT cells have been associated with a protective effect against GVHD (147, 148). Similar to NK cells, iNKT cells are more difficult to culture, transform and expand that T cells and repeated cell administration or adjuvant use of IL-2 would be necessary to achieve persistence and anti-tumor efficacy (144, 149). However, several groups are working to improve iNKT cell culture and expansion protocols, with recent advances potentially making this population an efficient and flexible platform for next-generation CAR therapies (88, 150).

## CAR T CELLS FOR BRAIN TUMORS

### Challenges Associated With Tumor Location in the Brain

Although solid tumors are challenging for CAR T cell therapy, GBM are endowed with specific hurdles. First, entrance of immune cells, including CAR T cells, into the CNS is usually low, since migration of these cells into the CNS is limited by the endothelial blood–brain barrier (BBB) and the epithelial blood–cerebrospinal fluid barrier (151). Second, high inter- and intratumoral heterogeneity is one of the hallmarks of GBM, making selection of tumor antigens for CAR T cell design more challenging. Finally, GBM displays an immunosuppressive environment induced by the tumor itself, by recruited immune cells and by standard radiochemotherapy treatments that hamper CAR T cell activity (152). Finally, there is a great limitation of relevant models in GBM to assess CAR T cell function, study resistant phenotypes and test combinatorial strategies (152).

### Making CAR T Cells Reach the Tumor in the Brain

The first challenge of CAR T cell therapies against GBM is to achieve active trafficking of the effector CAR T cells to the tumor site (153). In the published clinical trials, GBM patients were treated with anti-IL13Rα2, Her2, or EGFRvIII

CAR T cells, and investigators explored both local and systemic administration routes. The main achievement of these trials was to provide evidence of safety of anti-GBM CAR T cells and potential for clinical efficacy, with some durable responses reported (50, 51). However, the majority of patients treated did not experience clinical benefit. A critical analysis of the main factors contributing to the low efficacy of these trials pointed to the necessity to improve CAR T cell trafficking and engraftment (50). Relevance of the administration route was evidenced with one of the more remarkable results showing tumor regression in a patient receiving multiple infusions of IL13Rα2-specific CAR T cells. The patient was treated CAR T in a sequential manner, using first the intracavitary and then the intraventricular route. While the first treatment resulted in local tumor recurrence, intraventricular infusion caused regression of all CNS lesions (52). This correlates with pre-clinical brain tumors studies, in which evidence points to an increased anti-tumor activity after locoregional administration of CAR T cells (154–156).

Another way to enhance trafficking to the brain would be to engineer CAR T cells with specific chemokine receptors that improve infiltration into the tumor (157). As an example, anti-GBM CAR T cells targeting CD70 showed increased trafficking to the tumor site and a better anti-tumor activity after being transduced with CXCR1 or CXCR2 (158). CXCR1 and CXCR2 are receptors for IL-8 (CXCL8), an inhibitory chemokine involved in recruitment of tumor-associated neutrophils or myeloid derived suppressor cells (MDSCs) (159), tumor proliferation and angiogenesis (160). Furthermore, despite the fact that most of chemokines attracting T cells are downregulated in GBM, some are upregulated. Two examples are CCL17 and CCL22, two chemokines involved in T regulatory (Treg) recruitment (161), that can be used to attract CAR T cells if the latter are transduced with their cognate receptor, the CCR4 molecule. Another argument to justify transfection of CAR T cells with CCR4, which also holds true for CCR2, is based on their ability to bind to CCL2, a chemokine expressed in gliomas that has been demonstrated to recruit T cells to tumor site *in vivo* (162). Finally, other approaches point to overexpressing some of the chemokine receptors involved in T cell trafficking to inflammatory sites, such as CXCR3, owing to expression of its ligands, CXCL9 and CXCL10, in the GBM tumor microenvironment (TME) (163). Indeed, CXCR3 signaling through interaction with CXCL9 and CXCL10 has been shown to play a relevant role in tumor homing of effector T cells (164).

## *In vivo* Monitoring of Cellular Products and Treatment Efficacy

Monitoring persistence and functionality of CAR T cells is essential to improve effectiveness of anti-tumor therapy. In contrast to hematological malignancies, monitoring CAR T cells at the tumor site in solid tumors is usually more difficult and new strategies to follow and evaluate CAR T cells in the tumor and/or in periphery must be designed. Conventional brain imaging using computer tomography (CT) or magnetic resonance imaging (MRI) with contrast are commonly used for brain tumor diagnostic and disease follow-up. One of the issues associated with the use of MRI in patients treated with immunotherapy is a phenomenon known as pseudoprogression, i.e., increase of lesion sizes related to treatment, which simulates progressive disease (165). Pseudoprogression, which has been associated with favorable prognosis in some instances (166), is difficult to distinguish from true tumor progression. This was evidenced by a phase I dose-escalation trial with an anti-Her2 CAR. In the weeks following CAR T cell infusion, several patients showed a progression-like image with increase in peritumoral edema, but all survived more than 6 months, suggesting pseudoprogression and not true tumor progression (53). New imaging-based methods are therefore needed to more accurately follow CAR T cell expansion at the tumor site.

The *in vivo* detection of cell therapy products through $^{19}$F-based MRI after endocytosis of $^{19}$F-dense perfluorocarbon nanoemulsions, still in early development, is a promising option to monitor CAR T cell infiltration and survival in the tumor during clinical trials (167). Based on the high *in vivo* sensitivity of nuclear imaging methods, one interesting variant to $^{19}$F MRI cell detection are radionuclide-based imaging methods, mainly positron emission tomography (PET) and single photon emission computed tomography (SPECT) (168–170). Due to high sensitivity of PET and SPECT, higher than that of MRI techniques, much lower concentrations of radiolabeled compounds can be used, making possible to avoid interference with cell function and viability (168, 171). Using a $^{89}$Zr-labeled anti-ICOS antibody, it was shown that PET can be a useful tool for *in vivo* tracking of CAR T cells in an orthotopic murine tumor model of lymphoma (172). One disadvantage of PET and SPECT being that they have a limited spatial resolution, combination with CT provides a first approach to surpass this limitation (173). A first successful use of PET/CT to detect tumor-infiltrating CAR T cells was reported in a mouse model of an anti-GD2 CAR T cells, the vector used co-expressing a dihydrofolate reductase enzyme that generates an $^{18}$F-probe for PET (170). However, since PET-CT has disadvantages as well, such as errors during the co-registration of images and high radiation doses due to CT scans, approaches that combines nuclear imaging techniques with MRI are being developed (173, 174). PET-MRI and SPECT-MRI combine the high sensibility to visualize physiological process with the capacity to show anatomical structures and are already becoming one of most powerful imaging platforms to study CAR T cells (174). On the other hand, use of perfusion-weighted imaging approaches in the MRI field, both dynamic contrast enhanced and dynamic susceptibility contrast-enhanced seems to be able to differentiate between the effects of chemotherapy and radiotherapy on tumor progression in high-grade gliomas (175). Both methods have shown a relatively good accuracy in individual studies, but further investigation and standardization is needed until they can be used for CAR clinical trials (175). Another promising alternative, limited to patients with surgical re-interventions after CAR T cell treatment, is the *in-situ* analysis of CAR T cells. This uniquely allows to analyze the amount and phenotypical characteristic of CAR T cells that can successfully migrate to the tumor and evaluate, as a possible prognosis of tumor evasion, the

changes in CAR T cells antigen expression after treatment (54). In addition to MRI, another way to monitor tumor evolution under CAR T cell therapy is the detection of target antigen expression, using qPCR or immunohistochemistry on samples obtained from the tumor site (152). However, in anti-CD19 CAR T cell pre-clinical experiments, a decrease in surface CD19 expression was reported without a significant decrease in mRNA levels, leading to a debate about the reliability of qPCR measurements as surrogate of therapy efficacy against an specific antigen (176).

Although direct analysis of tumor evolution and detection of CAR T cells at the tumor site should ideally provide a direct correlation with functionality, CAR T cell assessment in the peripheral blood has some advantages as compared to reoperation or *in situ* measurements, being less invasive and safer. In this direction, analysis of the number of CAR T cells, absolute or in proportion to overall T cell populations, associated with expression of functional and exhaustion markers, shall provide an accurate picture of therapy efficacy. However, since, in GBM, intracranial administration CAR T is preferably used, it is necessary to know whether CAR T cells can reach and be detected in the periphery. A first report showed that CAR T cells administered intraventricularly, and, in lesser amounts, intratumorally, can reach the periphery, allowing to use flow cytometry to measure CAR T cells in that compartment (155). A recently developed approach makes use of liquid biopsies, which is a non-invasive technique that can be used to monitor CAR T cell persistence and tumor progression through analysis of circulating tumor (ct) or CAR DNA (177). Although initial reports showed that gliomas had lower levels of ctDNA compared to others tumors (178), recent reports pointed to the use of ctDNA or circulating cell-free DNA as a non-invasive measure of response to therapy in brain tumors (179, 180). Another recently developed platform is the detection of tumor mitochondrial DNA (tmtDNA), a technique that showed 3 times better detection rates as compared to ctDNA, and can be used in cerebrospinal fluid (CSF). tmtDNA appears to be a more sensitive method to analyze tumor change following CAR T cell treatment, and can allow overcoming limitations that other methods have shown in GBM (181). It is important to note that the anatomical structure of brain, especially the BBB, makes CSF a relevant fluid that might provide a more accurate picture of tumor treatment efficacy than plasma, including the measure of certain biomarkers (182).

## CAR T Addressing GBM Heterogeneity

Engineering successful CAR T cell therapies against GBM has two major prerequisites: (i) the choice of antigen, the goal being to target the high molecular heterogeneity inherent to GBM, and (ii) the modulation of the immunosuppressive TME to allow CAR T cell function. Analysis of clinical trials with CAR T cells for solid tumors suggests that improvements in cancer cell recognition as well as in CAR T cell persistence and activity, especially in an immunosuppressive TME, will lead to increased efficacy (183, 184). CARs that target multiple tumor antigens might allow enhancing tumor cell detection; however, increasing the number of antigens also increases the risk of "on-target off-tumor" effects that may cause serious damage to healthy tissues. Such toxicity has been observed with anti-CD19 CAR T cells, affecting dispensable B cells (185) but also brain mural cells, leading to neurotoxicity (186).

As many solid tumors, GBM displays a high intratumoral heterogeneity, with different tumor cell clusters showing differences at the genotype level (187). Single-cell transcriptomic showed that several cell types coexist in the same GBM sample, with a high degree of plasticity between the different states (188). Challenges in addressing GBM heterogeneity also lie in the fact that a small subset of tumor cells can actively sustain heterogeneity (189, 190) and that a relative small population of cancer stem cells is responsible for tumor recurrence (191, 192).

In addition to the need to target all cells within a tumor cell population, addressing antigen loss, which is a common risk when a single antigen is targeted, is required (193–195). In an attempt to predict efficacy of CAR T cells targeting two or three GBM antigens, one study applied a binominal mathematical model, using expression of three of the well-described GBM-associated antigens, Her2, IL-13Rα2, and EphA2. In primary GBM samples, the model predicted that targeting two of the three antigens would result in higher efficacy as compared to single antigen targeting, but that addition of the third antigen would not improve the outcome (193). Interestingly, in mouse models, authors compared two alternative strategies, one with a bispecific CAR and the other with a 1:1 pool of monospecific Her2- and IL-13Rα2-specific CAR T cells. Bispecific CAR T cells demonstrated a better anti-tumor activity and higher *in vitro* activation, with increased IFN-γ and IL-2 secretion, and improved cytolytic activity (193). These results encourage the development of a dual, both bispecific or tandem design CAR T cell therapy against Her2 and IL-13Rα2 in GBM (193, 194). Similar approaches to avoid antigen escape have been tested in breast cancer with CAR T cells targeting Her2 and MUC1 (196) and in B-ALL with CAR T cells targeting CD19 and CD22 (197). However, even if targeting two antigens can decrease the probability of tumor escape, this may not be enough in the case of highly heterogeneous tumors to reach complete remission (114). To enable targeting a wider proportion of GBM patients, a trivalent CAR targeting Her2, IL3Rα2, and EphA2 was generated. Trivalent CAR T cells displayed increased IFN-γ and IL-2 secretion after tumor recognition compared to monovalent and bivalent constructs, and the trivalent CAR therapy was able to eliminate nearly all tumor cells in an orthotopic patient-derived xenograft (PDX) mouse model (198).

In view of these results, developing a pool of CAR T cells with different antigen specificities would result in a flexible platform that could be adjusted to the antigen profile of each patient in terms of antigen expression. Using allogeneic cells would enable the generation of CAR T cells specific for antigens of choice or even a bank of "à la carte" CAR T cells specific to the major antigens expressed by a given tumor type (**Figure 3**). As mentioned above, different strategies are available to design CAR T cell therapies targeting multiple antigens, such as co-administration of two or more CAR T cell populations, each bearing a different antigen specificity, or the simultaneous expression of two, potentially three, CAR molecules in the same T cells, such as with bispecific and tandem CARs (113). In this regard, allogeneic CAR T cells is

a promising option for multi-targeting approaches. Whereas, a limited number of autologous T cells is usually available, allogeneic T cells can be obtained in high numbers, allowing manufacturing different CAR T cells populations. In addition, it allows compensating for the lower efficiency of transduction with viral vectors with higher packaging capacity, when a strategy using multiple CAR molecules is preferred. In addition, availability of multi-antigen targeting CAR T cells at the time of patient diagnostic will allow rapid administration and prevent the risk of manufacturing failure. Other options to target multiple antigens are under development and include the combination of CAR T cells and bispecific T cell engagers (BiTE), in the so-called CART.BiTE strategy (199). As an example, heterogeneous GBM were eradicated in mouse models using a combination of EGFRvIII-directed CAR T cells and a secreted BiTE targeting wild-type EGFR (200).

## CAR T Cells That Modify the TME

The GBM immunosuppressive TME has been regarded as one of the main obstacles to a successful CAR T cell therapy (51). GBM contains tumor and non-tumor cells that generate a hostile environment dampening T cell function and survival (201–203). First, at the cellular level, GBM cells are able to recruit and polarize immune cells to a regulator phenotype, the better described examples being Tregs, tumor-associated M2 macrophages and MDSCs (203, 204). Second, at the molecular level, cells in the GBM TME are able to express inhibitory ligands such as PD-L1, CD95-L, or non-classical MHC class-I proteins (205) and to secrete immunosuppressive cytokines such as TGF-β and IL-10 (206). Finally, at the metabolic level, TME cells are able to decrease relevant metabolites such as glucose *via* increased consumption, or to deplete relevant amino acids, such as tryptophan, *via* IDO1 (indoleamine 2,3-dioxygenase 1) secretion, limiting T cell function. These phenomena are in addition favored in hypoxic conditions, which is common in GBM (203, 207).

### Reshaping Immune Cells in the TME

One strategy to target immunosuppression at the TME of solid tumors using CAR T cells is based on the secretion of pro-inflammatory cytokines at the tumor site, with the aim to reprogram infiltrating immune cells while enhancing CAR T cell killing function. Cytokines tested include IL-7 (208–210), IL-12 (211–213) and IL-18 (214, 215). In GBM, pre-clinical data showed synergy of CAR T cells co-expressing IL-15 (195), IL-12, and IL-18 (216). IL-21 is another cytokine that is being considered due to its role in TME modulation (217). In fact, CAR T cells cultured *in vitro* with IL-21 showed a higher efficiency in controlling *in vivo* tumors (218). In addition, a pre-clinical study comparing CAR T cells secreting different γ-chain-cytokines (IL-2, IL-7, IL-15, and IL-21) found increased anti-tumor activity with all the cytokines tested, but effects were mediated through different mechanisms (208). Recently, transfecting CAR T cells with the IL-12β p40 subunit allowed production of IL-23 after activation, which led to an increase in cell proliferation and survival. This strategy resulted in enhanced anti-tumor activity, due to autocrine IL-23 signaling, in xenograft and syngeneic mouse models (219). A frequently reported CAR T cell design used to deliver cytokines and modulate the immunological balance at TME are TRUCKs ("T cell redirected for universal cytokine-mediated killing"). TRUCKs incorporate an NFAT (nuclear factor of activated T cells)-mediated signaling getting activated upon CAR engagement of its cognate antigen and leading to local secretion of a cytokine of interest (220). One limitation of the original TRUCKs was the necessity of transducing two vector constructs, but recently a modular "all-in-one" vector system was developed, resulting in enhanced cytotoxic activity and safety (216).

The increasing amount of results with CAR T cells secreting different cytokines suggest that selecting the appropriate cytokine could be a challenge and that, in some cases, combinations might be a better option. Since most cytokines are pleiotropic and can induce both immunostimulation or immunosuppression according to the immune contexture, their use is still in debate and more studies are needed to optimize their combination (183, 221). Also, as high levels of cytokine secretion is associated with two main CAR T cells toxicities—CRS and ICANS—the possible toxicity of additional cytokines, as we mention before for reshape TME, must be careful monitoring. Cytokines as IL-15 are strongly associated with ICANS, but direct neutralization of IL-15 or other pro-inflammatory cytokines must impair T cell activity, then alternative treatments directed to the inflammatory process itself could be more appropriate, including blockade of the signaling of IL-6, IL-1 or GM-CSF and the use of corticosteroids (222, 223).

Lastly, a new possibility to modify the TME is to target directly the immunosuppressive cells. CAR T cells targeting M2 macrophages are currently being developed using the most recent, and extensive, phenotypic characterization of these cells in GBM (224, 225).

### Resisting Immune Checkpoint Inhibition

The other main strategy to target immunosuppression at the TME is to avoid immune suppression signals by blocking them or turning them into activating ones, mainly based on decoy and chimeric switch receptors. Chimeric switch receptors, also called inverted receptors, are composed of a receptor for an immune inhibitory signaling molecule as extracellular domain, and of the cytoplasmic domain of an immunostimulatory molecule as intracellular domain, allowing to turn an inhibitory signal into a stimulatory one (157). An interesting use of this design combines the extracellular portion of PD1 with the intracellular signaling domain of the costimulatory molecule CD28. CAR T cells with this PD1CD28 switch receptor and specific for CD19, mesothelin or PSCA, showed increased cytotoxicity and improved tumor control in several established solid tumor models (226). Chimeric switch receptor were also designed by combining the IL-4R extracellular domain with the intracellular signaling domains of IL-7 or IL-21, resulting in CAR T cells with enhanced anti-tumor activity against IL-4$^{+}$ tumors (227, 228). Decoy, or neutralizing, receptors, including dominant-negative receptors, are also an approach that can be used to avoid TME immunosuppressive signals. In this case, receptors do not transmit any signal to the cell and can contribute to eliminate inhibitory cytokines from the TME. In a first attempt to use this strategy, CAR T cells were engineered to overexpress a truncated receptor for

PD1, without the transmembrane and intracellular signaling domains of the natural receptor, resulting in non-transduction of the signal upon binding and conferring resistance to PD-L1/2 immunosuppression. This led to an increase in effector functions and better tumor control in pre-clinical experiments (229). A similar approach was shown to be successful when targeting TGF-β *via* introduction of a dominant-negative TGF-β receptor in an anti-PSMA CAR. This construct resulted in increased proliferation rate and higher persistence of CAR T cells in mice, combined with a less exhausted phenotype, leading to enhanced anti-tumor activity (230). A recent report used anti-CD19 CAR T cells with concomitant expression of an IL-6 neutralizing receptor that reduced CRS by eliminating soluble IL-6, without affecting anti-tumor activity (231). In an alternative approach, genetic editing tools such as CRISPR/Cas9 can be used to disrupt immunosuppressive signals in the TME (232). PD1 gene (*Pdcd1*) disruption using CRISPR/Cas9 in anti-CD133 CAR T cells was shown to increase tumor killing *in vitro* while inhibiting *in vivo* tumor growth in a orthotopic glioma xenograft model (233). In a stimulating attempt to create universal allogeneic CAR T cells against EGFRvIII, the CRISPR/Cas9 system was also used to disrupt *Pdcd1*, together with the TRAC and β2-microglobulin genes. These triple negative CAR T cells showed improved persistence and anti-tumor activity in NSG mouse models of human GBM. Interestingly, this was observed only when locoregional administration was used, suggesting the relevance for a successful anti-GBM therapy of immune checkpoint signaling blockade and local infusion of CAR T cells (234).

### Targeting Metabolic Checkpoints

Since a hypoxic microenvironment is found in many solid tumors, and is considered a hallmark of GBM (235), some authors are considering turning it into an advantage. The best example is the development of a CAR containing sub-domains of hypoxia-inducible factor-1α to induce CAR T cell activation only under the low oxygen levels found in TME (**Figure 3**). These oxygen-sensitive CAR T cells showed a more tumor-localized function that enhanced efficacy, allowing the use of tumor associated antigens with lower risk of side effects (236). However, this strategy would still come with the risk of unanticipated CAR activation in regions of the human body bearing low oxygen levels, especially in patients with concomitant pathological conditions such as infections or inflammation (237).

## THE NEED TO HAVE IMMUNOCOMPETENT PRE-CLINICAL MODELS THAT REFLECT THE COMPLEXITY OF THE GBM MICROENVIRONMENT

Like any other therapy, CAR T cell therapy needs to be tested in pre-clinical models before reaching clinical trials. Available experimental models can be categorized in two main groups, both with advantages and disadvantages. On one side, reductionist models are being used to elucidate the specific role of molecules or biological processes, and make direct measures of functional effects such as cytotoxicity without most of the complex relations present in a live organism. On the other side, "close to reality" models aim at recapitulating the tumor and its interaction with other components of the organism such as the immune system, and are more focused on evaluating the probable efficacy of therapies (238) (**Figure 4**). CAR T cell cytotoxic functions can be directly tested using classical human GBM cell lines, such as U87MG and U251MG, but despite the fact that these experiments can provide a proof of concept, these models suffer from several limitations. Long-term *in vitro* culture induces significant changes at the genetic and transcriptional level, including loss of stemness attributes and differentiation to an astrocytic phenotype, diverging away from the characteristics of human GBM (239). Since human CAR T cells need to be tested in xenograft models and given that GBM cells lines are not optimal to recapitulate the heterogeneity of human tumors *in vivo*, many groups have moved to alternative models such as GBM stem cells (GSC), PDX or organoids (238). GSC culture conditions induce the preferential expansion of glioma stem cells and are a useful tool to study GBM biology, including *in vivo* tumor development and heterogeneity, and may also predict efficacy of some therapies (240). Human GBM-derived PDX models provide a more precise representation of tumor heterogeneity than other available models, including the ability to reproduce many of the interactions between tumor and the TME (241, 242). Due to these characteristics, PDX models, in particular when used orthotopically, appear to more closely recapitulate the real disease (243–246). However, after several *in vivo* passages, PDX tumors can also notably diverge from the original primary tumor, both at genetic level and at subpopulation composition (247, 248). In recent years, organoids have become another promising alternative to reproduce GBM in mouse models. Combining GSCs and iPSC, several researchers were able to generate highly heterogeneous 3D cultures, integrating neoangiogenesis and a hypoxic milieu and making these a clinically relevant option to test new anti-GBM therapies (240, 242, 249).

In addition to the challenges of any xenograft GBM model to represent tumor heterogeneity, immunosuppressed mouse models are not capable of reproducing the full interaction of tumor or CAR T cells with the immune system. As such, one of the most commonly used immunocompromised models, the NOD-scid/IL2R-$\gamma^{null}$ (NSG) mouse model, does not allow interrogating the effect of infiltrating immune cells (250, 251). In addition, interactions of CAR T cells with other immune or stromal cells, the potential "on-target off-tumor" effects and the generation of CRS cannot be modeled either (252). To overcome limitations in assessing CAR T cell interaction with other immune cells and simulating the CRS, important progress has been made to develop mouse models with a humanized immune system (253, 254). Through human hematopoietic $CD34^+$ stem cell transplantation in immunosuppressed mice, including NSG mice, many groups were able to develop mice incorporating many cellular components of the human immune system, including both myeloid and lymphoid lineages (255, 256). The humanized mouse field is in constant progress, with new mouse models being able to resemble more accurately the human

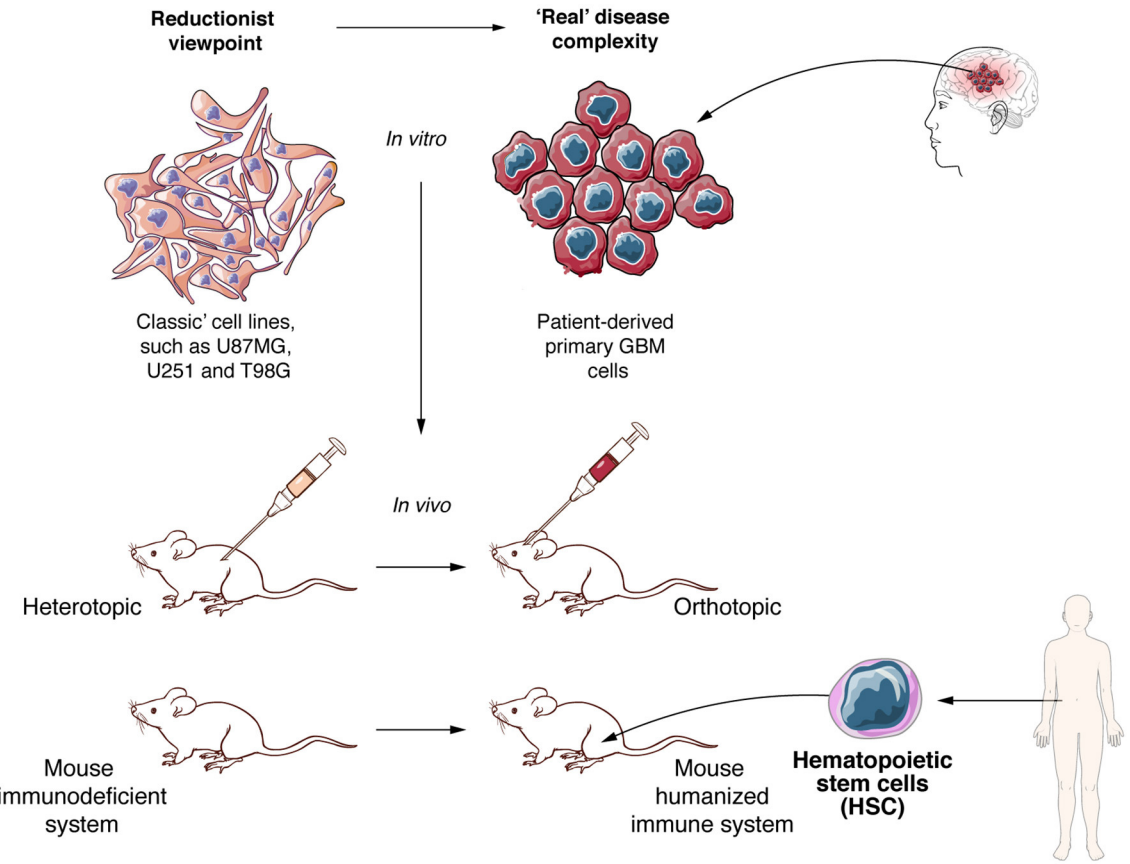


**FIGURE 4 |** To develop new and effective anti-GBM therapies better experimental models are required. Experimental *in vitro* and *in vivo* models need to move to more realistic settings. Patient derived primary cell lines and xenograft models, orthotopic tumor growth and mice with humanized immune system must become the standard to evaluate allogeneic CAR T cells against GBM.

immune system. As an example, NSG or NOD/Shi-scid/IL-2Rγ$^{null}$ (NOG) mice that express human cytokines such as G-CSF, GM-CSF, or IL-3, lead to better engraftment, generation and development of the human immune system after injection of human CD34$^{+}$ stem cells (257, 258). Another consideration in designing relevant GBM models is the inoculation site of tumor cells. Orthotopic engraftment must be preferred, as it confers an adequate environment to GBM development and better reflects the human disease (238). Finally, other animal models could be explored, such as immunocompetent dogs that spontaneously develop high grade gliomas and show some of the features of human GBM, such as tumor heterogeneity, in presence of a functional immune system (259, 260) (**Figure 4**).

## CONCLUSIONS AND PERSPECTIVES

CAR T cell based therapies are transforming the treatment of hematological malignancies and have the potential to do the same in solid tumors (184). However, despite showing some evidence of anti-tumor effect, CAR T cell therapies against GBM still need to prove their efficacy to become a viable and impactful therapeutic option (152). To this day, all 21 clinical trials (**Supplementary Table 1**, updated December 2020 in https://clinicaltrials.gov/) using CAR T against GBM are based on autologous CAR T cells. In addition to addressing the many obstacles raised by solid tumors and GBM in particular, using allogeneic T cell sources might be part of successful future strategies. Several advantages make allogeneic CAR T cells a relevant option for GBM patients (16). Allogeneic CAR T cells have the advantage of being ready to use and can be readily administered to patients from the moment of diagnosis. In addition, healthy donors provide high amounts of T cells, without loss of functionality due to exhaustion or suppression, which are commonly found in T cells derived from cancer patients (261) and particularly in GBM (262). However, using allogeneic CAR T cells comes with the need to overcome host immune rejection and to minimize GVHD (16). The first can be accomplished mainly through elimination of HLA molecules and the second by knocking-out the TCR or by using tumor site-specific activation strategies such as hypoxia-activated CAR T cells. Nonetheless, an important feature of allogeneic cells is that, as opposed to autologous T cells usually used to generate monovalent CAR T cells, allogeneic T cells can be used to generate several CAR T cell products with different antigen specificities or multivalent CAR T cells, enabling to overcome GBM heterogeneity. It could also

be used as a personalized therapy to target the set of antigens expressed by a given patient. Allogeneic CAR T cells could further be combined with modular CAR strategies to make "fully universal" CAR T cells that combine availability of allogeneic cells and flexibility of modular designs. As such, allogeneic CAR T cells would not only be able to target different GBM antigens but also to target the TME, offering a new promising field that could overcome the limitations of current CAR T cell strategies.

Today, there is virtually no limit to synthetic biology and cell engineering, providing a platform to develop new therapies against GBM. Together with the identification of new and highly tumor-restricted antigens, the development of more representative experimental models and improved imaging techniques to assess tumor response and CAR T cell features *in vivo* will be part of that therapeutic challenge. In the next decade, the neuro-oncology field will most probably witness the advent of allogeneic CAR T cells, engineered immune cell products endowed with multiple specificities and resistant to host rejection, hopefully allowing transition to improved patient outcome.

## AUTHOR CONTRIBUTIONS

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

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.640082/full#supplementary-material

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REVIEW
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# Harnessing Mechanisms of Immune Tolerance to Improve Outcomes in Solid Organ Transplantation: A Review

*Priscila Ferreira Slepicka, Mahboubeh Yazdanifar and Alice Bertaina* *

*Division of Hematology, Oncology and Stem Cell Transplantation and Regenerative Medicine, Department of Pediatrics, Stanford University School of Medicine, Stanford, CA, United States*

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***Correspondence:**
*Alice Bertaina*
*aliceb1@stanford.edu*

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Survival after solid organ transplantation (SOT) is limited by chronic rejection as well as the need for lifelong immunosuppression and its associated toxicities. Several preclinical and clinical studies have tested methods designed to induce transplantation tolerance without lifelong immune suppression. The limited success of these strategies has led to the development of clinical protocols that combine SOT with other approaches, such as allogeneic hematopoietic stem cell transplantation (HSCT). HSCT prior to SOT facilitates engraftment of donor cells that can drive immune tolerance. Recent innovations in graft manipulation strategies and post-HSCT immune therapy provide further advances in promoting tolerance and improving clinical outcomes. In this review, we discuss conventional and unconventional immunological mechanisms underlying the development of immune tolerance in SOT recipients and how they can inform clinical advances. Specifically, we review the most recent mechanistic studies elucidating which immune regulatory cells dampen cytotoxic immune reactivity while fostering a tolerogenic environment. We further discuss how this understanding of regulatory cells can shape graft engineering and other therapeutic strategies to improve long-term outcomes for patients receiving HSCT and SOT.

**Keywords: immune tolerance, hematopoietic stem cell transplantation, solid organ transplantation, innate immunity, adaptive immunity**

## INTRODUCTION

Solid organ transplantation is a lifesaving therapeutic strategy for numerous end-stage organ failures. The past 25 years have witnessed undeniable progress in preventing graft rejection and graft-versus-host-disease (GvHD), but these gains rely on lifelong use of immune suppressive (IS) drugs (1). Long-term IS regimens contribute to poor clinical outcomes by leading to severe side effects including cardiovascular diseases, hypertension, diabetes, nephrotoxicity, and an increased risk of cancer and infections (2). Even with IS drugs, graft loss occurs in half of patients within 15 years for histocompatibility leukocyte antigen (HLA)-mismatch kidney transplant recipients and within 25 years for those who are fully HLA-matched (3). Currently, many children who receive a SOT at a young age will need at least one additional transplant during their lifetime because of

inevitable loss of their graft caused by the combination of chronic rejection, infections, drug toxicity, and nonadherence (4).

Despite great advances in the induction of tolerogenesis in humanized mouse and classical preclinical models (5), there is still a large gap in translating this success to the bedside. Spontaneous operational tolerance remains rare, occurring in less than 5% of kidney and 20% of liver transplant recipients (6–9). Studies have found that some patients who have persistent graft acceptance with chronic IS drug use can become tolerant, allowing careful reduction and eventually full cessation of IS treatment. However, given current challenges in identifying biomarkers of graft rejection, removing IS—especially after kidney transplants—is risky and can lead to graft loss with consequent reductions in life expectancy (10–12). Enhancing long-term outcomes for patients of all ages requires new approaches to transplantation that can address these challenges.

In recent decades, investigators have focused on developing alternative approaches to induce immune tolerance toward the donor graft in transplant recipients. A standout example is the combination of allogeneic HLA-matched HSCT with SOT from the same donor (13). Despite promising results, over 70% of patients lack an HLA-identical sibling. For this reason, transplants from related full-haplotype mismatched (haploidentical) donors (14) and unrelated HLA-matched and mismatched donors have been performed to expand availability of this treatment protocol.

Successful allogeneic HSCT requires the development of immune tolerance towards both the donor and host allogeneic antigens. Induction of immune tolerance can prevent T-cell mediated graft-rejection and GvHD, which might lead to life threatening complications in HSCT recipients. Current approaches to prevent rejection and GvHD after HSCT primarily rely on pharmacological IS, either prior to or after HSCT. These approaches are limited by lack of antigen specificity, and the requirement for long-term therapy, which often leads to severe complications. Recent progress in understanding the mechanism of action of alloreactive and regulatory cell populations has led to the use of specific cell subsets to prevent/treat graft rejection and GvHD and induce immune tolerance. Peripheral tolerance after allogeneic HSCT may be achieved by several mechanisms, though blocking alloreactivity to the host human leukocyte antigens while preserving immune responses to pathogens and tumor antigens remains a challenge. Recently uncovered evidence regarding the mechanisms of post-HSCT immune reconstitution and tolerance in transplanted patients has allowed for the development of novel cell-based therapeutic approaches. These therapies are aimed at inducing long-term peripheral tolerance and reducing the risk GvHD, while sparing the graft-versus-leukemia (GvL) effect (15).

The use of sequential HSCT and SOT has resulted in meaningful improvements in kidney graft tolerance (16–24). With the addition of non-myeloablative conditioning, many HLA-matched recipients are able to taper and fully discontinue all IS drugs within two years after transplantation without GvHD or graft rejection (25). In HLA-mismatched recipients, though, achieving tolerance without IS has proven to be considerably more difficult and, when accomplished, has often come with heightened risks of GvHD and infections that can threaten graft survival (25–28). Although clinical studies have made strides in maintaining long-term organ engraftment with reduced IS regimens, there is an ongoing need to improve immune tolerance after sequential HSCT and SOT in order to completely eliminate the need for pharmacological IS and without potentially risky tradeoffs for patient outcomes.

A refined understanding of the mechanisms of immune tolerance creates opportunities for novel HSCT techniques-including graft engineering strategies- to optimize survival after SOT and enable a functional immune system that permanently accepts donor antigens without the need for IS. This review describes first key findings that influence our understanding of the cellular mechanisms involved in immune tolerance as well as the role of innate immunity in these regulatory processes. It then explores how development of new therapeutic strategies can harness this knowledge to more effectively induce tolerance, especially in the context of sequential HSCT and SOT.

## CELLULAR MECHANISMS OF *CONVENTIONAL* IMMUNE TOLERANCE

Immune tolerance is multifaceted and involves the interaction of different cells, listed in **Table 1**, that serve critical regulatory roles. While investigators have long worked to identify processes of tolerogenesis, advanced methods, including single-cell technologies, have expanded the mechanistic understanding of cells that are actively involved in the development of immune tolerance.

### 'Conventional' Treg Cells

In 1970, a seminal study by Gershon and Kondo (60) described a subset of T cells distinct from T helper (Th) cells that decreased the immune response. Twenty-five years later, these cells were named regulatory T cells (Tregs) in a study that found athymic mice inoculated with purified $CD4^+CD25^-$ T cells spontaneously developed autoimmune diseases (61) whereas the transfer of $CD4^+CD25^+$ cells inhibited CD4-mediated autoimmunity in lymphopenic mice.

The ontogeny of naturally emerged Tregs occurs in the thymus (tTregs) while other Tregs are converted or induced from $CD4^+CD25^-$ in the periphery (iTregs or pTregs, respectively) (62). tTregs are crucial for control of immune self-tolerance, allergy, and allograft survival. In mice and humans, tTregs comprise 2-10% of peripheral $CD4^+$ T cells (63, 64). Interleukin-2 (IL-2)-receptor α chain (CD25) is a cell surface marker that identifies Treg cells. Stimulation with TNF tumor necrosis factor (TNF) and IL-2 upregulates CD25 and activates Tregs (65); however, induction of CD25 expression in $CD25^-$ murine T cells is not sufficient to generate Treg suppressive function (66). Notably, activated memory and certain effector T cells (Teff) can also express CD25 (66). Thus,

**TABLE 1** | Features of conventional and unconventional immune regulatory cells.

| Cell type (Cell surface/ intracellular markers) | Plasticity (Cell surface/ intracellular markers) | Signaling factors to induce plasticity | Homing (Cell surface markers) | References |
|---|---|---|---|---|
| Tregs ($CD4^+CD25^+$ $CD127^{-/lo}$ | Th1-like Tregs (IFN-$\gamma^+$/T-$bet^+$/ $CXCR3^+$) | Th1-like: IFN-γ, IL-12, IL-27, IL-4, TGF-β, IL-2 | Gut (GPR-15) | (29–35) |
| Naïve Tregs ($CD45RA^+FoxP3^+$) | Th2-like Tregs (IL-$4^+$/IL-$5^+$/IL-$13^+$/ $GATA3^+$) | Th2-like: IL-4; IL-5 | Inflammation areas (CXCR3, LFA-1, VLA-4, CCR2, CCR5, CCR6, CCR8) | |
| *FOXP3*$^+$ effector non-Tregs ($CD45RA^-$ $FoxP3^{low}$) | Th17-like Treg (IL-$17A^+$/RORγ$t^+$) | Th17-like: IL-6, IL-21, IL-12, IL-23, TGF-β, IL-2, GATA3, IDO | Secondary lymphoid organs (CCR7, CD62L) | |
| Non-classic Tregs ($CD4^+CD25^+CD5^+CD38^{-/lo}CD45RA^+$) | Follicular regulatory T cells -Tfr ($CXCR5^+$/$Bcl6^+$/$ICOS^+$/$PD1^+$) | Tfr: IL-6, IL-21 | Skin (CCR4) | |
| Activated/effector Tregs ($CD25^{hi}CD127^{lo}CD45RO^+$ $CD45RA^-FoxP3^{high}$) | | | | (36–38) |
| Tr1 ($CD4^+$ $CD49b^+$ $LAG\text{-}3^+$ $CD226^+$) | Tr1 can be derived from | Th1 (TCR signaling, CXCL12, IL-12, IL-27) | Gut (GPR15, CCR9 – *in vitro* induced Tr1) | (39–43) |
| | Th1, Th2, Memory CD4+ T cell and Th17 | Th2 (TCR signaling) | Spleen (unknown) | |
| | | Memory CD4+ T cells (TCR signaling) | | |
| | | Th17 (IL-27, TGF-β) | | |
| Bregs -Transitional ($CD19^+CD20^+CD10^+$ $CD27^-$ $CD24^{high}CD38^{high}$) | Possible high plasticity | – | Inflamed skin | (44–46) |
| Bregs -Transitional TIM-$1^+$ ($CD19^+$ $CD24^{high}$ $CD38^{high}$ TIM-$1^+$) | | | | |
| Bregs - Memory/Mature ($CD19^+CD20^+CD10^-CD27^+$ $CD24^{high}CD38^-$) | | | | |
| γδTregs ($CD25^{low}$ CTLA-$4^{low}$ | Unknown for γδTregs | Th1-, Th2-like (pAg, IL-2, IL-4) | Kidney, Liver, Lung, Intestine (Vγ1, Vγ3, Vγ5) | (47–54) |
| $CD8^+$ - mouse renal allografts) | Vδ2 – High plasticity (Th1-, Th2-, Th9-, Th17-, Tfh-like cells) | Th9-like (IL15, TGF-β) | Gut (CD103, α4β7) | |
| | | Th17-like (pAg, Il-6, IL1g, TGF-β) | | |
| | | Tfh-like (pAg, IL-21) | | |
| Induced γδTregs ($FoxP3^+$) | | | | |
| NKT ($CD161^+$ TCR Vα24Jα$18^+$ $PLZF^+$) | NKT1 ($PLZF^{lo}$, T-$bet^{high}$, IFN-$\gamma^{high}$) | – | Liver (CXCR3, CXCR4) | (55–57) |
| | NKT2 ($PLZF^{high}$, T-$bet^{low}$, IL-4) | | Lung (CCR4) | |
| | NKT17($PLZF^+$, RORγ$t^+$/, IL-17) | | Spleen (CCR7, CXCR3-6) | |
| NKregs ($CD56^{bright}$ $CD16^{-/low}$ $NKp46^+$ Granzyme $B^{low}$ $Perforin^{low}$) | Unknown | | unknown | (58, 59) |

phenotype subsets of Tregs have been more precisely identified with other cell surface markers.

The identification of Forkhead Box P3 (*FOXP3)* as a master regulator of the Treg lineage commitment and differentiation has dramatically improved understanding of Treg biology (67–69). Loss-of-function mutations in human *FOXP3* cause Immunodysregulation Polyendocrinopathy Enteropathy X-linked (IPEX) syndrome, a rare and life-threatening immune disease (70). *FOXP3* mutation or deletion can also lead to loss of repression of oncogenes in some nonlymphoid cells, resulting in malignancies (71, 72). Early onset IPEX syndrome exclusively affects males and leads to fatal lymphoproliferative dysfunction in Tregs and subsequent severe autoimmunity (70, 73). The Treg specific demethylation of a highly conserved non-coding element within the *FOXP3* gene (Treg-specific demethylated region, TSDR) is required for *FOXP3* expression and can be used for Treg identification (74, 75). However, TSDR methylation status can vary; it is fully demethylated in tTregs, partially methylated in TGF-β polarized Tregs, and methylated in naïve cells (75–77). Accordingly, the methylation status of the *FOXP3* TSDR is a marker for the stability of *FOXP3* expression and Treg function during thymic differentiation, but it is not sufficient to isolate these cells. More recently, $CD4^+CD25^+$ $CD127^{-/lo}$ (IL-7R α chain) phenotype has been used for isolation and identification of Tregs (29–32).

During Treg thymic differentiation, *FOXP3* expression depends on the coordination of several factors, including T cell receptor (TCR) signaling, CD28 co-stimulation (78), cytokines (IL-2, IL-15, and IL-7), transcription factors (NFAT and ICOS) (79, 80), and the PI3K-mTOR signaling network (81). Notably, *FOXP3* can also be expressed in differentiating pTregs or in iTregs upon TCR stimulation with suboptimal co-stimulatory molecules. However, transient expression of *FOXP3* in Teff cells did not correlate with regulatory functions previously reported in Tregs, indicating that *FOXP3* may not be used as a marker solely for Tregs (82). TGF-β and IL-2 stimulation coupled with TCR

signaling and co-stimulatory molecules skew the differentiation of naïve CD4$^+$ T cells into Tregs. Mechanistically, IL-2 triggers the STAT5 signaling network and its downstream targets, including the expression of *FOXP3*, and polarizes CD4$^+$ T cell differentiation to Tregs rather than IL-17-producing effector T cells (Th17) (83, 84). Although human TGF-β-induced Tregs have a suppressive function *in vitro*, the transcriptomic landscape does not recapitulate tTregs, and its suppressive capacity is compromised *in vivo* in humanized GvHD mouse models (85).

Tregs can suppress autoimmunity directly through the release of cytokines (e.g. IL-10, IL-35, and TGF-β) or mediate cytotoxicity toward Teff *via* the production of proteases that induce cell apoptosis, such as granzyme and perforin, or galectins (86–91). Indirect mechanisms of suppression include: 1. recruitment of other cells, such as modulating antigen presenting cell (APC) function through cytotoxic T-lymphocyte antigen 4 (CTLA4), 2. expression of CD39/CD73 ectonucleotidases that convert ATP to immunosuppressive metabolites such as AMP and adenosine, 3. shifting a proinflammatory environment to anti-inflammatory (92, 93), and 4. outcompeting Teffs in IL-2 uptake by overexpressing CD25 (94) (**Figure 1**).

In the allogeneic transplant context, Treg signaling mechanisms are crucial for allograft survival because of their dampening of the immune response from Teff cells. As Tregs do not produce IL-2, their activation depends on the release of IL-2 by Teff cells (95). In the absence of Tregs, the binding of Teff TCR to alloantigen-major histocompatibility complex (MHC) and CD28 to CD80/CD86 activates Teff cells, leading to the secretion of IL-2 (86, 96). By autocrine mechanisms, IL-2 signaling triggers other T cells, causing activation, proliferation, and differentiation that can all lead to allograft rejection. However, activated Tregs secrete IL-10 and TGF-β that convert Teff cells into anergic cells, creating a tolerogenic environment. The expression of the co-stimulatory molecule CTLA4 on Tregs interacts with CD80/86 on dendritic cells (DCs) to suppress the immune response and contribute to allograft tolerance (97, 98).

Given their involvement in a multitude of immune responses, Tregs are considered a heterogeneous population with diverse

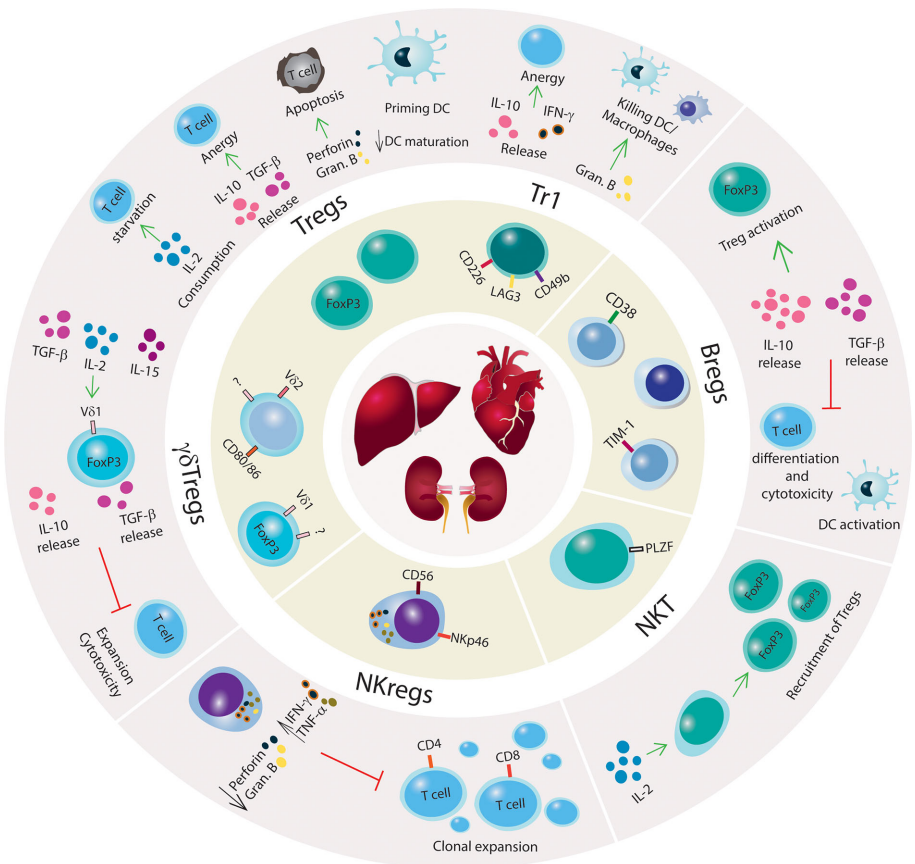


**FIGURE 1** | Mechanisms of immune tolerance to promote SOT engraftment and survival. Schematic illustration of regulatory innate and adaptive immune cells with a brief summary of mechanisms of immune suppression. Kidney, liver and heart are represented in the center of the figure, and regulatory immune cells (Tregs, Tr1, Bregs, NKT cells, NKregs, γδTregs) are shown surrounding the organs. The outer circle illustrates the main regulatory networks for each immune cell subset. Green arrows indicate promoting mechanisms, black arrows denote increase or decrease of cytokines production or biological processes, and red lines denote inhibitory networks.

functions and markers. Most of these cells can be categorized as naïve, *FOXP3*$^+$ effector non-Treg cells, or activated/effector Tregs with the latter being the most proliferative (Ki67$^+$) and suppressive (CTLA4$^{high}$) (33). Moreover, *FOXP3*$^+$ effector non-Treg cells produce pro-inflammatory cytokines such as IFN-$\gamma$ and IL-7 that have reduced immunosuppressive function and a high potential to become Teff. A further subset of human effector Tregs was identified using chemokine receptors and intracellular markers wherein T helper-like Tregs showed a memory-like phenotype (36). The migratory capacity (**Table 1**) and cytokine secretion of each subset offers crucial information in the graft tolerance context given that these cells have the ability to target specific tissue types, such as allografts or lymph nodes (99).

## Tr1 Cells

In contrast to tTregs, regulatory type 1 T cells (Tr1) are a subpopulation of memory CD4$^+$ T cells that can transiently express *FOXP3* upon activation; however, *FOXP3* expression in Tr1 cells is not constitutive or a requirement for Tr1 function and differentiation (100). Tr1 cells co-express integrin $\alpha$2 subunit (CD49b) and lymphocyte activation gene 3 (LAG-3), which facilitate the identification of Tr1 in the peripheral blood of tolerant patients (39). LAG-3 is mostly expressed on activated Tr1 cells while CD49b expression is constitutive. Other cell surface markers have been identified including Tim-3, PD-1, TIGIT, and CD39, but they are not exclusive to Tr1 cells (101). Another crucial difference between Tr1 and tTregs is the metabolic profile on which these cells rely; Tr1 cells depend on aerobic glycolysis (102) while *FOXP3*$^+$ Treg differentiation is associated with fatty acid oxidative phosphorylation (103).

Tr1 cell development, expansion, and function are independent of IL-2 and CD28 (104). The Tr1 cell mechanism of suppression is *via* secretion of TGF-$\beta$ and IL-10 in which IL-10 constitutively triggers Tr1 cells to release additional IL-10, creating a feedback loop (**Figure 1**). In the absence of IL-10, Tr1 cells lose their capacity to produce IL-10 but retain secondary mechanisms of immune suppression that are driven by the expression of granzyme B and CTLA-4 (105). Tr1 can release IFN-$\gamma$ but only low or absent levels of IL-2, IL-4, and IL-17 have been found in these cells. The activation of Tr1 cells is *via* cognate antigen binding by their TCR, which initiates the production of granzyme B and Tr1-mediated killing of DCs or macrophages (40, 106). Once activated, Tr1 cells perform bystander suppression. Tr1 cells also utilize suppressive mechanisms that are shared with *FOXP3*$^+$ Tregs including interactions of co-stimulatory molecules CTLA-4 with CD80 and PD-1 with PD-L1 (107).

IL-10 is essential for Tr1 cell function in humans and mice, but the signaling mechanism has not been fully elucidated. The STAT pathway has been suggested as the downstream target of IL-10 signaling in Tr1 cells. Studies have shown that STAT3 interacts with proteins associated with a glycolytic metabolic environment that favors Tr1 cell differentiation (102). High activation of STAT3 in T cells induces Tr1 differentiation (108), and the induction of IL-10 is STAT1- and STAT3-mediated (109). IL-27 has been described in mice and humans as a critical cytokine that promotes IL-10 secretion and Tr1 differentiation (110, 111). Mechanistically, IL-27 triggers STAT3, which activates B lymphocyte-induced maturation protein-1 expression (112). Under specific conditions, IL-27 also triggers c-Maf and AhR transcription factors to activate IL-10 transcription such that AhR also contributes to granzyme B expression in Tr1 cells (111, 113, 114). Other transcription factors necessary for IL-27-mediated induction of Tr1 include BAFT, IRF1, and ITK (115, 116).

## Single Cell Strategies to Identify Tregs and Tr1 Subpopulations

Previous studies have pioneered the investigation of cell surface markers to identify subsets of regulatory T cells. Given advances in single-cell strategies, the heterogeneity of regulatory T cells has been reflected in the stratification of these cells according to novel cell surface markers, intracellular markers, and transcriptomic signatures (34, 37, 38, 117, 118). For example, in a human T cell atlas study using single-cell RNA-seq, new evidence was reported for regulatory T cell ontogeny indicating that, in fact, there are two populations of Treg progenitors with specific transcriptional signatures in the human thymus (118). The cell population called Treg$_{(diff)}$ showed lower expression of *FOXP3* and *CTLA4* when compared to conventional Tregs. Another cell subset had features similar to Treg$_{(diff)}$ but not to Tregs. This population was referred to as T$_{agonist}$ and presented low *FOXP3* expression but high expression of a non-coding RNA (*MIR155HG*). The definition of these two populations opens new paths to investigate the functional roles of these recently identified progenitors and the mechanisms that skew Treg development to one progenitor or another. By understanding these features, it will be possible to improve the understanding of the post-transplantation scenario when Tregs from transplanted CD34$^+$ are differentiated in the thymus.

Human Tregs and Teffs from peripheral blood as well as from mouse *Foxp3*$^{GFP}$ lymphoid organs were sorted and analyzed in a scRNA-seq screening (117). In both species, a similar transcriptomic profile (*FOXP3, IL2Ra, IL2Rb, IKZF2, TNFRSF1B)* was shown to distinguish Tregs from Teffs using an expression profile associated with cell ontogenesis, cell function, and metabolic processes. Notably, the intensity of TCR signaling strongly influenced the clusters of Treg cells, suggesting multiple differentiation states in the Treg pool (117). However, approximately 55% of the human Treg cell cluster overlapped with Teffs, indicating that *FOXP3*$^+$Tregs with a CD4$^+$CD25$^+$CD127$^{lo}$ phenotype comprise a heterogeneous population with certain cells expressing an effector transcriptomic profile. In a scRNA-seq analysis of CD4$^+$ T cells from pancreatic intragrafts of mice treated with CD47 monoclonal antibodies (mAb), two subpopulations of Tregs with low proliferative capacity and a distinct transcriptomic network were identified in rejected grafts (119). These results indicate that Treg heterogeneity is susceptible to changes in the microenvironment caused by, for instance, mAbs.

In a single-cell mass cytometry (CyTOF) study, human peripheral blood mononuclear cells (PBMCs) and isolated CD4$^+$

T cells were analyzed for their cell surface and intracellular markers. Unsupervised high-dimension clustering analysis identified new subsets, their phenotypes, and the relationship among these cell subpopulations (37). In another single-cell CyTOF analysis of liver-transplanted children, a panel with 22 markers identified a remarkable enrichment for non-classic Tregs ($CD4^+$ $CD5^+$ $CD25^+$ $CD38^{-/lo}$ $CD45RA^-$) in tolerant recipients compared to patients under IS (34). Specifically, CD5 has been shown as a marker to promote extrathymic Treg development in response to self or tolerizing agents in the periphery (120–122), while lack of CD45RA indicates a memory phenotype in kidney transplanted patients (123). This shows that these induced Tregs in the periphery can have high plasticity to immune responses (120, 122) and be generated in a tolerogenic environment. The identification of new cell subtypes in tolerant patients can define novel diagnostic markers that will benefit other SOTs.

Similarly, CyTOF analysis of sorted Tregs from healthy donors showed a heterogeneous population of naïve Tregs that failed to express markers commonly reported for conventional Tregs such as CCR4, CD39, HLA-DR, ICOS, and CD147 (38). Interestingly, hierarchical analysis of naïve Tregs using CD31, CD103, and LAP markers showed subpopulations carrying a preprogrammed status, suggesting a transient state between naïve and fully differentiated Tregs. Thus, the heterogeneous population of Tregs identified in tolerogenic liver-transplanted recipients may have transient states that can contribute to prolonged graft survival. Current studies in lineage tracing and pseudotime analysis of single-cell data will provide valuable information about the biological trajectory for regulatory T cell specification.

Recently, Miragaia et al. (124) compared Tregs from murine and human non-lymphoid tissues to identify a conserved transcriptional signature for peripheral Tregs that have travelled across tissues. Two subpopulations of transient Tregs were found with tissue-specific gene signatures that had adapted toward either skin or colon tissues (124). Many factors have been previously reported to induce, maintain, and attract Tregs to the colon, including dietary antigens and the microbiota (125). Tr1 cells have also been reported in gut-related autoimmune disorders. Tr1 cells generated and expanded *in vitro* specific for ovalbumin (OVA-specific Tr1) have been previously tested in Crohn's disease and colitis (106, 126). In a clinical trial with OVA-specific Tr1 clones, patients ingested OVA-enriched diets to stimulate OVA-specific Tr1 cell migration to the gut. This study reported a decrease in tissue inflammation up to five weeks post-treatment and OVA-specific Tr1 immunoregulatory function *ex vivo*. Moreover, Tr1 cells induced *in vitro* can express gut-homing markers GPR15 and CCR9 (41), and Tr1 cells induced *in vivo* have been found in tolerant mouse models (39), indicating the migratory capacity of Tr1 cells.

Previous reports have extensively discussed therapies using Tregs and Tr1 to control and prevent GvHD (127, 128). The understanding of key molecular features in Tregs and Tr1 will improve therapeutic approaches and clinical protocols to mitigate GvHD and promote allograft survival. Results from more recent cutting-edge technologies will provide new insights into T regulatory networks, cell function, cellular states and plasticity, cell migration markers, and cell expansion and survival. Altogether, these studies highlight the critical importance of taking precautions in expanding Tregs based on specific phenotypes because these cells can carry subpopulations with effector function that may negatively impact allograft survival and function.

## Regulatory B Cells

Recent studies have identified potentially important contributions to tolerogenesis from humoral immunity, a section of the adaptive immune response. B cells are at the core of humoral immunity and are responsible for clonally producing antibodies, but immune regulatory function is less understood for B cells than for Tregs. In the bone marrow, various cytokines, chemokines, and transcription factors regulate B cell differentiation from hematopoietic stem cells. Premature B cells travel from the bone marrow to the spleen and secondary lymphoid tissues to mature and differentiate under antigen-dependent and independent phases of selection. Similar to T cells, B cell receptor (BCR) is expressed *via* V(D)J rearrangement during maturation and selection. The combination of the antigen recognition by BCR and a co-stimulatory signal (e.g., helper T cell binding) stimulates B cell proliferation into either plasma cells responsible for secreting antibodies or memory cells that have a high survival rate, high antigen affinity, and fast secondary response.

Studies have previously reported that the regulation of humoral immunity through either conventional mechanisms of immune suppression or B cell immunomodulatory functions can be crucial for the success of allograft transplant (**Figure 1**). Early evidence was found in 1970 when, upon B cell depletion, guinea pigs suffered severe and prolonged contact hypersensitivity responses, indicating a suppressive role of B cells toward T cell responses (129, 130). B regulatory cells (Bregs) have mostly been characterized by their capacity to secrete IL-10 and TGF-β; which curtail T cell differentiation and cytotoxic function. Briefly, the mechanism wherein Bregs also modulate T and Natural Killer (NK) cell apoptosis is *via* the production of granzyme B and FasL (131, 132). In humans, Bregs are phenotypically subdivided into multiple subsets including transitional $TIM\text{-}1^+$ cells expressing IL-10 and memory/mature (**Table 1**) (44–46). In studies with human kidney allografts, the imbalance of IL-10/TNF-α expression in Breg cells was correlated with kidney injury (44). Additionally, tolerant recipients with complete eradication of the IS regimen showed elevated numbers of naïve, memory, and total B cells, upregulation in co-stimulatory and inhibitory molecules, and a genomic signature toward tolerogenesis (133, 134).

# ROLE OF INNATE IMMUNITY IN PROMOTING TOLERANCE

Despite its fundamental role in immune defense, innate immunity can also involve regulatory functions. Specific subsets of cell types involved in innate immunity can contribute to graft tolerance or rejection after SOT.

## γδ T Cells With Regulatory Properties

The classic identification of T lymphocytes involves the expression of either αβ TCR or γδ TCR (γδ T cells), although pro-inflammatory T cells bearing both receptors have been identified in mice and humans (135). Compared to αβ T cells, γδ T cells have less variability in the V and J gene segments, but γδ TCR have vast variation in the rearrangement of the D genes. Although other subsets have been identified within the γδ T population, Vδ1 and Vδ2 T cells remain the most studied subtypes. In humans, peripheral γδ T cells comprise up to 5% of the T cell population with Vδ2 as the major subset, but γδ T cells can rapidly expand in response to viral infections like human cytomegalovirus (CMV), inflammation, and tumors. Although Vδ1 T cells exist in the blood, they predominantly reside in the mucosal epithelia of solid tissues including the liver, skin, and intestines.

In comparison to αβ T cells, γδ T cells directly recognize antigens independent of MHC haplotype. The Vδ1 TCR binds to stress-induced proteins, such as MHC-I related chain A or B which are often found on tumorigenic cells and in post-SOT biopsies. The Vδ2 TCR recognizes small non-peptide phosphorylated antigens (pAg), which are intermediates of the mevalonate pathway in eukaryotes and in the non-mevalonate pathways in prokaryotes. For example, isopentenyl pyrophosphate (IPP) can accumulate in tumor cells carrying a defective mevalonate pathway. Mechanistically, members of the butyrophilin receptor family (e.g. BTN3A1) in either APC or tumor cells bind to IPP *via* intracellular domains and undergo conformational changes in the extracellular domains that are recognized by γδ TCR, leading to the activation of Vδ2 T cells (136). Recently, pAg-mediated coupling of BTN2A1 and BTN3A1 was suggested as the stimulatory trigger of Vδ2 T cells (137). Notably, γδ T cell subsets can recognize antigens *via* the expression of receptors commonly found on NK cells, such as NKG2D, DNAM-1, NKp30, and NKp44 (138).

Besides their anti-tumorigenic and anti-infectious role, γδ T cells can exert immune suppressive functions. In 1989, Patel et al. (139) reported regulatory properties of a specific subset of γδ T cells (γδ Tregs) involved in inhibiting mitomycin-activated $CD4^+$ T cell to activate B cell maturation *in vitro*. The phenotypic identification of γδ Tregs has mostly been based on findings from functional assays *in vitro* and expression of markers previously reported for conventional Tregs. Peripheral γδ T cells from healthy donors have no detectable levels of *FOXP3* but show low expression of CD25 and CTLA-4. However, under IL-2, IL-15, and/or TGF-β stimulation, γδ Tregs can express *FOXP3*, release IL-10 and TGF-β; and inhibit the effector function of previously activated $CD4^+$ T cells (47–51) (**Figure 1**). Recently, γδ Tregs expressing CD73 that secrete IL-10 and TGF-β were identified in both the periphery and tumors of patients diagnosed with advanced metastatic breast cancer (140).

The expression of co-stimulatory molecules (e.g. CD80, CD86) and inhibitory molecules (PD-L1) on Vδ2 T cells and results from transwell assays have provided evidence of the cell-to-cell contact dependency for γδ Tregs immune suppressive function (48). However, no consensus has been achieved regarding the cell culture method to expand and activate regulatory mechanisms in γδ T cells. The variations include γδ T isolation strategies prior to or after cytokine stimulation, different types of cytokines stimulation, co-culture with either PBMCs (without the removal of conventional Tregs pool) or selectively activated $CD4^+$ T cells, anti-TCR γδ for activation, and presence or absence of pAgs (52). Although the largest population of γδ T cells carrying regulatory features are Vδ1 T cells, few studies have compared the immune regulatory function of Vδ1 and Vδ2 T subsets, and a comprehensive analysis of the suppressive capacity of γδ T subsets has not been clarified.

γδ T cells reside in several tissues where they can exert immune suppressive functions. For example, patients with active celiac disease had reduced levels of TGF-β-expressing γδ T cells, but patients on a gluten-free diet benefited from γδ Treg expansion and abrogation of Teff response (141). Additionally, the expansion of peripheral Vδ1 T cells in pregnant women and the production of IL-10 and TGF-β by γδ T cells in the uterus can promote a suppressive environment that is likely necessary for fetal-maternal interface to avoid rejection early in pregnancy (142, 143).

## NK and NKT Cells

Besides γδ T cells, NK and natural killer T (NKT) cells compose innate immunity. NK cells are known for exterminating tumor and virus-infected cells, and the term NKT cells derives from these cells' similarities with both NK and T cells. Like NK cells, NKT cells express surface markers such as CD161. Like T cells, NKT cells differentiate and mature in the thymus and, phenotypically, can be $CD4^+$, $CD8^+$, or $CD4^-CD8^-$. Although $CD4^-CD8^-$ is indicative of immature T cells, activated NKTs with this phenotype are fully competent to produce cytokines (IL-4 and IFN-γ). In mice and humans, another marker shared among NK, NKT, T, and γδ T cells is promyelocytic leukemia zinc finger (PLZF). In mice, PLZF, together with GATA-3, RORγT, and T-bet, can stratify subpopulations of thymic NKT cells (144). Notably, NKTs have limited diversity in αβ TCRs, especially in humans (Vα24Jα18); NKT cells are activated when NKT TCRs detect glycolipid Ags presented by CD1d molecules on APCs. In mice, subpopulations of NKT cells in different maturation stages have been identified by the expression of NK1.1 (145, 146).

Although some NKT permanently localize to the thymus (147), a subset migrates to other tissues. The largest accumulation is in the liver where these cells make up approximately 30% of the T lymphocyte population (148). NKT subpopulations sensitive to IL-15 and positive for the transcription factor T-bet express chemokine receptors (e.g. CXCR3 and CXCR6) that bind to ligands produced in the liver (e.g., CXCL9, CXCL16) (149). In an IL-2-dependent manner, NKT cells recruit and trigger Tregs to tissues (**Figure 1**), indicating a regulatory function for NKTs that is also crucial for tolerance in coupled stem cell and solid organ transplants (150). Recently, Zhou et al. (151) focused on single-cell analysis of human peripheric NKT cells to characterize the transcriptomic signatures in NKT subpopulations. By evaluating the gene expression of specific cytokines, one NKT subset showed an immune regulatory profile comprising $IL\text{-}2^+$, $IL\text{-}10^+$, $ICOS^+$, $IL\text{-}4^-$, $IFN\text{-}\gamma^-$, and $XCL^-$. In cancer studies, a

$CD4^+$ NKT population was reported with immune modulatory function (152, 153). In the context of allogeneic HSCT, low levels of $CD4^+$ NKT cells were correlated with the development of chronic GvHD in patients that received grafts from BMT (154).

NK cells distinguish between autologous and allogeneic cells *via* inhibitory receptors present on the cell surface that identify self-antigens and prevent cell lysis. For example, at later stages of maturation, NK cells express killer immunoglobulin-like receptors (KIRs) that bind to classic MHC-I. A NK tolerogenic marker is the heterodimer CD94/NKG2A that specifically recognizes HLA-E and is expressed at the early stages of NK differentiation (155). KIRs and CD94/NKG2A can be co-expressed at intermediary stages of differentiation, but to avoid autoreactivity, mature NKs selectively express one or the other (156). In humans, the receptor NKG2D recognizes stress-related ligands MICA and MICB, triggering NK cell toxicity (157). However, NKG2D is not exclusive to NKs as it is also expressed by γδ T cells and NKT cells. Other cytotoxic-related receptors found in NK are Nkp30 and Nkp46 (158).

As in other immune subsets, human NKs are heterogeneous with subpopulations mostly distinguished by different expression levels of CD56 and CD16. Terminally mature NKs with a cytotoxic phenotype are $CD56^{dim}CD16^+$ and are the vast majority of circulating NKs in the periphery. These mature NKs also have higher expression of KIR or CD94/NKG2A. In two single-cell transcriptomic analyses of NK cells from peripheral blood and bone marrow of healthy donors (159, 160), $CD56^{dim}CD16^+$ were reported as heterogeneous with only one subset (also $CD57^+$) showing a singular transcriptomic profile of terminally different NKs (high expression of *CX3CR1*, *TIM-3*, and *ZEB-2)*. Conversely, $CD56^{bright}CD16^{-/low}$ have an immature state and express NKG2A, but KIR is absent in these cells. In pseudotime trajectory analysis to determine lineage specification, $CD56^{bright}CD16^{-/low}$ were found as precursors of $CD56^{dim}CD16^+$ based on their transcriptomic profile (160). A transitional state between immature and terminal NKs was also reported and indicated the following developmental trace: $CD56^{bright}CD16^{-/low}$ cells to $CD56^{dim}$ $CD57^-$ and then $CD56^{dim}$ $CD16^+$ $CD57^+$.

The subset of $CD56^{bright}CD16^{-/low}$ cells secrete IFN-γ and TNF-α but express low to no levels of perforin and granzyme B, indicating a regulatory profile ($CD56^{bright}$ NKreg) rather than a cytolytic role (**Figure 1**). However, prolonged stimulation with IL-2 and IL-5 can activate $CD56^{bright}$ cells to become cytolytic and differentiate into $CD56^{dim}$ in a mechanism mediated by the STAT3 signaling network (161). $CD56^{bright}$ NK cells have been identified in an immune suppressive environment, such as in the uterus and periphery of pregnant women, leading to high response against viral infections and tumorigenesis as well as positively affecting successful full-term pregnancies (162, 163).

## THERAPEUTIC STRATEGIES TO IMPROVE IMMUNE CELL TOLERANCE

While immunosuppression has contributed to substantial improvements in graft survival in SOT, investigators have recognized the need for other mechanisms to promote transplant tolerance in order to avoid the implications of long-term IS administration. The combination of HSCT and SOT is an important development, but numerous challenges remain in optimizing graft survival without GvHD, excess risk of infection, or lifelong need for IS drugs. Applying the growing understanding of cells involved in immune tolerance can improve HSCT and SOT as a therapeutic strategy and lead to enhanced long-term patient outcomes.

### Tolerance in HSCT and SOT

Since the early 90s, Strober and collaborators have sought to develop the combination of haplo-HSCT with kidney transplant. Chimerism, the coexistence of both donor and recipient hematopoietic cells, is a critical mechanism for promoting tolerance in this approach. Chimerism that persists for at least six months after transplant is associated with improved kidney graft tolerance and effective immune response to infection (28, 164). Both HLA-matched and mismatched HSCT with SOT can achieve chimerism, but persistent chimerism that is believed to promote tolerance has been achieved more frequently in HLA-matched recipients (25).

Busque et al. (25) reported that 24 of 29 HLA-matched transplant recipients with stable mixed chimerism for at least 6 months were able to discontinue IS drugs within 6-14 months with no cases of GvHD and only one case of graft loss. Ten patients had mixed chimerism that persisted after cessation of IS drugs. The remaining patients lost mixed chimerism without IS, but only one experienced graft rejection (2, 25, 165, 166), suggesting that durable operational tolerance may be induced by prior mixed chimerism (28).

In contrast, HLA-mismatched HSCT and kidney transplant recipients have typically needed chronic IS drugs to avoid graft rejection and GvHD (26, 27, 165). HLA-mismatched patients with mixed chimerism 12 months post-transplant were able to taper to one IS drug (tacrolimus), but full cessation resulted in loss of chimerism and evidence of graft rejection that required reinstatement of single-agent tacrolimus. HLA-mismatched patients who do not develop mixed chimerism that lasts beyond three weeks after transplant were prone to engraftment syndrome and associated graft injury that occurred despite continued IS (25). Another approach in HLA-mismatched kidney transplants has been to induce tolerance with full rather than mixed chimerism. While this approach enabled 22 of 37 patients to discontinue IS therapy, there were two cases of GvHD, one of which was fatal and the other chronic (25, 28, 167). Side effects of an intensive conditioning regimen in this approach led to severe neutropenia and thrombocytopenia post-transplant, and two patients experienced graft loss due to infection (25, 28).

Multiple therapeutic strategies to avoid these difficult tradeoffs have been proposed and are being evaluated in preclinical and clinical studies. A promising approach involves HSCT graft engineering that capitalizes on a deepening understanding of regulatory cells to cultivate tolerance independent of IS drugs without GvHD or excess infection risk.

## Graft Manipulation to Optimize Sequential HSCT and SOT

In the context of hematologic malignancies, HSCT graft manipulation techniques have shown clear benefits, and many of these approaches could be applied and enhanced to improve combined HSCT and SOT. A breakthrough approach in hematologic diseases reported that using G-CSF to mobilize HSCs and hematopoietic stem cell progenitors ($CD34^+$) from the bone marrow of the donor allows infusion of more $CD34^+$ cells. Subsequent studies selectively depleted T cells *ex vivo* for obtaining a $CD34^+$-enriched graft ($>10\times10^6$ cells/kg) and reported successful and prolonged engraftment in more than 90% of adult patients (168). However, slow immune reconstitution due to lymphocyte absence in the graft increased the susceptibility of these patients to lethal infection. With the discovery of Tregs and of their translational application, grafts enriched for $CD34^+$ and co-infused with Tregs with a fraction of conventional T cells were infused in 43 conditioned patients with acute leukemia (169). These patients received no subsequent IS and had successful engraftment, but 15% developed acute GvHD, likely from the Teff cells in the graft.

In 2010, our group pioneered αβhaplo-HSCT (170), a new approach that eliminates the αβ T cells and $CD19^+$ B cells from the graft. By removing the T cell subsets responsible for GvHD, this graft manipulation approach dramatically reduces the risk of severe acute and chronic GvHD (170, 171). Another benefit of this strategy is the presence of NK and γδ T cells in the graft that can immediately respond against infections, reducing patients' morbidity and mortality. In fact, despite the removal of αβ T cells, the presence of mature donor-derived effector cells provides anti-infectious control while minimizing the risk of severe acute GvHD (172, 173). In both malignant and non-malignant disorders, αβhaplo-HSCT recipients have experienced excellent clinical outcomes including rapid immune reconstitution, low risk of infections, and low incidence of graft failure (170, 171, 174–176). As a result, αβhaplo-HSCT represents a potentially ideal approach for inducing a tolerogenic environment that enables successful SOT (177).

## Regulatory T Cells

Encouraging preclinical and clinical studies of Treg and Tr1 cells in autoimmune and inflammatory diseases (178, 179) suggest that regulatory T cell infusion could improve outcomes of SOT. In 2016, Todo et al. (180) published data from the first clinical trial with Tregs and liver transplanted patients (**Table 2**). Seven patients showed signs of transplant tolerance and were weaned off IS drugs starting at 6 months after SOT with complete withdrawal within 18 months. However, the same strategy failed in kidney transplanted patients (186). Although the cells transferred to these patients also carried Teff cells, this clinical trial is considered the first pilot study in humans of a strategy to induce allograft tolerance using Treg infusion.

Building on this pilot study, investigators have started turning to modified strategies for therapeutic Treg infusions. Expansion of human Tregs for clinical applications opened opportunities for the treatment of unwanted immune responses such as in autoimmunity and after transplantation. The identification of markers for subpopulations of Tregs (187) is allowing the isolation and removal of non-Tregs from the remaining Treg populations as part of cellular therapies for allograft tolerance. Additionally, manipulation of specific subsets of Treg effector cells may enable refining their immune suppressive functions (188, 189).

The advances in next generation sequencing-based strategies have been extended to evaluating TCR repertoire diversity and antigen specificity (190). T cell populations have multiple TCR clones resulting from previous and current exposure to antigens. The understanding of the TCR composition reflects prior infections, immunizations, and individual response to specific epitopes. For the transplantation field, clinical trials have evaluated polyclonal and donor antigen reactive Tregs (**Table 2**) to determine their therapeutic ability to promote a tolerogenic environment. Although patients were still under an immunosuppressive regimen, the analysis of donor-specific TCR repertoire from Tregs cultured with activated donor B cells separated nontolerant from tolerant kidney-transplanted patients (191). Moreover, tracking donor-specific Tregs repertoire may provide insights into stratifying patients according to the likelihood of successfully withdrawing immunosuppression. Growing evidence suggests that disease-relevant and antigen-specific Tregs offer advantages over polyclonal Tregs (192, 193). Donor Tregs have demonstrated better suppressive function towards alloreactive effector T-cells when compared to polyclonal Tregs, which can affect the number and purity of infused cells (194, 195). While expanded $CD4^+CD25^+$ Tregs have been used in clinical trials (196) with promising results in preventing GvHD, they are polyclonal, nonspecific and could induce universal immunosuppression. As a result, ongoing or recently completed clinical trials are focusing on purifying Tregs with or without alloantigen specificity (**Table 2**; LITTMUS, ARTEMIS, dELTA).

To improve tolerance in SOT recipients, other investigators explored the role of transient mixed chimerism (26). Previous observations showed that mild conditioning regimens can induce transient chimerism and tolerance, but myelosuppression was still required (197–200). The Trex001 Study (**Table 2**) will test an immunotherapy strategy to induce transient chimerism while reducing myelosuppression to promote a tolerogenic environment and prevent kidney rejection (184).

In kidney transplant recipients (**Table 2**; TASK), results from follow-up biopsies after two weeks and six months post-Treg infusion showed that no patient had a negative reaction to the Tregs, and no infections were observed (181). Interestingly, circulating Tregs peaked two weeks post-infusion and then declined until untraceable three months post-infusion. The ONE Study (**Table 2**) is a multi-center consortium testing the safety and feasibility of multiple Treg infusion protocols in kidney transplant recipients. Although the immunosuppression regimen is consolidated among the centers (tacrolimus, mycophenolate, and steroids for three months), differences include the clonality, donor origin, frozen or fresh cells, and expansion with or without co-stimulation. In the ONEnTreg13 trial, the infused nTregs became oligoclonal over time, favoring

**TABLE 2 |** Brief summary of ongoing or completed clinical trials combining immune cell infusion with solid organ transplantation.

| Clinical trial name and/or ID | Phase | Cells infused | Concentration of cells infused | Organ transplanted | Time of cell infusion | Reported outcomes | References |
|---|---|---|---|---|---|---|---|
| UMIN-000015789 | I/II | $CD4^+CD25^+Foxp3^+$ Treg enriched cells | $23.30 \pm 14.38 \times 10^6$ | Liver | 13 days post-SOT | Positive signs of transplant tolerance<br>Complete withdrawal of IS within 18 months | (180) |
| LITTMUS (NCT03577431) | I/II | Donor alloantigen reactive $CD4^+CD25^+CD127^{lo}$ Treg | $2.5\text{-}125 \times 10^6$ cells | Liver | Combined with SOT | Ongoing | – |
| LITTMUS (NCT03654040) | I/II | Donor alloantigen reactive $CD4^+CD25^+CD127^{lo}$ Treg | $90\text{-}500 \times 10^6$ cells | Liver | Combined with SOT | Ongoing | – |
| ARTEMIS (NCT02474199) | I/II | Donor alloantigen reactive Tregs | $300\text{-}500 \times 10^6$ cells | Liver | 2-6 years post-SOT | Recently completed | – |
| dELTA (NCT02188719) | I/II | Donor alloantigen reactive Tregs | $50 \times 10^6$ cells | Liver | 3 months post-SOT | Recently completed | – |
| TASK (NCT02088931) | I | Autologous polyclonal $CD4^+CD25^+CD127^{low}$ Tregs | $224\text{-}384 \times 10^6$ cells | Kidney | 6 months post-SOT | No negative reaction to infused Tregs<br>No infections | (181) |
| ONE Study (NCT02091232) | I | Tregs | – | Kidney | 7 days post-SOT | Completed | (182) |
| ONE Study/ ONETreg1 (NCT02129881) | I/II | Autologous Tregs | $1\text{-}10 \times 10^6$ cells/kg | Kidney | 5 days post-SOT | Ongoing | – |
| ONE study/ ONEnTreg13 (NCT02371434) | I/II | Autologous, polyclonally expanded $CD4^+CD25^+Foxp3^+$ Tregs | $0.5 \times 10^6$ cells/kg or $1 \times 10^6$ cells/kg or $2.5\text{-}3 \times 10^6$ cells/kg | Kidney | Post-SOT | No rejection<br>Tapering of Immunosuppression drug for >70% of the patients<br>No infections | (183) |
| ONE Study/ darTREGs (NCT02244801) | I | Donor alloantigen reactive Treg | $300\times 10^6$ cells/kg | Kidney | Post-SOT | No rejection | (182) |
| Trex001 Study (NCT03867617) | I/II | Autologous *in vitro* expanded Tregs ($CD45RA^+CD4^+CD25^{high}CD127^{low/neg}$) | $0.3\text{-}1.5 \times 10^6$ cells/kg | Kidney | 3 days post-SOT | Ongoing | (184) |
| STEADFAST | I/IIa | Autologous Antigen-Specific CAR-Treg | $25 \times 10^6$ cells | Kidney | Post-SOT | Ongoing | – |
| TOL-1 (NCT02560220) | I | Peripheral Blood Mononuclear Cells (MICs) | $1.5 \times 10^6$ or $1.51\times 10^8$ MICs/kg | Kidney | 2 or 7 days before SOT | Persistent high frequencies of Bregs<br>No rejection | (185) |

specific TCR repertoires selectively to alloantigens and potentially helping a tolerant environment post-SOT (183). As the numbers of Tregs in circulation decreased after a month of infusion, the study hypothesized that these cells homed to the graft. Although the protocol of the multicenter ONE Study was considered safe, the infusion of nTregs was insufficient to completely remove the three IS drug treatment post-SOT (182). These results indicate the need for strategies to improve Treg cellular therapy.

Collectively, these completed and ongoing trials will offer valuable information about safety, therapeutic strategy, and the most suitable time point in which to infuse Tregs after SOT.

## Engineering Tregs

Gene therapy to engineer Tregs offers another intriguing approach for HSCT and SOT, and major advances in designing Treg cell therapies and various gene editing methods are comprehensively discussed by Ferreira et al. (201).

Antigen-specificity in regulatory T cells could be obtained through the TCR or gene transduction of a chimeric antigen receptor (CAR). HLA-A mismatching is one of the critical factors affecting graft outcome; therefore, targeting HLA-A *via* antigen-specific Tregs may be a promising method of inducing tolerance (202, 203). MacDonald et al. (195) generated CAR Tregs expressing an HLA-A2-specific CAR (A2-CAR), which maintained Treg phenotypes and stability and could suppress CD8 T cell proliferation *in vitro*. They demonstrated that CAR Tregs were more potent than Tregs expressing an irrelevant CAR in preventing GvHD in a xenogeneic mouse model receiving $HLA\text{-}A2^+$ human PBMC (195). Their results imply that the "off-target" effects of CAR-expressing Tregs is not different from the polyclonal Tregs. Nevertheless, Treg suppressive response is more likely to be induced *via* CAR than *via* TCR because it requires fewer target antigens. Moreover, the CAR Treg strategy allows for a lower number of Tregs that in turn decrease the off-target toxicity (195).

Dawson et al. (204) showed that the insertion of the wild type CD28 co-stimulatory domain is essential to the effective function of CAR Tregs *in vitro* and in an HLA-A2-mismatched xenoGvHD mouse model. Notably, RNA-seq analysis of CAR Tregs highlighted that stable expression of Helios and ability to suppress CD80 expression on DCs were major predictors of an effective *in vivo* performance (204). By incorporating in silico analysis, this comprehensive study showed that humanization of scFvs decreased cross-reactivity to several HLA-A allelic variants but could alter affinity and antigen specificity of CAR. This highlights the importance of testing multiple CARs to identify the optimal constructs. Determining allo-antigen specificity of Tregs is critical in the transplantation context to ensure precise targeting of allogeneic cells, tissues, and organs. Tregs expressing the optimal humanized A2-CARs showed rapid trafficking and persistence in HLA-A2-expressing allografts, migrated to draining lymph nodes, prevented HLA-$A2^+$ cell-mediated xenogeneic GvHD, and effectively suppressed rejection of human HLA-$A2^+$ skin allografts (205).

Adoptive transfer of A2-CAR Tregs was utilized to prevent rejection of human skin allograft in mice (202). A2-CAR Tregs potently suppressed the allogeneic responses of delayed-type hypersensitivity and prevented rejection of HLA-A2-positive human skin grafts for over 40 days, an effect attributed to A2-CAR Tregs homing to skin grafts and long-term persistence (202). In a similar study, CAR Tregs exhibited a greater suppressive function than ΔCAR Tregs (lacking CD28-CD3ζ domain) or polyclonal Tregs *in vitro* and ameliorated the alloimmune-mediated skin injury (203). These studies demonstrate that human CAR Tregs specific for HLA-A2 are more protective than polyclonal Tregs in humanized skin transplants. Altogether, these studies lay the foundation for developing HLA-specific CAR Tregs as adoptive cell therapy for autoimmune diseases and SOT.

To further extend CAR technology to Treg application in mice, Pierini et al. (206) showed that Tregs with transient expression of mAbCAR (engineered FITC-targeted-CARs activated with FITC-conjugated mAbs) promoted suppressive function once incubated with FITC-mAbs *in vitro* and *in vivo* and induced homing of mAbCAR Tregs to specific cells and organs (206) Adoptive transfer of mAbCAR Tregs reduced allograft responses such as GvHD, prolonged MHC-mismatched pancreatic islet allograft survival, and increased alloantigen-specific tolerance to secondary skin grafts (206). Although this strategy is promising, FITC could induce immunogenicity in humans, a limitation that can be resolved with the use of clinically safe antibody-tagged systems. Nevertheless, these findings highlight the flexibility of the mAbCAR Treg approach and suggest benefits in its application in transplantation to induce tolerance while controlling GvHD.

Although promising results were described with CAR Tregs in preclinical studies, there are several concerns surrounding the translation of these approaches to human HSCT and SOT. First, immune-deficient NSG mice lack the complexity of the human immune system, which may affect interpretation of data regarding tolerance and safety. Second, adoptively transferred CAR Tregs are only present at the initial phase after transplantation, which increases the chance of graft rejection (207). Third, obtaining clinically relevant numbers of CAR Tregs that can survive long-term in SOT patients is challenging. Indeed, IS drugs may reduce the number of CAR Tregs in liver and kidney transplants, which could impact CAR Treg efficacy. In 2019, Sangamo Therapeutics, Inc. (UK) started the STEADFAST clinical trial (**Table 2**) to evaluate CAR Treg therapy for the prevention of immune-mediated rejection following HLA-A2 mismatched kidney transplant in end-stage renal disease. This trial will soon provide information about the short-term safety and tolerability of CAR-Tregs as well as insights into the impact of CAR Tregs that can be incorporated into future SOT clinical trials.

## B Cell Strategies

In the allograft context, antibody-mediated rejection (AMR) is a leading cause of graft loss (208, 209). The activation of long-lived plasma cells and B cells releases donor-specific antibodies (DSA) that bind to the endothelium of the allograft. This binding triggers the recruitment of NK cells, neutrophils, and macrophages, leading to a series of inflammatory events, cytotoxicity, and cellular necrosis (210, 211). The outcome is severe endothelial injury, platelet aggregation, thrombotic microangiopathy, and the eventual loss of allograft function. For this reason, influencing B cell activity to facilitate tolerance can directly influence the success of SOT.

Importantly, the choice of initial IS regimen can influence the overall differentiation profile of B cells (212). This, in turn, can impact the variety and quantity of specific Bregs after SOT. For example, sirolimus significantly expanded Bregs and *FOXP3*$^+$ Tregs one month after liver transplant (213), but this effect was not observed for tacrolimus. Transcriptomic studies coupled with flow cytometry analysis have shown that Bregs express inhibitory/co-stimulatory molecules, such as PD-L1, CTLA-4/CD80, and CD86 (214–216), known to promote Treg function including dampening of Teff response. Although treatment with belatacept, a CTLA-4-immunoglobulin fusion protein, was first developed to target T cells, low levels of BAFF were detected in tolerant patients (217). The results from a 10-year follow-up trial showed that the numbers of Breg cells as well as *FOXP3*$^+$ Tregs were constitutively elevated in patients treated with belatacept (218). This provides evidence that the combination of strategies targeting multiple levels of immunosuppression can benefit transplant recipients.

In a phase I clinical trial, modified immune cells (MICs) were stimulated with the alkylating agent mitomycin C, resulting in immature donor-derived DCs with high immune modulatory capacity (**Table 2**; TOL-1) (185). Although these patients were under steroid regimen, circulating Bregs were present in high numbers one month post-transplant with a persistent and significant increase in Breg frequencies two years later. The allograft function was normal, and, as the patients showed unmodified levels of Tregs compared to pretransplant and pretreatment levels, the effectiveness of the treatment could be associated with the tolerogenic capacity of Bregs. Other therapeutic strategies targeting molecular regulators of Breg function, such as TIM-1, histone deacetylase, and the STAT3

network pathway, can have meaningful impact in inducing humoral-mediated immune suppression in tolerant allograft recipients (219).

Therapies with mAbs in kidney, liver, and heart recipients have shown efficacy in mitigating graft loss and improving long-term outcomes. In renal-transplant patients, *de novo* and increased pre-formed DSA correlated with high levels of the B cell survival factor, BAFF, and elevated rates of AMR (220). Concordantly, in a phase II clinical trial, treatment with an anti-BAFF mAb (belimumab) after SOT reduced the formation of *de novo* DSA, dampened the number of active memory B cells, and expanded Breg cells (221). In another study, treatment with alemtuzumab, an anti-CD25 antibody, correlated with good clinical outcomes including expansion of transitional Bregs one year after kidney transplant (222). In heart allograft recipients, a single dose of rituximab pre-transplant was sufficient to support B cell differentiation, but a second dose at 15 days post-transplant accelerated graft rejection and led to poor outcomes (223). Taken together, these results indicate that clinical protocols using mAbs can modulate the humoral response with potential benefits in transplant recipients, and further studies will provide optimization of drug choice, dosage, and timing.

## γδ T Cell Strategies

Preclinical and observational studies have reported γδ Treg function in SOT survival and homeostasis. In mouse models of kidney and liver transplant, the enrichment of peripheral $CD8^+$ γδ T cells was positively correlated with graft tolerance as these cells secreted suppressive cytokines (e.g. IL-10 and IL-4) toward Th1 responses (53). Moreover, IL-4 dampened Vδ2 T cell function and increased the IL-10-secreting Vδ1 T cell population (224).

The ability of γδ T cells to control viral infections is important in the transplantation setting as HCMV infection is a major complication in transplant recipients. Interestingly, enrichment of cytotoxic Vδ1 T cells with an effector memory phenotype has been found in αβhaplo-HSCT (225) and kidney recipients (226) positive for CMV. In fact, HCMV reactivation was resolved one year after kidney transplant in patients with elevated $V\delta2^-$ T cells. To amplify the benefits of γδ T cells, Vδ2 T cells from αβhaplo-HSCT recipients can be expanded under zoledronic acid (Zol) treatment *in vitro* whereupon these cells show an effector memory phenotype and aggressive cytolytic capacity against leukemia cells (225). In 43 pediatric leukemia patients transplanted with αβhaplo-HSCT, multiple Zol infusions were safe and improved overall survival, potentially due to the promotion of strong cytotoxicity against leukemia cells from Vδ2 T cells (227). In previous studies, Zol-activated Vδ2 T cells infused in patients with solid tumors reestablished a γδ T cell reservoir and halted cancer progression (228). Notably, γδ Treg cells can be induced *in vitro* under Concanavalin A treatment (47), indicating its potential function for γδ Treg expansion *ex vivo*.

In pediatric liver transplant recipients, the increase in Vδ1/Vδ2 could indicate successful long-term tolerance (229). Reduced incidence of GvHD was associated with increased levels of $CD27^+$ Vδ1 T cells in patients who received allogeneic HSCT (230). This study also reported that G-CSF can significantly increase donor γδ Tregs *in vivo* and *in vitro*, suggesting that the choice of mobilization agent can influence the immunosuppressive environment of the graft. However, a clear phenotype to identify and isolate γδ Tregs is still under investigation, which limits the understanding of the molecular mechanisms of suppression and the appreciation of findings in clinical tolerance. Despite an absence in the literature regarding approaches to engineer or expand γδ Tregs for clinical applications, other recently developed strategies for genetic modifications of γδ T cells may propel future studies in γδ Tregs (231).

## NK and NKT Cell Strategies

In light of the importance of NK and NKT cells for both immune defense and tolerance, these cells have a potentially impactful role in successful transplant outcomes. NK and NKT cell infusions to prevent GvHD are under preclinical investigation (127). In previous studies of post-HSCT recipients, expansion of NKT cell subpopulations positively correlated with GvHD mitigation (232). $CD56^{bright}$ NKreg cells positive for NKp46 have been found to be related to a low incidence of GvHD and have been used as a marker in clinical studies (58). In fact, NKp46 receptors are the drivers of NK response to eliminate HCMV-infected DCs. In clinical trials of chronic HCMV-infected patients who received liver transplants, IS administration was removed for half of the recipients (233). The tolerogenic environment was associated with the expansion of $CD8^+$ T cell expressing regulatory markers (CTLA-4, TIM-3, PD-1) and the upregulation of genes downstream of IFN-γ signaling (ISG15, IRF1/7/9), suggesting an immune response that includes NK cell mechanisms. Low levels of non-cytolytic NKregs were associated with chronic GvHD 100 days post-HSCT in HLA-matched recipients enrolled in the ABLE/PBMTC1202 study (58, 59). An ongoing clinical trial (NCT03605953) is testing the feasibility of expanding and injecting donor $CD4^-$ NKT cells post-allogeneic HSCT to promote graft versus leukemia (GvL) while reducing the risk of GvHD.

In the graft manipulation approach of αβhaplo-HSCT, donor NK cells ($30\text{-}40 \times 10^6$ per kg) were included in cell infusion for children with acute myeloid and acute lymphocytic leukemia (170, 176). To improve the clinical outcome in terms of GvHD and GvL, the infused NK cells were selectively chosen according to their alloreactivity based on KIR/KIR-ligand model, KIR B haplotype, size of NK alloreactive subset, and high expression of NKp46 and NKG2C (170, 234). While the NK alloreactivity was not observed to be crucial for the overall GvL effect, NK cells were believed to reduce GvHD and short-term infection risk.

NK cell alloreactivity plays a major role in SOT as the graft can be recognized by NKs under a "missing self" mechanism which potentially leads to graft rejection (235). KIR-HLA mismatch has been shown to negatively impact short- and long-term survival of kidney grafts (236). However, to date, no clinical consensus has been reached regarding the use of KIR-ligand as a predictive model for transplantation outcome because other NK receptors can also mediate alloreactivity and tolerance.

Solid organ transplant recipients may also benefit from infused or recently differentiated NKreg cells. In other clinical studies, immature NK cells ($CD56^{bright}NKG2A^{high}KIR^{low}$) derived from hematopoietic stem cell differentiation were identified in the first weeks post-HSCT (237–239). These results have important implications in the context of SOT as immature NK cells may carry an NKreg subpopulation that will offer a tolerogenic environment to improve graft survival. Moreover, understanding the peak of the noncytolytic NKregs pool, as well as of other regulatory cells, can offer the best timing of SOT post-HSCT. Further studies identifying NKT cell phenotype and function will provide valuable information for understanding the cytotoxic and regulatory role of NKTs subsets, especially in the context of HSCT and SOT.

In recent years, there has been substantial investigation to develop off-the-shelf products for cell therapy. Although the synthesis of CAR-NKs is challenging, CAR-NK cells have the potential to become universal therapies. Preclinical and clinical studies have shown promising safety and efficacy for CAR-NK in cancer immunotherapies and in reducing GvHD (240). To date, the feasibility of CAR NKreg tolerogenic potential for SOT has not been tested. Understanding the mechanisms and phenotypes of NKreg cells will enable development of targeting strategies using CAR or CRISPR-Cas9 gain-of-function to create a tolerogenic environment and reduce graft loss.

## CONCLUSION

The transplantation tolerance field has dramatically advanced over recent decades to improve organ engraftment and survival and abate the mortality and morbidity caused by IS. Despite major advances, widespread tolerance in SOT has not yet been achieved without dependence on IS regimens. Several preclinical studies have confirmed the feasibility for inducing transplantation tolerance; however, there remains a gap in translating these findings to the clinic.

One major challenge is represented by the variability in outcomes depending on the type of solid organ transplant. To prevent graft loss, immunosuppression regimens are proportional to the likelihood of graft rejection for specific organs. Allogeneic skin transplants are the most complex model of transplantation due to high immunogenicity and high numbers and varieties of APCs (241). Intestine transplants are also at high risk of rejection while heart, kidney, and liver transplants carry a lower risk. Given its function in metabolism and detoxification, the liver receives and processes large quantities of bacteria and dietary products and, accordingly, has a persistent, well-regulated immunoregulatory property. The benefit of low hepatic immunogenicity is to offer systemic immune tolerance and successful engraftment for a co-transplanted organ, such as liver and kidney co-transplants (242, 243). Mechanistically, liver-resident macrophages and hepatic myeloid and plasmacytoid DCs produce and secrete IL-10 and prostaglandins which reduce the expression of co-stimulatory receptors on APCs and compromise the activation of Teff cells (244–249). Myeloid populations provide additional regulatory mechanisms to prevent $CD4^+$ T cell activation *via* IL-10, TGF-β; and IDO (250). In preclinical studies of kidney and heart transplants, host DCs rapidly replace donor DCs within days post-transplant and are associated with graft rejection (251–253). The depletion of graft DCs was reported to delay ongoing acute rejection. Thus, the diversity in immunogenicity across tissues poses a challenge in predicting the outcome of SOTs that apply similar transplantation strategies.

Despite these difficulties, pairing allogeneic HSCT with SOT is a promising approach. Besides dramatically increasing the chance of finding a suitable donor for the organ transplant, combining allogeneic HSCT with SOT can positively impact allograft survival and overall clinical outcomes. More recently, modifications in HSCT and SOT protocols have successfully decreased or eliminated IS administration for select patients. Further improvements are needed to consolidate and expand these results. In HSCT, graft manipulations, such as αβhaplo-HSCT, have successfully minimized IS administration while contributing to the prolonged survival of pediatric and adult patients (170, 174, 254). A deeper understanding of regulatory and suppressive immune mechanisms has vast applicability in inducing tolerance in transplant patients and bringing αβhaplo-HSCT and other techniques into SOT. In the era of single-cell data, novel regulatory subsets have been more comprehensively studied in their transcriptomic, epigenomic, and immuno phenotypic profile, providing new avenues for amplifying immune tolerance. By recapitulating or increasing cellular regulatory networks, engineering strategies to manipulate immune cells *in vitro* for subsequent infusion can prolong tolerance post-HSCT which may offer a suitable window for SOT and allograft survival. Taken together, technological advances and ongoing clinical trials in these areas will appreciably change the field of transplantation tolerance.

## AUTHOR CONTRIBUTIONS

PFS and AB outlined and wrote the manuscript. PFS designed the figure and the tables. MY reviewed the literature and wrote about engineered CAR-Tregs. PFS, MY and AB edited and approved the final version of this manuscript. All authors contributed to the article and approved the submitted version.

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# Immunomonitoring of Stage IV Relapsed Neuroblastoma Patients Undergoing Haploidentical Hematopoietic Stem Cell Transplantation and Subsequent GD2 (ch14.18/CHO) Antibody Treatment

*Christian Martin Seitz[1,2], Tim Flaadt[1], Markus Mezger[1], Anne-Marie Lang[1,2], Sebastian Michaelis[1], Marie Katz[1], Desireé Syring[1], Alexander Joechner[1,3], Armin Rabsteyn[1,2], Nikolai Siebert[4], Sascha Troschke-Meurer[4], Maxi Zumpe[4], Holger N. Lode[4], Sile F. Yang[3], Daniel Atar[1], Anna-Sophia Mast[1], Sophia Scheuermann[1], Florian Heubach[1], Rupert Handgretinger[1,2], Peter Lang[1,2†] and Patrick Schlegel[1,2,3,5,6*†]*

[1] Department of Pediatric Hematology and Oncology, University Children's Hospital, Eberhard Karls University Tuebingen, Tuebingen, Germany, [2] Cluster of Excellence iFIT (EXC 2180) "Image-Guided and Functionally Instructed Tumor Therapies", University of Tuebingen, Tuebingen, Germany, [3] Cellular Cancer Therapeutics Unit, Children's Medical Research Institute, Westmead, NSW, Australia, [4] Department of Pediatric Hematology and Oncology, University Medicine Greifswald, Greifswald, Germany, [5] School of Medical Sciences, Faculty of Medicine and Health, University of Sydney, Sydney, NSW, Australia, [6] Department of Pediatric Hematology and Oncology, Westmead Children's Hospital, Westmead, NSW, Australia

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**Edited by:**
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***Correspondence:**
*Patrick Schlegel*
*patrick.schlegel@sydney.edu.au*

*[†]These authors have contributed equally to this work*

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Haploidentical stem cell transplantation (haplo SCT) in Stage IV neuroblastoma relapsed patients has been proven efficacious, while immunotherapy utilizing the anti-GD2 antibody dinutuximab beta has become a standard treatment for neuroblastoma. The combinatorial therapy of haplo SCT and dinutuximab may potentiate the efficacy of the immunotherapy. To gain further understanding of the synergistic effects, functional immunomonitoring was assessed during the clinical trial CH14.18 1021 Antibody and IL2 After haplo SCT in Children with Relapsed Neuroblastoma (NCT02258815). Rapid immune reconstitution of the lymphoid compartment was confirmed, with clinically relevant dinutuximab serum levels found in all patients over the course of treatment. Only one patient developed human anti-chimeric antibodies (HACAs). In-patient monitoring revealed highly functional NK cell posttransplant capable of antibody-dependent cellular cytotoxicity (ADCC). Degranulation of NK cell subsets revealed a significant response increased by dinutuximab. This was irrespective of the KIR receptor–ligand constellation within the NK subsets, defined by the major KIR receptors CD158a, CD158b, and CD158e. Moreover, complement-dependent cytotoxicity (CDC) was shown to be an extremely potent effector-cell independent mechanism of tumor cell lysis, with a clear positive correlation to GD2 expression on the cancer cells as well as to the dinutuximab concentrations. The *ex vivo* testing of patient-derived effector cells and the sera collected during dinutuximab therapy demonstrated both high functionality of the

newly established lymphoid immune compartment and provided confidence that the antibody dosing regimen was sufficient over the duration of the dinutuximab therapy (up to nine cycles in a 9-month period). During the course of the dinutuximab therapy, proinflammatory cytokines and markers (sIL2R, TNFa, IL6, and C reactive protein) were significantly elevated indicating a strong anti-GD2 immune response. No impact of FcGR polymorphism on event-free and overall survival was found. Collectively, this study has shown that in-patient functional immunomonitoring is feasible and valuable in contributing to the understanding of anti-cancer combinatorial treatments such as haplo SCT and antibody immunotherapy.

**Keywords: neuroblastoma, immunomonitoring immunotherapy, GD2 antibody therapy, haploidentical allogeneic stem cell transplantation, antibody-dependent cellular cytotoxicity, complement-dependent cytotoxicity**

## INTRODUCTION

Neuroblastoma is the most common pediatric extracranial solid cancer, accounting for 12% of childhood cancer deaths (1). It arises from cells of the sympathetic nervous system (2). In high-risk neuroblastoma, defined by the presence of metastatic diseases in children older than 12 or 18 months or the MYCN amplification (MNA) in patients of any age, the prognosis is especially dismal, with a 5-year survival of only 40% (3). The current standard therapy consists of a multimodal treatment approach that encompasses a surgical resection or a biopsy, an intensive course of high-dose chemotherapy (six cycles), and another surgical intervention with complete resection of the primary tumor if possible. In addition, tumors with diagnostic $^{123}$I-meta-iodobenzylguanidine ($^{123}$I-mIBG) uptake may receive a $^{131}$I-meta-iodobenzylguanidine ($^{131}$I-mIBG) targeted radiation therapy prior to subsequent autologous stem-cell rescue as consolidation therapy and isotretinoin for minimal residual disease (MRD) therapy (4–6). In recent years, immunotherapy has demonstrated promising clinical efficacy. Monoclonal antibodies (mAbs) against the disialoganglioside GD2, an antigen highly expressed on most neuroblastoma cells, with a much lower expression on physiological human tissues including neurons, skin melanocytes, and peripheral sensory nerve fibers, have been developed and intensively studied in clinical trials (7, 8). The chimeric antibody ch14.18, dinutuximab, in combination with granulocyte–macrophage colony-stimulating factor (GM-CSF) or interleukin-2 (IL2) has significantly improved the 2-year event-free survival by 20% and the overall survival by 10% compared to standard therapy in high-risk patients (9). Substantial toxicities observed included pain, fever, allergic reactions, and capillary leak syndrome, which were in part attributed to the use of GM-CSF and IL2. In Europe, ch14.18 was re-cloned in Chinese Hamster Ovary (CHO) cells and designated as ch14.18/CHO (dinutuximab beta) to reflect the molecular difference in the glycosylation pattern compared to ch14.18. In a multicenter, randomized, phase 3 trial (HR-NBL1/SIOPEN), dinutuximab beta has shown to improve the 5-year event-free survival rate by 15% and overall survival by 14% over a historic cohort (10, 11). The results of this trial contributed to the approval of dinutuximab beta in the European Union for the treatment of neuroblastoma. In contrast to MRD settings, the clinical activity of dinutuximab beta has only been demonstrated in combination with high dose IL2 against relapsed and refractory diseases (12), while the single agent activity of dinutuximab beta has not been addressed in clinical trials yet. We have demonstrated that antibody-dependent cell-mediated cytotoxicity (ADCC) by NK cells significantly contributes to the clinical activity and improved event-free survival in patients treated with dinutuximab beta (13). However, repetitive high-dose cytotoxic therapy and tumor-mediated immune editing may lead to dysfunctional immune cells, devoid of therapeutic activity of dinutuximab beta in heavily pretreated neuroblastoma patients. We have shown that haploidentical stem cell transplantation (haplo SCT) utilizing CD3/CD19 depleted G-CSF-mobilized peripheral blood stem cell grafts is a feasible strategy to establish a novel and functional cellular immune system (14). Notably, NK cells were demonstrated as the predominant immune cell population in the early phase of immune reconstitution after haplo SCT. Together, these findings led to the rationale to initiate the "phase I/II feasibility study using ch14.18/CHO antibody and subcutaneous interleukin 2 after haploidentical stem cell transplantation in children with relapsed neuroblastoma" (NCT02258815). Here, we report on the results of the immune monitoring and evaluate the cooperative activity of immune reconstitution and targeted redirection by dinutuximab beta.

## PATIENTS, MATERIALS, AND METHODS

### Ethical Statement

All procedures involving human participants were in accordance with the ethical standards of the institutional and national research committees, competent authorities, and the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The treatment was conducted according to the clinical trial "CH14.18 1021 Antibody and IL2 After Haplo SCT in Children With Relapsed Neuroblastoma" registered at ClinicalTrials.gov (NCT02258815). Informed consent was obtained from all individual participants or their parents or legal guardians.

## Cell Lines and Culturing Conditions

All cell lines including LAN-1 (ACC 655), LS (ACC 675), SK-N-AS (CRL2137), SH-SY5Y (CRL-2266) were purchased from ATCC or DSMZ (LS) and maintained in complete RPMI 1640 (Biochrom) media or DMEM (Gibco) supplemented with 10–20% of heat-inactivated fetal bovine serum (Biochrom), 2 mM L-glutamine, and 1 mM sodium pyruvate (Biochrom) according to cell culturing instructions. All media contained 100 units/ml of penicillin and 100 µg/ml of streptomycin (Biochrom).

## Flow Cytometry

### Monitoring of Lymphoid Immune Compartment and Activation State of NK Cells

For the calculation of absolute cell numbers per µl, differential blood counts of patients were measured on ADVIA®120-Siemens in the routine hematologic laboratory and calculated accordingly. Reconstitution of lymphocytes and the activation state of NK cells were monitored by flow cytometry using three different combinations of antibodies. BD Multitest™ T cells: $CD3^+$ FITC (clone SK7), $CD45^+$ PerCp (clone 2D1), $CD4^+$ APC (clone SK3), and $CD8^+$ PE (clone SK1). BD Multitest™ NK/B cells: $CD3^+$ FITC (clone SK7), $CD45^+$ PerCp (clone 2D1), $CD56/CD16^+$ PE (clones NCAM16.2/B73.1), $CD19^+$ APC (clone SJ25C1). Non-activated NK cells were defined as $CD56^+CD16^+CD69^-$; activated NK cells were identified by $CD56^+CD16^+CD69^+$ immunophenotype using the combination of $CD3^+$ FITC (clone SK7), $CD45^+$ PerCp (clone 2D1), $CD56/CD16^+$ PE (clones NCAM16.2/B73.1), and $CD69^+$ APC (clone FN50). Data acquisition was performed by the stem cell laboratory of the GMP facility (University of Tuebingen) using standard protocols. A total of >10,000 cell events were acquired on a BD FACSCalibur™ and analyzed by the CELLQuest™ software.

### CD107a-Based Degranulation Assay

The frequency of degranulation by NK cells within the PBMCs was quantitated by multi-parameter flow cytometry after 6 h incubation at 37°C and 5% $CO_2$ in a Heracell incubator. RPMI 1640 (Biochrom) cell culture media containing 10% FBS (Gibco), 2 mM L-glutamine (Biochrom) were used. Effector cells (PBMCs) cultivated without target cells were defined as a negative control for background activation. Cells stimulated with phorbol-12-myristate-13-acetate (PMA) at 200 ng/ml and ionomycin at 4 µM (Sigma) served as an internal positive control. In the testing conditions, PBMCs were stimulated by coincubation with indicated tumor cell lines with and without GD2-mAb ch14.18/CHO at 1 µg/ml.

CD107a-APC (clone H4A3) antibody (Biolegend) was added directly to the tubes. Monensin (Golgi-Stop, BD Biosciences) was added at a final concentration of 10 µg/ml. According to a standard protocol, PBMCs were stained with CD3 PerCP (SK7), CD56-PECy7 (clone HCD65), CD16 AF700 (clone 3G8), CD158b PE (clone DX27), and CD158e BV421 (clone DX9) (all these antibodies are from Biolegend) as well as CD158a FITC (clone HP-3E4, BD Biosciences) for 10 min at 4°C. After washing, cells were resuspended in 0.5% paraformaldehyde (Sigma) until multi-color flow cytometric analysis was performed on a LSRII instrument (BD Biosciences). A total of >50,000 cell events were acquired and analyzed using FlowJo 10.7.1 software.

### GD2 Antigen Expression Screening on Neuroblastoma Cell Lines

GD2 expression was measured on a BD™ LSR II flow cytometer using primary labeled GD2 PE (clone 14G2a) mAb and a mouse IgG2a, k PE mAb as isotype control. Antibody staining was done according to standard operating procedure at 4°C in PBS buffer. Staining of tumor cells using primary labeled mAbs compared to isotype control defined antigen positivity. The Median Fluorescence Intensity Ratio (MFIR) was calculated by MFI GD2 PE mAb divided by MFI IgG2a, k PE mAb. Data analyses were performed using FlowJo 10.7.1 software.

## Analysis of Antibody Serum-Levels

Validated detection of ch14.18/CHO in patient samples was performed using the triple-ELISA strategy (limit of detection in serum samples: 58 ng/ml ch14.18/CHO) as previously described (15, 16). The anti-idiotype mAb ganglidiomab (17) was used as capture mAb. Briefly, patient serum samples were first analyzed using the "low sensitivity" ELISA with a detection range of 3.0–25 µg/ml ch14.18/CHO. Then, samples containing ch14.18/CHO levels lower than 3 µg/ml were subjected to reanalysis with the "intermediate sensitivity" ELISA (detection range: 0.5–3.1 µg/ml). Finally, samples with ch14.18/CHO concentrations below 0.5 µg/ml were reanalyzed with the "high sensitivity" ELISA with a detection range of 0.058–1.0 µg/ml (18).

## Analysis of Human Anti-Chimeric Antibody

To analyze HACA development in patients treated with ch14.18/CHO, a validated ELISA allowing specific detection of anti-ch14.18/CHO Ab in patient serum was performed as previously described (19).

## Quantification of Cytokine Levels IL2, IL6, TNFα, And C-Reactive Protein in Patient Serum

For determination of secreted cytokines, patient samples were collected at indicated time points. The quantification was performed by the institute for clinical chemistry and laboratory medicine according to high-standard pharmaceutical protocols undergoing external validation.

## Analysis of NK-cell Activity Cytotoxicity

The cytolytic activity of patient PBMCs was analyzed in a 2h-DELFIA-EuTDA cytotoxicity assay (PerkinElmer/USA) according to the manufacturer's instructions. A PBMC to neuroblastoma cell line ratio (E:T ratio) 5:1 with or without GD2-mAb-ch14.18 at 1 µg/ml was used. Experiments were analyzed in triplicates using six replicate wells for maximum release (target cells treated for 20 s with ultrasonic homogenizer). The specific lysis was calculated according to the formula: (test release − negative control release)/(maximum release − negative control release) × 100%. The fluorescence intensities were measured on a VICTOR-II-multi-label-reader (Wallac/Finland) as described previously (20).

### Generation Lentiviral Vectors Encoding Firefly Luciferase-mCherry/GFP

Lentivirus (LV) was produced in Lenti-X$^{TM}$ 293T (Clontech) after lipofection (Lipofectamine 3000, Thermo Fisher) of a second generation packaging plasmid, a VSV-G envelope plasmid and the indicated transfer plasmid. LV containing supernatants were concentrated using the Lenti-X concentrator (TaKaRa) and cryopreserved.

### Generation of Luciferase Expressing Cell Lines

Transfer plasmids, based on a third generation lentiviral vector plasmid, containing firefly luciferase and mCherry or GFP were kindly provided by Irmela Jeremias, Helmholtz Center Munich, Germany (21). LV particles were generated as described above. Cell lines were transduced at a MOI of three. Transgene expression was confirmed by flow cytometry using the co-expressed fluorescent protein. Transduced cells were enriched by bulk fluorescence-activated cell sorting (FACS).

## Luciferase-Based Cytotoxicity Assay

Tumor cells were plated in RPMI 1640-based complete media (see above) at 50,000 cells per well in 96-well flat bottom white plates (Greiner bio one). Synthetic D-luciferin (Sigma Aldrich) was added at 4 µg/ml. Effector cells and/or patient sera were plated at an effector to target ratio (E:T) of 5:1. The total volume per well was 200 µl. GD2-mAb ch14.18/CHO was used at 1 µg/ml unless indicated otherwise. Plates were incubated in a HERAcell incubator (Heraeus) at 37°C, 95% humidity and 5% $CO_2$. Plates were measured using the Wallac Victor 1420 Multilabel Counter (Perkin Elmer) at 37°C after 24 h. Lysis was determined by the relative luminescence of the testing condition to a dilution series of target cells (100, 75, 50, 25, 10, and 0%) according to standard controls.

## Sample Collection for FcGR Polymorphisms and KIR Genotyping

EDTA-anticoagulated whole blood samples (5–10 ml) were collected from stem cell donors before haploidentical SCT at the University of Tuebingen, Germany. DNA was extracted using standard methodologies based on spin column technologies (Qiagen) and frozen at −20°C until further analysis.

## Analysis of FcGR Polymorphisms

The analysis of FcGR3A 158-F/V (rs396991) was carried out as proposed by Dall'Ozzo and colleagues (22). Briefly, 5 µl of Sybr Mix was added to 2 µl of PCR-grade water (Peqlab), 1 µl of genomic DNA [final concentration: 10 ng], and 1 µl of FcGR specific primers [each 5 pmol], respectively. After an initial denaturation step for 1 min at 95°C, 35 PCR cycles of 3 s at 95°C and 20 s at 59°C were run on the CFX96 Real-Time PCR Detection System (Biorad). By post-amplification melting curve analysis, the V allele (melting point 83°C) and F allele (melting point 88°C) were distinguished. Restriction fragment length polymorphism (RFLP) assays were applied to analyze FcGR2A 131-H/R (rs1801274) polymorphism (Jiang et al., 1996). A 366 bp region was amplified by GoTaq DNA Polymerase (Promega), digested with BstUI (H/H 343 bp and R/R 322 bp), and separated by agarose gel electrophoresis.

# STATISTICS

For statistical analysis GraphPad Prism 8.4.3 (GraphPad Software Inc., La Jolla/CA, USA) was used. For comparing two groups, the t-test was used. For comparing three or more groups, the one-way-ANOVA test and *post-hoc* Tukey were used. P-values below 0.05 were defined significant.

# RESULTS

In this study, we examined patients with histologically confirmed Stage IV neuroblastoma at relapse post standard therapies, who were treated between 2010 and 2017 in a prospective multicenter Phase I/II trial (NCT02258815) with a combination of haploidentical HSCT and consecutive GD2 dinutuximab beta (ch14.18) mAb therapy administered with IL-2. Conditioning regimen included fludarabine (40 mg/m²), thiotepa (10 mg/kg), melphalan (70 mg/m²) as well as anti-thymocyte globulin (ATG, Fresenius) 30 mg/kg on days −12 to −9. Grafts were T- and B-cell depleted by CD3 and CD19 *via* magnetic-activated cell sorting from G-CSF-mobilized apheresis from haploidentical donors, as previously described (14, 23). Mycophenolate mofetil (1,200 mg/m²/day) was applied as posttransplant GVHD-prophylaxis until day +30 if residual T cells in the graft exceeded $2.5 \times 10^4$/kg BW. GD2 mAb therapy was initiated between day +60 and day +180 posttransplant if patients showed no signs of GvHD and required no immunosuppressive medications. The protocol consisted of six consecutive 4-week cycles at 20 mg/m$^2$ dinutuximab beta (ch14.18/CHO), which was administered as a continuous intravenous infusion over a period of 8 h per day on the first 5 days of each cycle. IL2 (Aldesleukin) was administered during the cycles 4 to 6 on the days +6, +8 and +10 of the corresponding cycle at $1 \times 10^6$ IU/m²/d subcutaneously (s.c.), only in patients with no signs of severe acute GvHD (Grades 3–4) or extensive chronic GvHD. Clinical details will be described in a separate publication.

## Immune Reconstitution Post Haploidentical HSCT, Dinutuximab Beta Serum Levels, and the Development of Neutralizing Human Anti-Chimeric Antibodies

To assess the requirements for cooperative antitumoral immune activation, as envisioned in the study design, immune reconstitution as well as pharmacokinetics of dinutuximab beta was monitored. A total of n = 36 eligible patients were included in the analysis. Absolute cell counts per microliter blood (mean ± SEM) were calculated from flow cytometric frequencies (%) and total lymphoid cells derived from the patients' whole blood counts. Haploidentical HSCT was followed by rapid NK-cell reconstitution. The "NK cell wave" peaked at day +14

posttransplant with a median cell count of 413 (108 to 1,424) $CD56^+CD16^+$ cells/µl in the peripheral blood. T-, B- and NK-cell reconstitution was within the expected ranges for CD3/CD19 depleted grafts, with a median of 256 (34 to 923) $CD3^+$, 120 (13 to 396) $CD4^+$ and 140 (6 to 555) $CD8^+$ as well as 246 (61 to 771) $CD19^+$ cells/µl, and 423 (32 to 1,278) $CD56^+$ cells/µl at 6 months posttransplant and full recovery at the first year after haploidentical HSCT in most patients. The time point of T cells representing the main lymphoid population was reached at approximately day +150, as demonstrated in **Figure 1A** by absolute numbers (cells/µl) and % cell subsets for T cells and NK cells (**Figure 1A**).

Administration of dinutuximab beta resulted in sufficient serum levels, peaking at approximately day 5 of each cycle, after a total infusion of 100 $mg/m^2$ was completed. Serum levels were measured by triple-ELISAs as previously described (15). A minimum of 1 and a maximum of $n_{cycle\ 1} = 15$, $n_{cycle\ 3} = 14$, $n_{cycle\ 4} = 12$ and $n_{cycle\ 6} = 13$ patient sera were measured. The serum levels of ch14.18/CHO remained above the *ex vivo* evaluated effective concentration >1 µg/ml over the course of the protocol until all cycles were completed for cycles 1–6 (**Figure 1B**). As some patients even received nine cycles of dinutuximab beta, it is most likely that these patients achieved a clinically relevant serum concentration of ch14.18/CHO for a total of 9 months as an immunologic antitumor consolidation treatment posttransplant, which could strongly mediate both antibody-dependent cellular cytotoxicity (ADCC) as well as complement-dependent cytotoxicity (CDC).

Since dinutuximab beta is a chimeric mAb, patients were monitored for human anti-chimeric antibody (HACA), capable of neutralizing therapeutic activity of mAbs. Neutralizing HACA antibodies were measured by ELISAs as previously described (19). There were no detectable HACA prior to initiation of ch14.18/CHO treatment in all evaluated patients. Solely in one patient, indicated by red dots, HACAs were detected at relevant levels in cycle 3 at 8 µg/ml and cycle 4 at 2 µg/ml. No further data on the course of HACA is available for this patient. The following number of patients was tested during the indicated cycles— $n_{prior\ to\ cycle\ 1} = 32$, $n_{cycle\ 3} = 37$, $n_{cycle\ 4} = 36$, $n_{cycle\ 6} = 28$, $n_{cycle\ 9} = 14$ (**Figure 1C**).

## Elevation of Proinflammatory Cytokines and NK Cell Activation as a Consequence Of Dinutuximab Beta Immunotherapy

To demonstrate the functional activity of the newly established haploidentical immune system in combination with GD2 mAb therapy, biomarkers for inflammation and NK cell activation were monitored. Administration of dinutuximab beta resulted in a highly significant and temporally cohesive induction of the proinflammatory cytokines measured on day 1 prior to start of

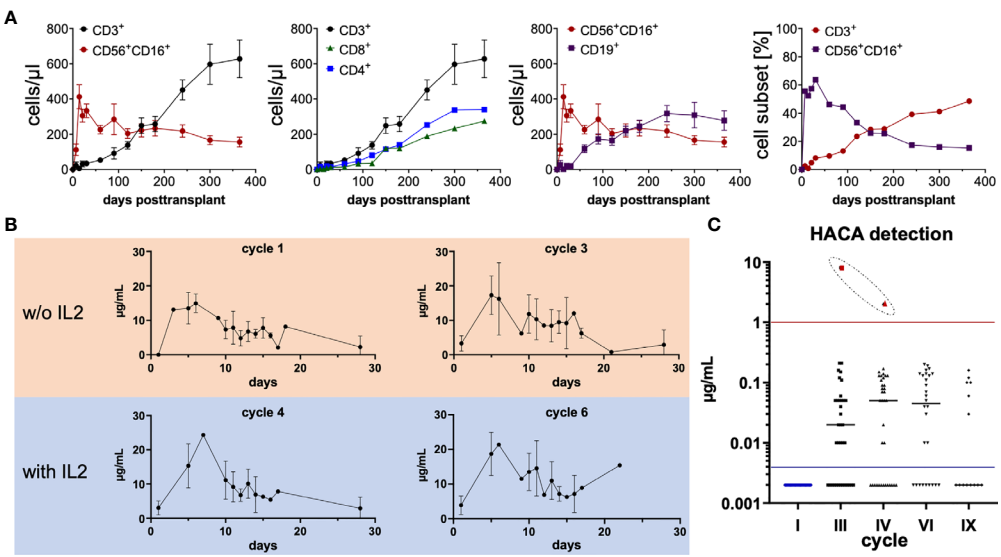


**FIGURE 1** | Lymphoid immune reconstitution posttransplant, ch14.18/CHO patient serum levels as well as serum levels of human anti-chimeric antibodies (HACA). **(A)** Posttransplant lymphoid immune reconstitution of $CD3^+$ T cells ($CD4^+$ and $CD8^+$), $CD19^+$ B cells, and $CD56^+CD16^+$ NK cells was assessed by flow cytometry at indicated time points. A total of n = 36 eligible patients were included in the analysis. Absolute cell counts per microliter blood (mean ± SEM) were calculated from flow cytometric frequencies (%) and total lymphoid cells derived from the whole blood counts of patients. In the right panel the mean frequencies [%] of $CD3^+$ and $CD56^+CD16^+$ are shown. **(B)** ch14.18/CHO patient serum levels at indicated time points during cycles 1, 3, 4, and 6 were measured by triple-ELISA as previously described (15). A minimum of one and a maximum of n = 15 in cycle 1, n = 14 in cycle 3, n = 12 in cycle 4, and n = 13 in cycle 6 serum of patients were measured. Basically in all ch14.18/CHO treatment cycles, a continuous relevant serum concentration above 1 µg/ml was sustained, facilitating a strong immunologic anti-tumor effect and consolidation by complement-dependent and cellular-dependent cytotoxicity throughout the course of antibody therapy (>6 months). **(C)** Neutralizing HACA antibodies were measured by ELISA as previously described (19). Only in one patient (red dots), HACAs were detected at relevant levels, indicated by the red horizontal line, in cycle 3 and cycle 4. No further data for this patient is available since no additional testing was performed in this patient afterwards. In cycle 1 n = 32, cycle 3 n = 37, cycle 4 n = 36, cycle 6 n = 28, cycle 9 n = 14 patients were evaluated. In order to display the 0 values in the logarithmic scale, 0 was substituted by 0.002 below the detection threshold (indicated by the blue horizontal line).

ch14.18/CHO infusion and on day 5 of any treatment cycle (**Figure 2**). In general, patient values for the assessed inflammatory markers were above normal values on day 5. The number of value pairs corresponded to the treatment cycles ranging from one up to a maximum of nine cycles per patient. The means of soluble IL2 receptors on day 1 ($IL2^{day\ 1}$) and on day 5 ($IL2^{day\ 5}$) were respectively 1,062 IU/ml (range 284–4,109) and 2,046 (range 11–7,406) [standard value 300–900 IU/mL] $n_{IL2}$ = 232 ($p < 0.0001$) (**Figure 2A**). The mean of $TNF\alpha^{day\ 1}$ was 13.3 pg/ml (range 1.8–34.6 pg/ml), *versus* $TNF\alpha^{day\ 5}$ at 18.5 (range 4–37.90 pg/ml) [standard value 0–25 IU/ml] $n_{TNF\alpha}$ = 127 ($p < 0.0001$) (**Figure 2B**). The mean of $IL6^{day\ 1}$ was 4.7 pg/ml (range 1.1–37.1 pg/ml) *versus* $IL6^{day\ 5}$ at 241 pg/ml (range 2.1–43,469 pg/ml) [standard value < 5 pg/ml] $n_{IL6}$ = 207 ($p < 0.0001$) (**Figure 2C**). The mean maximum CrP value per cycle was 8.2 mg/dl (range 0.1–39.71 mg/dl) [standard value < 0.5 mg/dl] $n_{CrP}$ = 198 ($p < 0.0001$) (**Figure 2D**). Analysis of NK cells freshly isolated from patient peripheral blood demonstrated a significant increase in the expression of the activation marker CD69 on NK cells during the course of treatment from day 1 to day 5 of dinutuximab beta ($n_{cylces\ 1–3}$ = 47, $p < 0.0001$; $n_{cylces\ 4–6}$ = 36, $p < 0.0001$). This increase was especially prominent in $CD16^+$ NK cells, indicative of stimulation *via* Fc-binding as a surrogate for active ADCC (**Figures 2E, F**). Statistical analyses were performed by a two-tailed paired t-test on the paired values of one patient.

## Comparison of Early (Cycles 1–3) *Versus* Later (Cycles 4–6) Dinutuximab Beta Treatment Cycles and the Impact Of Subcutaneous IL2 Application on Proinflammatory Cytokines and NK Cell Activation State

Notably, neither an increase in inflammatory cytokines including IL2, TNFα, IL6, CrP, nor an increase of NK-cell activation $CD69^+$ positivity was observed when comparing cycles 1 to 3 (without s.c. IL2 application) to cycles 4 to 6 (with additional s.c. IL2 application) as illustrated in **Figure 3**. For the comparison between the treating conditions without IL2 (cycles 1–3) and with IL2 s.c. application (cycles 4–6), the data from cycles 7–9 were excluded. The difference for IL2 (**Figure 3A**) on day 1 was not significant ($n_{cycles\ 1–3}$ = 62 *vs.* $n_{cycles\ 4–6}$ = 46, p = 0.17), but on day 5 the sILR level was significantly higher during cycles 1–3 without s.c. IL2 application ($n_{cycles\ 1–3}$ = 62/$n_{cycles\ 4–6}$ = 41, p = 0.0057). There was no difference in TNFα levels on day 1 ($n_{cycles\ 1–3}$ = 67/$n_{cycles\ 4–6}$ = 55, p = 0.71) or day 5 ($n_{cycles\ 1–3}$ = 63/$n_{cycles\ 4–6}$ = 47, p = 0.24) (**Figure 3B**). Further, no difference was found in IL6 levels (**Figure 3C**) on day 1 ($n_{cycles\ 1–3}$ = 129/$n_{cycles\ 4–6}$ = 97, p = 0.99) or on day 5 ($n_{cycles\ 1–3}$ = 125/$n_{cycles\ 4–6}$ = 86, p = 0.39). In line with higher sILR levels in cycles 1–3 compared to cycles 4–6, there was a significant increase in CrP ($n_{cycles\ 1–3}$ = 99, $n_{cycles\ 4–6}$ = 99, $p < 0.0001$) (**Figure 3D**). NK cell

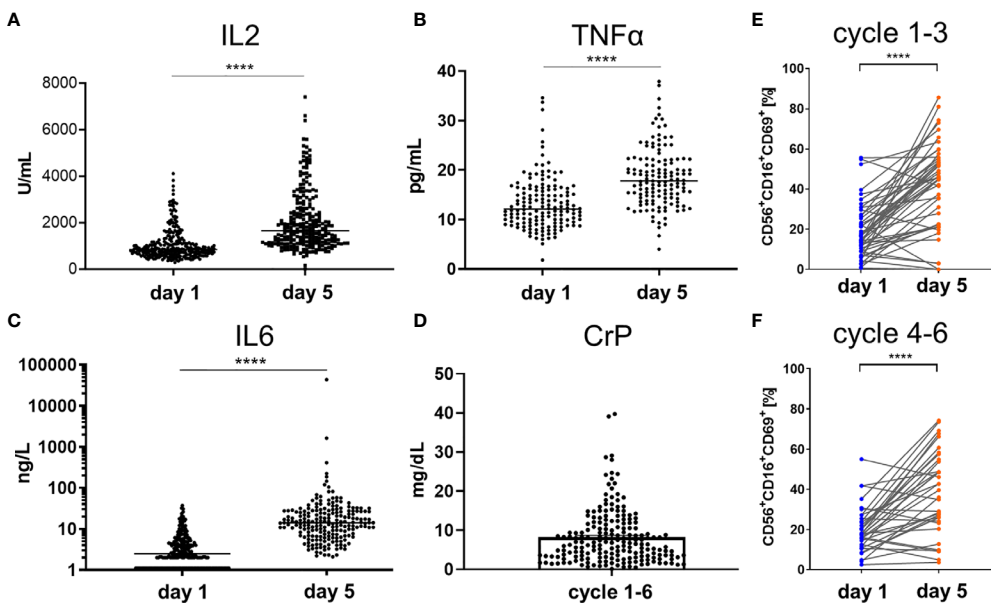


**FIGURE 2** | Elevation of proinflammatory cytokines and activation of NK cells by ch14.18/CHO antibody infusion therapy. The infusion of ch14.18/CHO induced robust immune response measured by means of secretion of proinflammatory cytokines **(A)** IL2, **(B)** TNFα, **(C)** IL6, and **(D)** CrP in serum of patients or lithium heparin plasma as well as by the percentage increase in number of activated NK cells **(E, F)**. Cytokines and NK cells were measured on day 1 prior to the start of antibody infusion and on day 5 of cycles 1 to 9. The maximum level of CrP per cycle is shown for the cycles 1–6 in **(D)**. Every single dot represents an independent single value per cycle and patient used from cycles 1–6 **(A–D)** but as indicated in **(E)** cycles 1–3 and **(F)** cycles 4–6. For the comparison of **(A)** IL2 n = 232, **(B)** TNFα n = 137, **(C)** IL6 n = 207 was available in pair values and for **(D)** CrP n = 198 single values were used. NK cell immunophenotype was assessed by flow cytometry and was defined as the $CD56^+CD16^+$ and $CD3^-$ subset of lymphoid cells. The early activation marker CD69 was used to distinguish resting ($CD69^-$) from activated ($CD69^+$) NK cells $n_{cylces\ 1–3}$ = 47, $n_{cylces\ 4–6}$ = 36. Statistical analysis was done by two-tailed paired t-test. P-values below 0.05 were defined significant. **** = <0.0001.

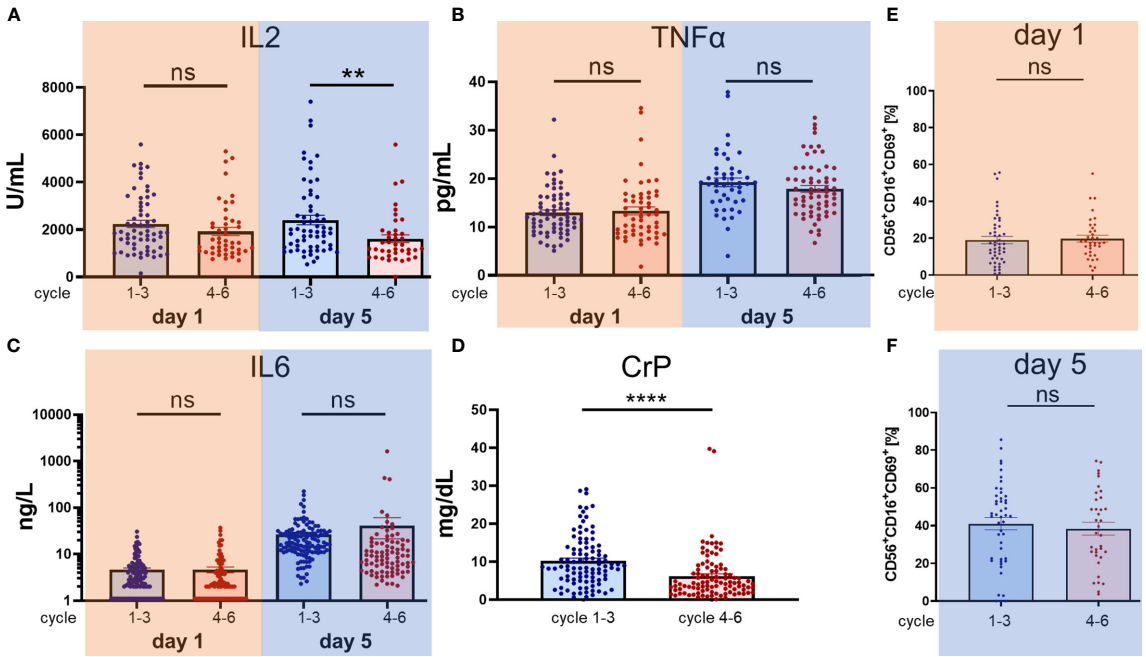


**FIGURE 3** | Comparison of cytokine secretion and activation of NK cells during cycles 1–3 without IL2 administration and cycles 4–6 with low dose subcutaneous IL2 administration. In contrast to cycles 1–3 starting as early as 60 days post haploidentical HSCT, in cycles 4–6 at days 6, 8, and 10 subcutaneous IL2 is administered at 1E06 IU/m$^2$/day. Consequently, we performed a systematic analysis and comparison of cytokine secretion and the activation of NK cells between the cycles 1–3 *versus* the cycles 4–6. We compared the levels of the proinflammatory cytokines **(A)** IL2, **(B)** TNFα, **(C)** IL6 and **(D)** CrP in serum of patients or lithium heparin plasma and the percentage increase in number of activated NK cells **(E, F)**. Every single dot represents an independent single value per cycle and patient used from cycles 1–6 **(A–D)** but as indicated in **(E)** cycles 1–3 and **(F)** cycles 4–6. For the comparison of **(A)** IL2 day 1 $n_{cycles\ 1–3} = 62/n_{cycles\ 4–6} = 46$, day 5 $n_{cycles\ 1–3} = 62/n_{cycles\ 4–6} = 41$, **(B)** TNFα day 1 $n_{cycles\ 1–3} = 67/n_{cycles\ 4–6} = 55$, day 5 $n_{cycles\ 1–3} = 63/n_{cycles\ 4–6} = 47$, **(C)** IL6 day 1 $n_{cycles\ 1–3} = 129/n_{cycles\ 4–6} = 97$, day 5 $n_{cycles\ 1–3} = 125/n_{cycles\ 4–6} = 86$ was available in pair values and for **(D)** CrP n = 99 single values were used. NK cell immunophenotype was assessed by flow cytometry and was defined as the $CD56^+CD16^+$ and $CD3^-$ subset of lymphoid cells. The early activation marker CD69 was used to distinguish resting ($CD69^-$) from activated ($CD69^+$) NK cells, day 1 and 5 $n_{cycles\ 1–3} = 47$, $n_{cycles\ 4–6} = 36$, day 1 p = 0.78, day 5 p = 0.58. Statistical analysis was done by two-tailed unpaired t-test. P-values below 0.05 were defined significant. ** = <0.01, **** = <0.0001. ns, not significant.

immunophenotype was assessed by flow cytometry and was defined as the $CD56^+CD16^+$ and $CD3^-$ subset of lymphoid cells (**Figures 3E, F**). The early activation marker CD69 was used to distinguish resting ($CD69^-$) from activated ($CD69^+$) NK cells, with days 1 and 5 $n_{cycle\ 1–3} = 47$, $n_{cycles\ 4–6} = 36$, day 1 p = 0.78, day 5 p = 0.58. Statistical analysis was done by two-tailed unpaired t-tests for IL2, TNFα, IL6, and NK activation marker $CD69^+$. Only the daily evaluated laboratory marker CrP was available in a complete data set to perform a paired analysis using the paired t-tests. P-values below 0.05 were defined as significant.

## Evaluation of NK Cell Mediated ADCC and CDC Utilizing Dinutuximab Beta

### Degranulation of NK Cells (CD107a Assay)

NK cell degranulation and cytolytic activity are shown in **Figure 4**. To prove the specific cytolytic activity of NK cells recruited to tumor cells by dinutuximab beta as a result of ADCC and CDC, patient derived-PBMCs acquired post haploidentical HSCT and patient sera post dinutuximab beta infusion and the condition 1 µg/ml ch14.18/CHO were analyzed *ex vivo*. Specific activities against two established neuroblastoma cell lines LS and LAN-1 were assessed. Patient-derived PBMCs were co-cultured with target cells in the presence of patient serum or dinutuximab beta at 1 µg/ml. First, degranulation, measured by CD107a (LAMP-1) positivity as a strong indicator of NK cell activation and cytolysis, by patient-derived PBMCs in the presence of patient serum or dinutuximab beta was analyzed in a 6 h flow cytometric based kill assay. The results demonstrated significant increases in cytolysis in the conditions with GD2 antibody in the presence of patient serum and 1 µg/ml ch14.18/CHO for the cell line LAN-1, but only in the condition with 1 µg/ml ch14.18/CHO for the cell line LS [**Figures 4A–D** n = 12 independent experiments and donors; $p_{LAN-1} = 0.0112$ (without mAb *versus* serum), $p_{LAN-1} < 0.0001$ (1 µg/ml ch14.18/CHO *versus* serum *versus* without mAb). $p_{LS} > 0.99$ (without mAb *versus* serum), $p_{LS} < 0.0001$ (1 µg/ml ch14.18/CHO *versus* without mAb and *versus* serum)] (**Figures 4A, B**).

Moreover, the single inhibitory killer cell immunoglobulin-like receptor (KIR) positive NK cell subsets CD158a/KIR2DL1, CD159b/KIR2DL2, and CD158e/KIR3DL1 were compared with regard to the overall degranulation capacity to address the impact of the KIR receptor ligand model (KIR R/L) in neuroblastoma. The comparison of NK subsets *versus* LAN-1 revealed no significant differences within the groups ($p_{w/o\ mAb} = 0.60$, $p_{serum} = 0.93$, $p_{ch14.18} = 0.08$) (**Figure 4C**). In **Figure 4D**,

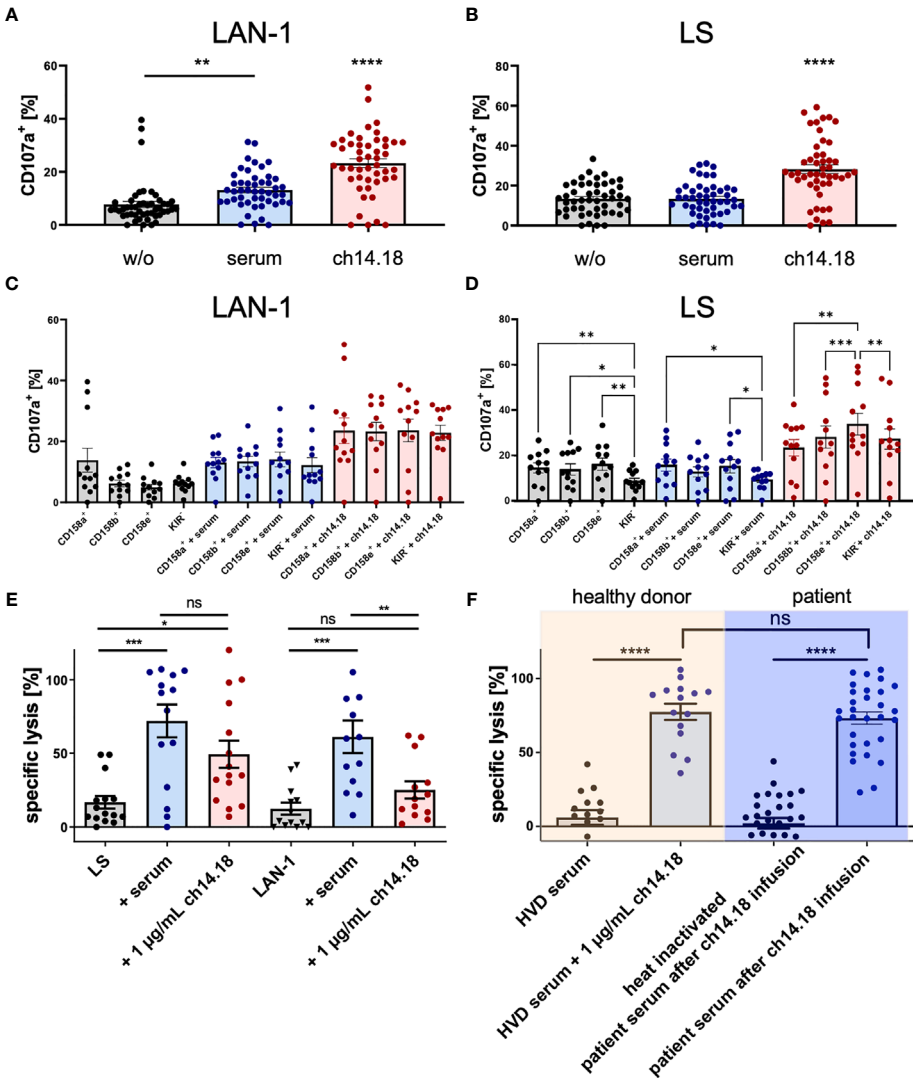


**FIGURE 4** | Degranulation of patient NK cells and cytolysis mediated by ch14.18/CHO. Patient PBMCs acquired post haploidentical HSCT were used to test the capacity of degranulation, measuring CD107a (LAMP-1) expression as a strong indicator of NK cell activation and cytolysis targeting the neuroblastoma cell lines LAN-1 and LS in a 6 h flow cytometry based kill assay **(A–D)**. Besides testing the overall degranulation capacity, NK cell subsets distinguished by the major inhibitory killer cell immunoglobulin-like receptors (KIR) CD158a/KIR2DL1, CD159b/KIR2DL2 and CD158e/KIR3DL1 were analyzed for interpopulation differences in CD107a positivity and were evaluated in the context of the KIR receptor ligand model. Additionally, a 2 h-DELFIA-EuTDA release cytotoxicity assay was used to evaluate the cytolysis of tumor cells by patient PBMCs *via* ADCC and CDC **(E, F)**. The testing conditions **(E)** comprised I) PBMCs *versus* tumor, II) PBMCs *versus* tumor and patient serum (after infusion of ch14.18/CHO), and III) PBMCs *versus* tumor and 1 µg/ml ch14.18/CHO. Moreover, cytolysis of the neuroblastoma cell line LAN-1 by PBMCs from healthy volunteer donors (HVDs) compared to patient PBMCs was assessed. **(F)** The comparison included the conditions I) [HVD PBMCs plus HVD serum] *versus* tumor, II) [HVD PBMCs plus HVD serum plus 1 µg/ml ch14.18/CHO] *versus* tumor, III) [patient PBMCs plus heat inactivated patient serum] *versus* tumor, and IV) [patient PBMCs plus patient serum] *versus* tumor. Data shown in **(A–D)** represent mean of (n = 12) independent experiments and different donors in triplicates, respectively. Data shown in **(E)** represent mean of ($n_{LAN-1}$ = 15; $n_{LS}$ = 13) and **(F)** represent single values of ($n_{HVD}$ = 5; $n_{patients}$ = 10) independent experiments and different donors in triplicates, respectively. Statistical significance was determined by one-way ANOVA and Tukey *post-hoc* test. P-values below 0.05 were defined significant. * = <0.05, ** = <0.01, *** = <0.0001, **** = <0.0001. ns, not significant.

thecomparison of NK subsets *versus* LS was illustrated with a significant difference found—$p_{w/o\ mAb}$ = 0.0006 (in the *post-hoc* test $p_{CD158a+\ vs.\ KIR-}$ = 0.0092, $p_{CD158b+\ vs.\ KIR-}$ = 0.39, $p_{CD158e+\ vs.\ KIR-}$ = 0.0068), $p_{serum}$ = 0.0028 (in the *post-hoc* test $p_{CD158a+\ vs.\ KIR-}$ = 0.0131, $p_{CD158e+\ vs.\ KIR-}$ = 0.0489), $p_{ch14.18}$ = 0.0004 (in the *post-hoc* test $p_{CD158a+\ vs.\ CD158e+}$ = 0.003, $p_{CD158b+\ vs.\ CD158e+}$ = 0.0004, $p_{CD158e+\ vs.\ KIR-}$ = 0.0082).

## EuTDA Release Cytotoxicity Assay

Additionally, a 2 h-DELFIA-EuTDA release cytotoxicity assay was used to evaluate the cytolysis of patient PBMCs (**Figures 4E, F**). The testing conditions comprised: I) PBMCs *versus* tumor, II) PBMCs *versus* tumor and patient serum (after infusion of ch14.18/CHO), and III) PBMCs *versus* tumor and 1 µg/ml ch14.18/CHO. Moreover, cytolysis of the neuroblastoma cell

line LAN-1 by PBMCs from healthy volunteer donors (HVDs) compared to PBMCs of patients was assessed. In contrast to degranulation, as a marker for NK-cell activation, patient serum and the condition with 1 µg/ml ch14.18/CHO mediated significantly higher lysis with patient-derived PBMCs compared to the condition without mAb ($p = 0.0002$). The condition patient serum *versus* 1 µg/ml ch14.18/CHO indicated a trend (ns) that complement-dependent cytotoxicity (CDC) contributes to total cell lysis (**Figure 4E**). To further substantiate this observation, specific lysis mediated by patient sera, acquired post dinutuximab beta infusion, was compared to serum of untreated healthy volunteer donors, untouched or substituted with dinutuximab beta, and heat inactivated patient serum. Clearly, specific lyses in the absence of effector cells requires both dinutuximab beta and a functional complement system ($p = 0.0001$), demonstrating CDC as a substantial mechanism of dinutuximab beta-mediated activity (**Figure 4F**). Data shown in **Figures 4A–D** represent mean of ($n = 12$) independent experiments and different donors in triplicates, respectively. Data shown in **Figure 4E** represent mean of ($n_{LAN-1} = 15$; $n_{LS} = 13$) and **Figure 4F** represent single values of ($n_{HVD} = 5$; $n_{patients} = 10$) independent experiments and different donors in triplicates, respectively. Statistical significance was determined by one-way ANOVA and Tukey *post-hoc* test.

## Impact of GD2 Expression on Dinutuximab Beta-Mediated ADCC and CDC

GD2 expression on neuroblastoma tumors may vary individually from patient to patient. To further decipher the contribution of ADCC and CDC to target-antigen specific antitumoral activity, patient-derived PBMCs and sera were studied in 24 h luciferase-based cytotoxicity assays (LCAs) using neuroblastoma cell lines with high (LAN-1 and LS) and low or absent (SK-N-AS and SH-SY5Y) GD2 expression (**Figure 5A**). GD2 expression was measured by flow cytometry using primary labeled GD2 PE (clone 14G2a) mAb and a mouse IgG2a, k PE mAb as isotype control. The median fluorescence intensity (MFI) was used to calculate the MFI ratio (MFIR) by the MFI GD2 PE mAb divided by MFI isotype control IgG2a, k PE mAb. The relative GD2 expression was MFIR LAN-1 = 112.9 > MFIR LS = 46.8 > MFIR SK-N-AS = 1.3 > MFIR SH-SY5Y = 1.

Patient-derived PBMCs acquired post haploidentical HSCT were used to test the direct cellular cytotoxicity and ADCC at an effector to target ratio E:T 5:1. Specific lysis was assessed in the conditions I) PBMCs without mAb *versus* tumor, II) PBMCs plus patient serum *versus* tumor, III) PBMCs plus 1 µg/ml ch14.18/CHO mAb *versus* tumor. In **Figure 5B** PBMCs mediated significantly improved target cell lysis in the presence of dinutuximab beta and patient sera; post dinutuximab beta infusion strictly depended on target-antigen expression ($n = 15$, $p_{LAN-1} < 0.0001$, $p_{LS} < 0.01$, $p_{SK-N-AS} < 0.05$, $p_{SH-SY5Y} < 0.005$). The same antigen specificity was found for CDC (**Figure 5C**). CDC was tested in a coincubation experiment of human serum from healthy volunteer donors (HVDs) at different concentration levels of added ch14.18/CHO mAb or heat-inactivated serum of patients or serum of patients *versus* the indicated neuroblastoma cell lines *without* adding any effector cells. ch14.18/CHO antibody titration experiments demonstrated a threshold for CDC induction *ex vivo* at levels of dinutuximab beta approximately 500 ng/ml in the presence of human serum. Importantly, serum levels of dinutuximab beta in patients were more than two log-fold higher during treatment cycles, indicating highly sufficient conditions for CDC in patients, functionally confirmed by patient sera-mediated lysis. Again, heat inactivation prevented effector cell lysis by patient sera, confirming CDC activity. Data shown in (**Figure 5C**) represent ($n = 3$) independent experiments of ($n = 15$) different donors. Statistical significance was determined by one-way ANOVA and Tukey *post-hoc* test. ADCC is antibody-dependent cellular cytotoxicity, and CDC is complement-dependent cytotoxicity.

## Impact of Fc$\gamma$ Receptor Polymorphisms on Patient Outcome

In addition to functional patient monitoring, the relevance of genetic preconditions with regard to polymorphisms in the Fc-gamma-receptor (FCGR2A and FCGR3A) genes were studied in the haploidentical transplant setting. Among the 33 donors analyzed, n = 9 (27%) were VV homozygous, n = 10 (30%) were VF, and n = 14 (42%) were FF homozygous for rs396991 (FcGR3A). Analysis of rs1801274 (FcGR2A) revealed n = 5 patients (15%) homozygous for HH, n = 24 (73%) heterozygous for HR, and n = 4 (12%) homozygous for RR. These data are in line with the expected genotype frequencies for European individuals available at NCBI (HapMap-CEU). Patients homozygous for low-affinity polymorphisms were assigned to the low-affinity cohort; patients homozygous for high-affinity polymorphisms or heterozygous were assigned to the high-affinity cohort as previously described (13). Studying the impact of polymorphisms rs1801274 and rs396991 in *FCGR2A* and *-3A* genes on EFS and OS, no statistical association was found, respectively. No association of Fc-gamma-receptor polymorphism and KIR content on survival was found.

# DISCUSSION

Haploidentical HSCT has evolved from being a well-acknowledged treatment procedure in high-risk leukemias in need of an allogeneic stem cell transplantation (23–25) to being an efficacious treatment for Stage IV relapsed high-risk neuroblastoma patients. Long-term remission can be achieved in a proportion of patients with a tolerable side effect profile (14). In case of *ex vivo* graft manipulation procedures (enrichment of stem cells or T-cell depletion), NK cells have been shown to rapidly reconstitute and contribute to the reduced relapse rates in the early posttransplant period (26–28). Further, the combination of the chimeric antibody ch14.18 dinutuximab with the granulocyte–macrophage colony-stimulating factor (GM-CSF) or interleukin-2 (IL2) has demonstrated significantly improved 2-year event-free survival in high-risk neuroblastoma patients (9). In order to maximize the treatment effects, the combination of haplo HSCT and GD2 antibody therapy in neuroblastoma has been evaluated in the clinical trial (NCT02258815) registered at (clinicaltrials.gov).

Here, we report on the immunomonitoring results of our phase I/II study and evaluate the feasibility of haplo HSCT in

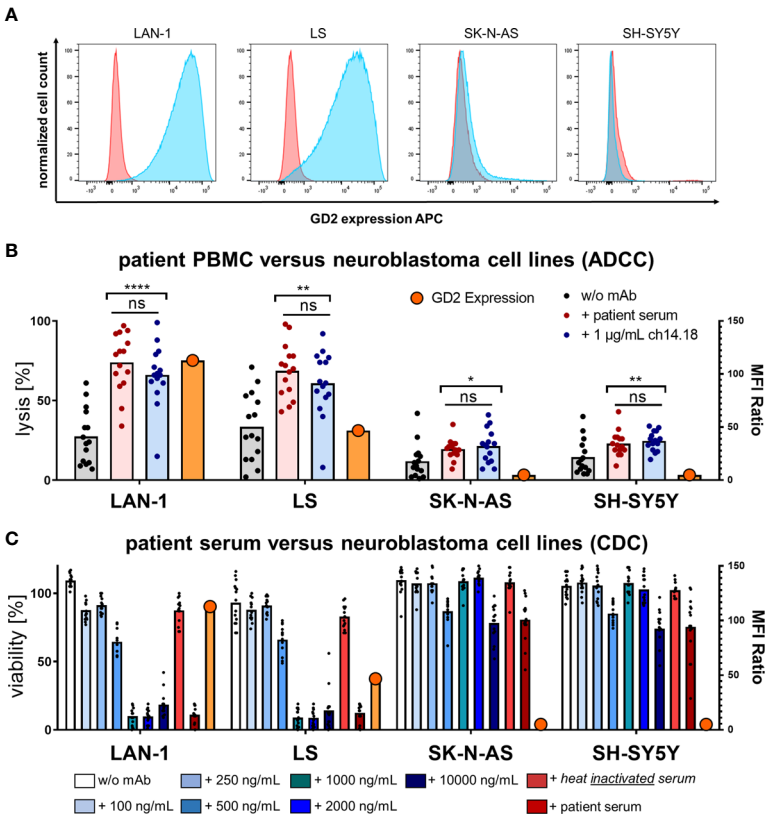


**FIGURE 5** | GD2 expression and cytolysis of neuroblastoma cell lines *via* ADCC and CDC by PBMCs and serum of patients. **(A)** GD2 expression of the neuroblastoma cell lines LAN-1, LS, SK-N-AS, and SH-SY5Y was measured by flow cytometry using primary labeled GD2 PE (clone 14G2a) mAb and a mouse IgG2a, k PE mAb as isotype control. The median fluorescence intensity (MFI) was used to calculate the MFI ratio (MFIR) by the MFI GD2 PE mAb divided by MFI isotype control IgG2a, k PE mAb. Representative univariate histograms are shown. From left to right panels the relative GD2 expression declines MFIR LAN-1 = 112.9 > MFIR LS = 46.8 > MFIR SK-N-AS = 1.3 > MFIR SH-SY5Y = 1. **(B)** Patient PBMCs acquired post haploidentical HSCT were used to test the direct cellular cytotoxicity and ADCC at an effector to target ratio E:T 5:1 of PBMCs *versus* the indicated neuroblastoma cell line in a 24 h luciferase-based kill assay. Specific lysis was assessed in the conditions I) PBMCs without mAb *versus* tumor, II) PBMCs plus patient serum *versus* tumor, III) PBMCs plus 1 µg/ml ch14.18/CHO mAb *versus* tumor. **(C)** CDC was tested in a coincubation of human serum from healthy volunteer donors (HVDs) at different levels of concentrations of added ch14.18/CHO mAb or heat-inactivated serum of patients or serum of patients *versus* the indicated neuroblastoma cell lines in a 24 h luciferase-based kill assay without adding any effector cells. Data shown in **(B)** represent mean of triplicates of (n = 15) independent experiments; data shown in **(C)** represent (n = 3) independent experiments of (n = 15) different donors in **(B, C)**, respectively. Statistical significance was determined by one-way ANOVA and Tukey *post-hoc* test. P-values below 0.05 were defined significant. ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; HVD, healthy volunteer donor. * = <0.05, ** = <0.01, **** = <0.0001. ns, not significant.

combination with anti-GD2 antibody (dinutuximab beta) treatment in children with Stage IV relapsed neuroblastoma. Detailed clinical outcomes, including toxicities and survival, are the subject of a distinct publication and will be reported separately. In line with the scientific rationale for this combinatorial approach, haplo HSCT led to a rapid and robust establishment of a functional cellular immune system in this cohort of heavily pretreated patients. Especially NK-cell recovery, a prerequisite for ADCC, was seen early after haplo HSCT, with a peak at approximately day +14 and a significant increase in cell numbers throughout the six cycles of dinutuximab beta therapy, which is consistent with our previous findings in haplo HSCT (14, 29–31). The long-term consolidation treatment with dinutuximab was dosed in the range of regimens with proven objective response rates in high-risk and refractory neuroblastoma patients (9, 32) who continuously showed relevant dinutuximab serum levels over the course of treatment. Despite the risk for the development of neutralizing antibodies under dinutuximab treatment during the use of chimeric antibodies (18), there was only one patient who was tested positive for HACAs in the presented cohort. Besides the quantitative assessment of serum mAb levels, objective functional activity of dinutuximab was measured in patients. Administration of dinutuximab beta led to a highly significant increase in activated NK cells and elevated serum marker of inflammation (IL2, TNFα, IL6, and CrP) which is in line with previous findings in the autologous setting (10). Additional administration of IL-2 neither boosted NK-cell activity nor increased the inflammatory response which may result from the massive cytokine secretion induced by the antibody infusion itself, thus giving no rationale for IL-2 application in subsequent studies, which have been proposed in a trial with continuous long-term infusion of dinutuximab (33).

To complement in-patient monitoring, NK-cell activity was analyzed *ex vivo*. As shown before, dinutuximab enhances NK-cell function (34). This was shown with patient-derived NK cells demonstrating potent cytokine secretion, degranulation, and cytotoxicity in combination with dinutuximab beta or patient serum against GD2+ target cells *ex vivo*, underscoring the combinatorial functionality. No significant correlation between cytotoxic activity and KIR receptor–ligand mismatch was found yet on a genetic level. Erbe et al. have shown contradictory data that KIR receptor and ligand interaction on a genetic level can negatively impact on the clinical outcome in the treatment with dinutuximab (35). In addition to ADCC, CDC was identified as a relevant mechanism of dinutuximab beta-mediated cytotoxicity with a clear correlation to antigen expression (GD2). In the autologous setting, we have previously demonstrated that neuroblastoma patients with high-affinity FCGR2A, -3A and stimulatory KIR2DS2 show higher levels of ADCC and improved event-free survival (13). Since haplo HSCT allows donor selection based on possibly beneficial donor characteristics, survival data were correlated to polymorphisms in *FCGR2A* and *-3A* genes as well as KIR-gene content score (36). In contrast to the LTI study (13), in our cohort, haplo HSCT using donors with high affinity FcGR3A or FcGR2A did not have any impact on event-free survival and overall survival in neuroblastoma Stage IV relapsed patients with subsequent GD2 targeted ch14.18/CHO antibody therapy. Further, KIR content did not have an impact on patient outcome. Since no correlation was found, no recommendations for donor selection based on the evaluated variables are possible at this stage. As mentioned above, the impact of the KIR receptors and ligand interaction appears to be unsolved (35).

In summary, we can state that haplo HSCT in combination with targeted immunotherapy utilizing dinutuximab is feasible. Haplo HSCT can serve as a safe and reliable tool to strengthen the cellular immune system in heavily pretreated patients. Moreover, haplo HSCT can provide a platform for cellular immunotherapy, generating highly functional effector cells for NK- or T-cell based therapies. This study has important implications for the future therapy of neuroblastoma patients.

## DATA AVAILABILITY STATEMENT

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

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Tuebingen. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## AUTHOR CONTRIBUTIONS

CM: interpretation of the data, drafted the manuscript, and approved the final revision. TF, A-ML, MM, SM, MK, DS, AJ, AR, NS, ST-M, MZ, HL, DA, A-SM, SS, and FH: acquisition, analyzed and interpreted the data, and approved the final revision. SY and RH analyzed and interpreted the data, and approved the final revision. PL: designed study, analyzed and interpreted the data, drafted the manuscript, and approved the final revision. PS: designed study, acquisition, analyzed and interpreted the data, drafted the manuscript, and approved the final revision. All authors contributed to the article and approved the submitted version.

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PERSPECTIVE
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# Novel Immune Cell-Based Therapies to Eradicate High-Risk Acute Myeloid Leukemia

*Roberto Limongello[1†], Andrea Marra[1†], Antonella Mancusi[1], Samanta Bonato[1], Eni Hoxha[1], Loredana Ruggeri[1], Susanta Hui[2,3], Andrea Velardi[1] and Antonio Pierini[1*]*

[1] Institute of Hematology and Centre of Haemato-Oncology Research (CREO), University and Hospital of Perugia, Perugia, Italy, [2] Department of Radiation Oncology, City of Hope Medical Center, Duarte, CA, United States, [3] Beckman Research Institute of City of Hope, Duarte, CA, United States

Adverse genetic risk acute myeloid leukemia (AML) includes a wide range of clinical-pathological entities with extremely poor outcomes; thus, novel therapeutic approaches are needed. Promising results achieved by engineered chimeric antigen receptor (CAR) T cells in other blood neoplasms have paved the way for the development of immune cell-based therapies for adverse genetic risk AML. Among these, adoptive cell immunotherapies with single/multiple CAR-T cells, CAR-natural killer (NK) cells, cytokine-induced killer cells (CIK), and NK cells are subjects of ongoing clinical trials. On the other hand, allogeneic hematopoietic stem cell transplantation (allo-HSCT) still represents the only curative option for adverse genetic risk AML patients. Unfortunately, high relapse rates (above 50%) and associated dismal outcomes (reported survival ~10–20%) even question the role of current allo-HSCT protocols and emphasize the urgency of adopting novel effective transplant strategies. We have recently demonstrated that haploidentical allo-HSCT combined with regulatory and conventional T cells adoptive immunotherapy (Treg-Tcon haplo-HSCT) is able to overcome disease-intrinsic chemoresistance, prevent leukemia-relapse, and improve survival of adverse genetic risk AML patients. In this *Perspective*, we briefly review the recent advancements with immune cell-based strategies against adverse genetic risk AML and discuss how such approaches could favorably impact on patients' outcomes.

**Keywords: HR-AML, poor outcome, adoptive immune therapies, CAR-T, HSCT, Treg-Tcon**

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***Correspondence:**
*Antonio Pierini*
*antonio.pierini@unipg.it*

*†These authors have contributed equally to this work and share first authorship*

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## INTRODUCTION

High risk (or adverse risk) acute myeloid leukemias (HR-AML) include a number of clinical and biological AML subsets which are usually characterized by poor response to conventional treatments and dismal long-term survival, even when conventional allogeneic hematopoietic stem cell transplantation (allo-HSCT) is performed (1). Such AML category is characterized by high-risk cytogenetics [i.e., complex and/or monosomal karyotypes, chromosomes 3, 5, 7, and 17 aberrations) and/or by specific genetic signatures (including mutations in *TP53, RUNX1, ASXL1*, and *FLT3* genes (2)] that confer an aggressive phenotype and often chemoresistance. Moreover, a

large proportion of patients affected by secondary AML (sAML) (3) and therapy-related leukemias (tr-AML) (4) converge into the HR-AML category. sAML is characterized by distinct molecular features, frequently involving the aberrant displacement of spliceosomal machinery (*SRSF2, SF3B1, U2AF1*, and *ZRSR2*), epigenetic modifiers (*ASXL1, EZH2, BCOR, RUNX1*), and cell-cycle regulators (*TP53*) (2). Despite the fact that next-generation sequencing (NGS) analyses have recently shed some light on the genetic complexity of these AML subsets, deep knowledge on leukemogenesis of each specific biological entity is currently lacking. Thus, targeted therapeutic approaches are still missing. While several drugs have been recently approved for the treatment of adult AML, they have only shown to slightly influence the fatal course of HR-AML patients. Such expanding *armamentarium* includes small molecules (e.g., FLT3 inhibitors, Midostaurin and Gilteritinib; isocitrate-dehydrogenase type 1 and 2/IDH1-2 inhibitors, Ivosidenib and Enasidenib; the Bcl2-inhibitor, Venetoclax) and new-generation cytotoxic treatments, like CPX-351 (5). Indeed, CPX-351 received Food and Drug Administration (FDA) 2019 approval for the treatment of tr-AML or AML with myelodysplasia-related changes (AML-MRC). Furthermore, emerging tailored strategies against mutant *TP53* (i.e., APR-246, Pevonedistat) (6–9) are providing encouraging yet preliminary evidences that may support their use in this high-risk setting. Since the achievement of durable remissions and the prevention of disease relapse remain major issues in the treatment of these patients, many research efforts have been directed towards a deeper understanding of mechanisms regulating relapse biology, with a major focus on immune system perturbation.

Immune-based adoptive cell therapies (ACTs) rely on the infusion of immune cells that aim to kill the tumor. These therapeutic platforms are revolutionizing treatment of blood neoplasms (**Figure 1**) and are challenging traditional drug interventions (10). In recent years, important advances have been made in developing novel effective immunotherapies (immune-checkpoint blockade, ACT, and vaccines) to overcome tumor-induced T-cell exhaustion and immune escape (10). Chimeric antigen receptor T-cells (CAR-T cells) are a form of ACT that has already demonstrated to be an effective treatment of various aggressive cancers, including

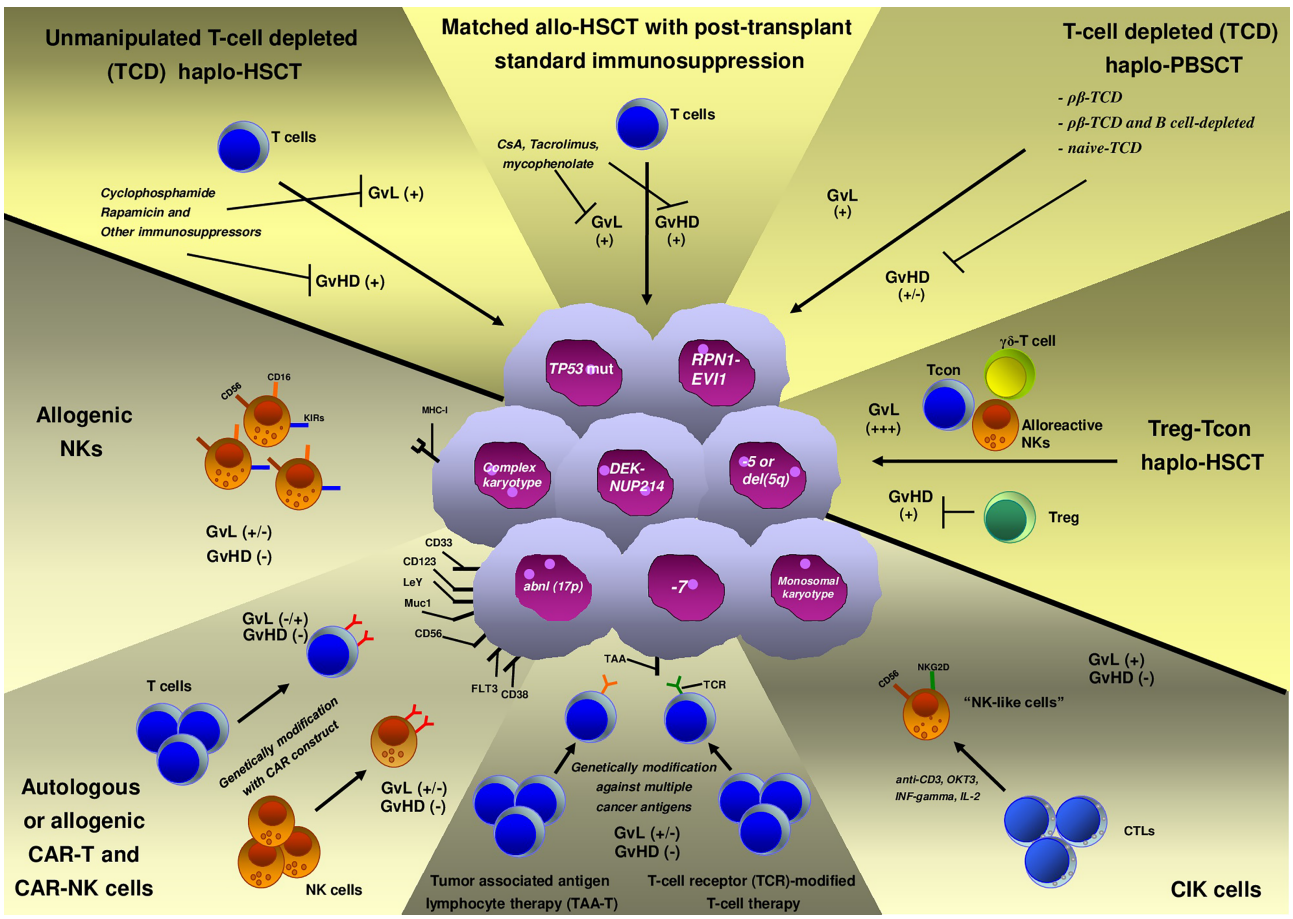


**FIGURE 1** | Available immune cell-based therapies for high-risk acute myeloid leukemia (HR-AML).

subsets of advanced leukemias (11–13). Beyond this, a plethora of other immune cell-based approaches are currently under investigation in blood tumors, including CAR-natural killer (NK) cells, cytokine-induced killer cells (CIK), and NK cells, as well as novel forms of CAR-T cells (dual CAR-T and multi-CAR-T cells).

Allo-HSCT and especially HLA haploidentical allo-HSCT (haplo-HSCT) may serve as "discovery platforms" that can help to reveal the complex interplay between AML and the immune microenvironment and to set the base for pioneering studies of AML immune-targeting (**Figure 1**). However, conventional transplantation strategies have limited impact on HR-AML outcomes, as survival curves rarely exceed 30–35% (14–16). In order to improve such outcomes, novel allo-HSCT strategies that exert more potent antileukemic activity need to be developed. Adoptive immunotherapy with conventional T cells (Tcons) and regulatory T cells (Tregs) is an innovative strategy that has been built to overcome disease chemoresistance and boost T-cell immunity, while preserving host tissues from graft-*versus*-host disease (GvHD) damaging (17). In particular, Treg infusion in the absence of other forms of immune suppression allows for T cell-mediated killing of leukemic blasts. Thus, such approach resulted in prolonged and stable disease remission in the vast majority of HR-AML patients, as we have preliminarily observed in a *proof-of-concept* retrospective study (18).

In the present *Perspective*, we will review molecular mechanisms underlying HR-AML biology that drive disease relapse, as well as the potential impact of the newly developed approaches with target therapies on patients' outcomes. We will describe immune-based strategies against HR-AML in ongoing trials and discuss how refined transplantation approaches with adoptive immunotherapy might represent the "ultimate" therapeutic option for a definitive eradication of HR-AML.

## Molecular Genetics of HR-AML

HR-AML includes many distinct biological entities, often characterized by an aggressive phenotype and intrinsic resistance to conventional treatments (2). HR-AML are not well defined in World Health Organization (WHO) classification, but this definition is widely used in clinical risk-adapted algorithms and also in the evaluation of the results from clinical trials. The major subgroup within this category consists of *AML with high-risk cytogenetics*, a subset of AML with different pathologic and clinical features that include the following:

- AML with complex karyotype (CK): its definition is not clear yet; however, it might be identified by the presence of ≥3 chromosomal abnormalities not included in defined WHO categories, and not associated with favorable prognosis (2, 19, 20)
- AML with monosomal karyotype (MK): it is defined by the presence of at least two autosomal monosomies or one single autosomal monosomy in combination with at least one structural abnormality (21)
- AML bearing specific chromosomal aberrations: it is defined by the presence of specific genetic abnormalities such as inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2,MECOM(EVI1)*; -5 or del(5q); -7; -17/abn(17p) (2)

Specifically, CK AML have been recently proposed to be further divided into *typical* and *atypical* cases by the presence (or the absence, respectively) of 5q, 7q, and/or 17p losses (22). *Typical* CK AML category bears *TP53* mutations (almost absent within the *atypical* subgroup) more frequently and, thus, it is associated with poorer outcomes (compared to *atypical* cases). On the other hand, *atypical* CK AMLs are characterized by different mutational onco-prints and more frequently display mutations of RAS pathway–associated genes, *NPM1* and/or *FLT3* genes (22). Functional transcriptomic analyses of CK AML highlighted an elevated genomic instability with aberrant activation of DNA damage response and cell-cycle checkpoint pathways (23, 24). Although at very initial stages, genomic analyses of MK AML cases have consistently demonstrated that abnormalities involving chromosomes 5 [-5, or del(5q)], 7 (-7), and 17 (-17/abn(17p) are frequent in this setting, and strongly coupled to *TP53* pathogenic mutations (25). However, other molecular pathways are implicated in MK AML pathogenesis and rely on a peculiar mutational signature targeting *NOTCH1* (rarely reported in AML), *BCOR/BCORL1*, or *RUNX1* genes (25). Interestingly, MK AML (as well as CK AML) are commonly associated with a catastrophic mutational phenomenon, namely, chromothripsis, that is promoted by clustered genomic rearrangements that result in multiple oncogenic hits and tumor-suppressors' inactivation (26). Eventually, such genomic events lead to the development of a highly proliferative disease. Specific aberrations involving chromosomes 3, 5, and 7 also clustered within HR-AML category. While chromosomes 5 and 7 aberrations are a common cytogenetic feature of trAML (4) and sAML that developed from previous myelodysplasia or myeloproliferative neoplasms (27), abnormalities involving chromosome 3 [inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2,MECOM(EVI1)*] could be related to a distinct (usually *de novo*) clinical-biological entity (2, 28). *TP53* mutations and aberrant RAS pathway activity (*NRAS, KRAS, PTPN11, NF1*) are common features of trAML (27), as well as of *EVI1*-rearranged (*EVI-r*) AML (28, 29). Importantly, the latter is characterized by typical morphologic features (dysplastic megakaryocytes, multilineage dysplasia, and normal/elevated blood platelet counts) and driven by distinct molecular programs (like *MECOM* and *IKZF1*). *EVI-r* AML is associated with very poor overall survival (OS) (28, 29).

Furthermore, the 2017 European LeukemiaNet (ELN) adverse-risk category (2) also comprises specific WHO-defined genetic entities, which include wild-type *NPM1* and *FLT3*-ITD$^{high}$, mutated *RUNX1*, and mutated *ASXL1*. The molecular pathogenesis of each distinct genetic entity is very poorly understood, and future studies are needed to investigate such biological complexity.

## Ongoing Adoptive Cell Therapies

The ability of leukemic blasts to evade immune surveillance has been recognized as a major mechanism of leukemia relapse after allo-HSCT (30). The novel use of immune therapeutics that aims

to redirect the immune system against malignant blasts is now considered a new powerful tool to eradicate leukemia. Indeed, novel cellular immunotherapies with chimeric antigen receptor (CAR) T cells for B-lymphoproliferative disorders have recently achieved promising results (31) and generated great enthusiasm in the scientific community. As a matter of fact, a great number of studies are now emerging with the goal to provide similar effective treatments for various hematologic neoplasms. Cell therapy for AML is more complex than for lymphoid malignancies because myeloid leukemia-specific targets still need to be well identified. Many adoptive cell strategies are under investigation (**Table 1**) and are object of several clinical trials (45).

## Natural Killer (NK) Cells Adoptive Immunotherapies

NK-cells are a subset of peripheral blood lymphocytes that are innately able to kill malignant cells through different mechanisms based on the balance between activatory and inhibitory signals. Interaction between major histocompatibility complex class I (MHC-I) molecules with killer immunoglobulin receptors (KIRs) on NK cells plays a major role in regulating NK cell function and activity. NK-cells kill leukemia cells when a mismatch between KIRs and their ligand on target cells is present (46). Such activity was demonstrated in T cell-depleted HLA-haploidentical transplant setting by the Perugia group and referred to as "NK cell alloreactivity" (47). The absence of any sort of pharmacologic immune suppression in TCD haplo-HSCT allowed for leukemia killing by alloreactive NK cells. On the other hand, the use of conventional immune suppressives to prevent GvHD in other transplant platforms may limit NK cell alloreactivity and its clinical effect (48). Further, NK cells may be dysfunctional and fail to kill AML blasts in case of abnormal phenotype, decreased degranulation level, and low INF-gamma and TNF-alfa production (32, 49, 50). For many years, NK cell adoptive transfer has been investigated as a possible approach to treat HR-AML. Studies showed donor-derived allogeneic NK-cells achieved durable complete remission in ~33% of HR-AML patients. Such studies proved infusion of a high number of NK-cells to be safe and well-tolerated. Indeed, donor NK cells appear not to cause any GvHD (33–35, 51). Moreover, donor NK-cells are able to persist and expand *in vivo* after infusion. On the other hand, while promising, allogeneic NK cell adoptive transfer has still limited efficacy, with generally low overall response rate. To overcome such limitations and boost NK cell *in vivo* function, different protocols and schemes that aim to generate and activate NK-cells are under evaluation and further studies are needed to establish the most effective approach.

## Cytokine-Induced Killer (CIK) Cells

CIK cells derived from cytotoxic T lymphocytes (CTL) that are *in vitro* activated by anti-CD3, OKT3, INF-gamma and subsequently expanded with IL-2. Other than T cell markers, they express surface protein similar to NK-cells, such as CD56, the inhibitory NK receptors, and the natural killer group 2 member D (NKG2D) receptor, one of the most important receptors involved in NK-mediated cancer cell killing (45). In clinical trials, CIK cells have been generated both from autologous and allogeneic lymphocytes and have been infused in combination or not with different strategies of allo-HSCT. Even if the results of early trials were disappointing (37), last studies are more encouraging (36, 38, 39, 52, 53). CIK cell transfer resulted in stable complete remission in ~60% of patients with AML. No significant infusion-related toxicities and a very low rate of acute GvHD were observed after CIK cell infusions. No studies focused on HR-AML, so that the efficacy in this setting still remains to be determined.

## Chimeric Antigen Receptor (CAR) T and NK Cells

CAR-T cells are genetically engineered T cells to express a variable heavy and light chains ($V_{HL}$) on cell surface with high specificity for malignant cell antigens (54). Despite the great enthusiasm that followed the CAR-T cell success in the treatment of acute B-lymphoblastic leukemia/lymphoma (B-ALL/LBL) and forms of B-cell lymphoma, generation of CAR-T cells against myeloid leukemic blasts is challenging because of the absence of leukemia-specific target antigens. In fact, AML antigens are often widely expressed by other hemopoietic cells or tissues. While *in vitro* studies and xenografts demonstrated the effectiveness of anti-CD33 and anti-CD123 CAR-T cells (42, 55), clinical efficacy on AML is still to be confirmed. CD33 is a transmembrane receptor expressed on >90% of blasts, but unfortunately also on multilineage hematopoietic progenitors and myelomonocytic precursors. It was still validated as therapeutic target based on the efficacy of gemtuzumab ozogamicin, a drug-conjugated monoclonal antibody against CD33. Preliminary data of anti-CD33 CAR-T cells are not encouraging (41). CD123 is a transmembrane subunit of the IL-3 receptor expressed on 100% of AML cells, and its expression is increased in FLT3-mutated AML. *In vitro* ed *in vivo* (xenograft) preliminary data showed an increased cytokine release and decreased tumor burden using anti-CD123 CAR-T cells. FLT3 receptor is typically expressed on myeloid blasts, independent of FLT3 mutational status. Anti-FLT3 CAR-T cells showed *in vitro* ed *in vivo* promising antileukemic effect (56, 57). Moreover, these seem to be less toxic on normal hematopoiesis than the anti-CD33 counterpart. Many other potential targets are now under evaluation. Lewis antigen (LeY) is overexpressed on myeloid blasts in comparison to normal tissues. A trial of autologous CAR-T cells targeting LeY showed a biological response (~60% of patients), but relapse occurred within 2 years (40). An ongoing clinical trial (NCT03222674) evaluates the feasibility, safety, and efficacy of multi-CAR-T cell therapy that targets different AML surface antigens (Muc1/CLL1/CD33/CD38/CD56/CD123) in patients with relapsed/refractory AML. Another phase I study (NCT04156256) evaluates the safety and tolerability of CD123-CD33 dual CAR-T in patients with relapsed and/or refractory AMLs.

In alternative to CAR-T cells, CAR-NK cell therapy ideally combines the specific targeting provided by CARs with the NK cell ability to kill AML blasts in the absence of relevant systemic toxicity. Indeed, CAR-NK cells showed promising results with no important toxicity in lymphoma patients (58). Mouse preclinical models suggest that CD123 CAR-NK cells may be effective in AML (59). Clinical-grade CAR-NK cells can be

**TABLE 1 | |** Selected published studies of immune cell-based strategies other than allogeneic transplantation for high-risk acute myeloid leukemia (HR-AML).

| Type of immune cell-based therapy | Study design | AML patient cohort included in the study | Outcomes | ClinicalTrials.gov or others identifier |
|---|---|---|---|---|
| **Natural killer (NK) adoptive immunotherapy** | Prospective trial of NK cells from haploidentical KIR-ligand–mismatched donors after fludarabine/cyclophosphamide chemotherapy, followed by IL2. | 17 adult acute myeloid leukemia (AML) patients (pts) in CR1[a] | CR[b] = 9/16 pts (56%). (1 patient died due to infection). Follow-up duration: 6–68 months. | NCT00799799 (32) |
| | Phase 1 non-randomized open-label, dose-escalation trial of CNDO-109-Activated allogeneic NK Cells. | 12 adult AML pts in CR1[a] not eligible for allo-HSCT[c] and at high risk for disease recurrence. | CR[b] = 3/12 pts (25%). Follow-up duration: 32.6–47.6 months. | NCT01520558 (33) |
| | Phase 1 dose-escalation trial of membrane-bound interleukin 21 (mb-IL21) expanded donor NK cells infused before/after haploidentical allo-HSCT[c]. | 8 adult high-risk AML pts in morphologic remission. | CR[b] = 7/8 pts (88%). Follow-up duration: 7.9–15.9 months. | NCT01904136 (34) |
| | Phase 2 trial of donor NK lymphocyte infusion (NK-DLI) after haploidentical allo-HSCT[c]. | 8 pediatric and adult AML pts of a cohort of 16 pts with high-risk leukemia and highly malignant solid tumors. | Relapse rate = 4/8 pts (50%). CR[b] rate and follow-up duration were not specifically detailed for AML cohort. | NCT01386619 (35) |
| **Cytokine-induced killer (CIK) cells** | Phase I study of allo-CIK cells in pts with blood tumors relapsed after allo-HSCT[c]. | 4 adult AML pts. | Response in 0/4 pts (0%). | N/A[f] (36) |
| | Prospective enrolling study of allo-CIK in pts with high-risk leukemias relapsed after cord-blood transplantation. | 4 adult AML pts, including 2/4 R/R[e] AML and 2/4 in CR2[d]. | PR[g] in 1/4 pts (25%). Follow-up duration: ~4 months. | N/A[f] (37) |
| | Retrospective study of allo-CIK administered after allo-HSCT[c] in pts with high-risk leukemias. | 5 adult AML pts (n=5). | CMR[h] = 4/5 pts (80%). Follow-up duration: 6.9–16 months. | N/A[f] (38) |
| | Phase I/II clinical trial of autologous CIK in pts with AML. | 13 adult AML pts in CR[b]. | CR[b] = 6/13 (46%). Follow-up duration: 38–50 months. | NCT00394381 (39) |
| **Chimeric antigen T (CART) cells** | Phase I study of autologous CAR anti-LeY T-cell therapy for AML. | 4 adult R/R[e] AML pts, including 3 pts treated in cytogenetic minimal residual disease, and 1 pt in progressive disease. | CR[b] = 1/4 (25%). Follow-up duration: 23 months. | CTX 08-0002 (40) (Australia) |
| | Phase I/II study of autologous CD33-directed CAR-T cells (CART-33) for the treatment of R/R[e] AML. | 1 adult AML pt. | Partial remission (PR) = 1/1. Follow-up duration: 3 months. | NCT01864902 (41) |
| | Interventional open-label pilot study of RNA-redirected anti-CD123 autologous T-cell in patients with R/R[e] AML. | 5 adult AML pts. | CR[b] = 0/5. All patient progressed at day 28. | NCT02623582 (42) |
| | Single-center phase I dose-escalation study of a single infusion of autologous NKG2D-CART cells without lymphodepleting conditioning in subjects with AML. | 7 adult AML pts, including 3 with CK, 3 with *TP53* mutation, and 4 secondary AML. | No objective response. | NCT02203825 (43) |
| **Chimeric antigen natural killer (CAR-NK) cells** | Phase I study of CD33-CAR NK-92 cells in R/R[e] AML pts. | 2 adult and 1 adolescent AML pts. | 2/3 pts achieved CR[b]. Relapse occurred in the 2 pts, ~4 months after CAR-NK cells infusion | NCT02944162 (44) |

*[a]CR1, first complete remission; [b]CR, complete remission; [c]Allo-HSCT, allogeneic hematopoietic stem cell transplantation; [d]CR2, second complete remission; [e]R/R, relapsed/refractory; [f]N/A, not available; [g]PR, partial response; [h]CMR, complete molecular remission; [i]CK, complex karyotype.*

manufactured from multiple sources (e.g., peripheral blood mononuclear cells, umbilical cord blood, hematopoietic progenitors, induced pluripotent stem cells), including the recently introduced CAR NK-92 cells, which consist of a modified CAR-engineered form of the NK-92 cell line. Such cell line represents an easily manageable and cost-effective tool for large-scale production of CAR-NK cells. Conversely, few drawbacks should be taken into account when using such strategy for CAR-NK cell manufacturing: i) failure of an *in vivo* expansion, due to lethal irradiation before infusion; ii) lack of NK-cell activating molecules (CD16 and NKp44); iii) potential *in vivo* tumorigenicity (60). The first-in-human clinical trial using CD33 CAR-NK cells derived from engineered NK-92 cells on three relapse/refractory extramedullary AML patients had no encouraging results (1/3 reached a transitory complete remission of 4 months) (44). Other trials with CD33 CAR-NK cells are under investigation (NCT02892695, NCT02944162). Such studies will help to clarify whether combinatorial strategies can provide antileukemic activity in the absence of relevant toxicity. While there was no specific focus on HR-AML in these preliminary studies and no clear studies showed HR-AML to be particularly sensitive to immune killing, the development of an effective anti-AML CAR-T or CAR-NK cell approach might provide a potent tool for reducing relapse in this high-risk disease.

### Other Adoptive Cell Therapies in the Near Future

T cells can be engineered to target different tumor-associated antigens that are frequently expressed in advanced AML blasts and other hematological neoplasms. Preliminary results of such tumor-associated antigen lymphocyte therapy (TAA-**T**) for different relapsed hematologic malignancies after allo-HSCT (11 patient, Hodgkin's lymphoma n=2, B-ALL n=3, AML n=5, and 1 HR-AML post 2$^{nd}$ allo-HSCT) showed that 80% of patients (4/5) with AML achieved a stable complete remission (61). This study also suggested TAA-T to be safe and tolerable (only one patient showed a liver GvHD; no cytokine release syndrome or neurotoxicity was observed). These preliminary promising data suggest that TAA-T therapy may be a feasible option for preemptive treatment of relapse after allo-HSCT for HR-AML, but further clinical studies are needed to ascertain its feasibility and efficacy in this setting. T-cell receptor (TCR)-modified T-cell therapy is a novel emerging strategy using the anti-tumor effect of genetically modifying T cells through the transduction of TCR genes against several cancer antigens (62, 63). The impact of this therapy against specific leukemic antigens is still under investigation. This therapy seems also very safe (64). The *in vitro* and *in vivo* preliminary studies on B-malignancies are very promising (65).

## Allo-HSCT Strategies: Is There Room for the Cure of High-Risk Acute Myeloid Leukemias?

Allo-HSCT is the only treatment modality that can provide a long-term survival benefit for HR-AML (**Table 2**), although current conventional transplantation strategies have scarce effect on HR-AML outcomes, with a maximum 2-year OS of 30–35% and a higher relapse rate when compared to other cytogenetic risk categories (14–16).

### HLA-Matched Allo-HSCT

A multicenter study of HLA-matched allo-HSCT that employed various immunosuppressive strategies for GvHD prophylaxis (cyclosporine A, tacrolimus, and T-cell depletion) compared a total of 584 patients carrying HR-AML in first complete remission (CR1) from 151 transplantation centers. It showed a median 3-year OS of 45% (range 38–52%), 37% (range 31–44%), and 31% (range 22–41%) in patients undergoing matched sibling donor (MSD), HLA-well-matched and partially-matched unrelated donor (MUD) transplantation, respectively. Myeloablative or reduced-intensity conditioning (RIC) regimens were used. Cumulative incidence (CI) of relapse at 3 years was 37% for MSD, 40% for well-MUD, and 24% for partially-MUD, while 3-year relapse-free survival (RFS) was 42, 34, and 29%, respectively. No significant differences in relapse were observed among the various cytogenetic subsets (66). Another retrospective multicenter study that involved more than 500 transplantation centers reported outcomes of 1,342 patients with CK-AML. Increased risk of relapse correlated with age, secondary AML, active disease at transplant, and the presence of deletion/monosomy 5. High tumor burden before transplant negatively impacted on post-transplantation outcomes. Indeed, 2-year CI of relapse for patients in CR and with active disease at transplantation was 47 and 64%, respectively. A very short OS at 2 years post-transplantation was observed in a subgroup of patients carrying deletion or monosomy 7 and deletion or monosomy 5 (29 and 20% respectively *vs* 42% in control groups without 7 and 5 deletion/monosomy). No significant survival benefit was observed between fully myeloablative conditioning and RIC regimen for patients with CK AML (34 and 28%). RFS rate was 39.9, 33, and 18.3% for patients ages <40, 40 to 60, and >60 years, respectively (14). Such studies demonstrate that the high relapse incidence after transplant in HR-AML patients is the major limitation of the procedure. Such outcomes urge the development of novel transplantation approaches.

### Haplo-HSCT

The recent advancements in T-cell manipulation and in GvHD prophylaxis make haplo-HSCT a valuable transplantation strategy to overcome intrinsic chemotherapy resistance of high-risk leukemias. Haplo-HSCT procedures can be mainly divided in two major categories: T-cell depleted (TCD) peripheral-blood stem cells (PBSCs) haplo-HSCT and unmanipulated haplo-HSCT.

Unmanipulated haplo-HSCT relies on pharmacologic GvHD prophylaxis, and it is now adopted worldwide. The use of G-CSF-primed grafts (67, 77), post-transplant high-dose cyclophosphamide (PT-Cy) in combination with other immunosuppressive drugs (78, 79), and post-transplant rapamycin (80), are different approaches that have been tested in this setting. While such strategies help to keep non-relapse mortality (NRM) acceptable, disease relapse remains a major

**TABLE 2 |** Selected published strategies of allogeneic hematopoietic stem cell transplantation (allo-HSCT) for high-risk acute myeloid leukemia (HR-AML).

| Allo-HSCT strategy | Study design | AML patient cohort in the study | Conditioning regimen | Graft-*versus*-Host Disease (GvHD) prophylaxis | Outcomes | Ref. |
|---|---|---|---|---|---|---|
| **HLA-matched allo-HSCT** | Retrospective multicenter study of URD[a] and MSD[b] allo-HSCT in patients (pts) with high-risk acute myeloid leukemia (HR-AML) in CR1[c]. | 584 adult HR-AML pts:<br>- CK[d]: 32%<br>- -7/del(7q): 25%<br>- Others: 43% | MAC[e]:<br>- MSD[b]: n=183<br>- URD[a]: n=252<br>RIC[f]:<br>- MSD[b]: n=252<br>- URD[a]: n=106 | ATG[g]:<br>- MSD[b]: n=18<br>- URD[a]: n=96<br>CsA[h]:<br>- MSD[b]: n=155<br>- URD[a]: n=137<br>Tacrolimus:<br>- MSD[b]: n=40<br>- URD[a]: n=191<br>T-cell depletion:<br>- MSD[b]: n=20<br>- URD[a]: n=29<br>Others/missing:<br>- MSD[b] n=11<br>- URD[a]: n= | 3-year OS°:<br>- MSD[b]=45%<br>- HLA-well-matched URD[a]= 37%<br>- Partially-matched URD[a]=31%<br>Median follow-up:<br>- MSD[b]: 61 months<br>- URD[a]: 35 months<br>3-year TRM[i]:<br>- MSD[b]=21%<br>- HLA-well-matched URD[a]=26%<br>- Partially-matched URD[a]=47% | (66) |
| | Retrospective multicenter study of MSD[b], MUD[p], and MMUD[q] allo-HSCT in CK[d] AML pts. | 1,342 adult CK[d] AML pts:<br>- 357 with -7/del(7q)<br>- 259 with -5/del(5q) | MAC[e]: n=739<br>RIC[f]: n=603 | T-cell depletion: n=665 | 2-year OS[m] = 36.8%<br>2-year NRM° = 17.6% | (14) |
| **HLA-haploidentical allo-HSCT (Haplo-HSCT)** | Prospective multicenter trial of G-CSF-primed grafts for haploidentical allo-HSCT in pts with blood neoplasms. | 45 adult AML pts:<br>- 34 standard-risk AML<br>- 11 HR-AML<br>In HR-AML group:<br>- 2 pts in CR3[n]<br>- 9 pts with active disease | MAC[e]: n=64<br>RIC[f]: n=16 | ATG[g]<br>CsA[h]<br>Methotrexate<br>Mycophenolate<br>Basiliximab | 18-month LFS[x] = 44% | (67) |
| | Retrospective multicenter study of unmanipulated haploidentical allo-HSCT in patients with AML. | Within the entire AML cohort:<br>- 99 pts in CR[l]<br>- 51 pts with active disease150 adult AML pts:<br>- 95 HR-AML | MAC[e] | CsA[h]<br>Mycophenolate | 4-year OS[h] = 57% | (68) |
| | Retrospective single-center analysis of MSD[b] *vs* URD[a] *vs* HRD[r] allo-HSCT for pts >60 years with AML. | 94 adult AML pts:<br>- 28 HR-AML<br>Within the entire AML cohort:<br>- 80 pts in CR[l]<br>- 14 with active disease | In HRD[r] allo-HSCT:<br>MAC[e]: n=0<br>Non-MAC[e]: n=9<br>RIC[f]: n=24 | In HRD[r] allo-HSCT:<br>Post-transplant cyclophosphamide<br>CsA[h]<br>Mycophenolate | 4-year TRM[i] = 20%<br>2-year OS[h] = 55%<br>2-year TRM[d] = 24%<br>2-year GRFS[e] = 32% | (69) |
| | Prospective trial of TCR[s] HRD[r] allo-HSCT in pts with blood neoplasms, compared with a retrospective cohort of pts treated with TCD[t] haplo-HSCT. | 65 pts:<br>- 42 AML/MDS | TCR[s] group (n=32):<br>- MAC[e]: n=26 | In TCR:<br>Post-transplant cyclophosphamide,<br>Tacrolimus, | 1-year OS[m:]<br>- TCR[s] = 64% | (70) |

*(Continued)*

**TABLE 2** | Continued

| Allo-HSCT strategy | Study design | AML patient cohort in the study | Conditioning regimen | Graft-*versus*-Host Disease (GvHD) prophylaxis | Outcomes | Ref. |
|---|---|---|---|---|---|---|
| | | | - RIC[f]: n=6<br>TCD[t] group (n=33):<br>- MAC[e] | Mycophenolate<br>In TCD:<br>ATG[g] | - TCD[t]= 30%<br>1-year TRM[i]:<br>- TCR[s] = 16%<br>- TCD[t] = 42% | |
| | Prospective trial of α/β TCD[t] HRD[r] allo-HSCT without ATG in children with chemorefractory AML. | 22 AML:<br>- 9 HR-AML<br>- 10 primary refractory<br>- 12 R/R[u] AML with active disease | MAC[e] | Bortezomib and tocilizumab +/– abatacept | 2-year OS[m] = 53%<br>2-year EFS[v] for HR-AML = 44%<br>TRM[i] = 9% | (71) |
| | Retrospective analysis in children with HR-AML in CR[l] receiving α/β TCD[t] HRD[r] allo-HSCT or MUD[p]. | 73 HR-AML:<br>- 59 pts in CR1[c]<br>- 14 pts ≥ CR2[w] | MAC[e] | 36 pts ATG[g], tacrolimus and methotrexate<br>47 pts ATG[g], Bortezomib and rituximab | 3-year OS[m]: 74%<br>OS[m]:<br>- MUD[p] = 64%<br>- haplo-HSCT = 86%<br>GRFS[z]:<br>- MUD[p] = 49%<br>- haplo-HSCT = 70%<br>TRM[i]:<br>- MUD[p] = 14%<br>- haplo-HSCT = 5% | (72) |
| | Prospective trial of α/β TCD[t] and B cell-depleted HRD[r] allo-HSCT in children with AL. | 80 AL:<br>- 24 CR[l] (CR1[c]=16, CR2[w]=8)<br>- 4 HR-AML | MAC[e] | ATG[g] | For entire cohort:<br>5-year OS[m] = 72%<br>5-year CRFS[y] = 71%<br>5-year TRM[i] = 5%<br>For AML sub-cohort:<br>5-year LFS[x] = 68% | (73) |
| | Retrospective multicenter comparative analysis of URD[a]- or α/β TCD[t] HRD[r] allo-HSCT in children with AL. | 342 AL:<br>- MUD[p]: 127<br>- MMUD[q]: 118<br>- HRD[r]: 98<br>105 CR[l] AML:<br>- MUD[p]: 43<br>- MMUD[q]: 32<br>- haplo-HSCT: 30 | MAC[e] | In HRD[r] allo-HSCT:<br>α/β[+] and CD19[+] negative selection + ATG[g] | For α/β TCD[t] haplo-HSCT AL cohort:<br>5-year probability of OS[m] = 68%<br>5-year LFS[x] = 62%<br>5-year CRFS[y] = 59%<br>TRM[i] = 9%<br>Cumulative incidence of relapse for AML sub-cohort = 21% | (74) |
| | Prospective single-arm clinical trial of naïve TCD[t] peripheral blood stem cells grafts for adult pts with high-risk leukemia. | 35 Adult high-risk leukemia:<br>- 10 AML | MAC[e] | Tacrolimus | 2-year OS[m] = 78% | (75) |
| | Prospective single-center trial of adult AML pts undergoing HRD[r] allo-HSCT combined with regulatory and conventional T cells adoptive immunotherapy | 50 adult AML pts:<br>- 20 HR-AML<br>- 42 CR[l]<br>- 8 with active disease | Age-adapted MAC[e] | None | 29-month OS[m] = 77%<br>CRFS[y] = 75%<br>CRFS[y] (for HR-AML) = 72%<br>TRM[i] = 21%<br>Cumulative Incidence of relapse: 4% | (76) |

*[a]URD, unrelated donor; [b]MSD, matched sibling donor; [c]CR1, first complete remission; [d]CK, complex karyotype; [e]MAC, Myeloablative conditioning regimen; [f]RIC, Reduced-intensity conditioning regimen; [g]ATG, anti-thymocyte immunoglobulin; [h]CsA, cyclosporin; [i]TRM, transplant-related mortality; [l]CR, complete remission; [m]OS, overall survival; [n]CR3, third complete remission; [o]NRM, non-relapse mortality; [p]MUD, matched unrelated donors; [q]MMUD, mismatched unrelated donors; [r]HRD, haploidentical related donor; [s]TCR, T-cell replete; [t]TCD, T-cell deplete; [u]R/R, relapsed/refractory; [v]EFS, event-free survival; [z]GRFS, GvHD-free, relapse-free survival; [w]CR2, second complete remission; [x]LFS, leukemia-free survival; [y]CRFS, chronic GvHD-free, relapse-free survival.*

concern, especially when non-myeloablative conditioning regimens are used (81). A study of unmanipulated G-CSF-primed haplo-HSCT showed a 1-year CI of NRM of 36% and a CI of relapse of 21% at 1 year and 28% at 5 years respectively, with a 3-year probability of OS and RFS in 44 and 30%, respectively, in high-risk patients (> second CR or active disease) with hematologic malignancies (including HR-AML) (67). Haplo-HSCT with PT-Cy is now the most widely adopted haplo-HSCT platform, thanks to acceptable rates of acute and chronic GvHD, low NRM, no need of graft manipulation and contained costs. On the other hand, relapse rates are still disappointing in HR-AML patients. In fact, subanalyses showing outcomes of patients with adverse genetic risk AML reported relapse rates up to 50% (14). Because of such limitation and with the goal of reducing leukemia relapse, high-intensity myeloablative conditioning regimens have been employed. *Chiusolo P et al.* (68) and *Devillier R et al.* (69) showed a CI of AML relapse of 24% at 4 years and 25% at 2 years, respectively. Further studies will be needed to evaluate if such strategies are effective in subsets of HR-AML patients.

### T-Cell Depleted Haplo-HSCT

In the last 20 years, several strategies of *ex vivo* T-cell depletion (TCD) have been tested to improve outcomes of acute leukemia patients who underwent haplo-HSCT. While traditional TCD procedure based on positive selection of CD34+ cells was associated with delayed immune reconstitution and increased risk of NRM (70, 82), more recent strategies are directed towards the preservation of immune subsets that improve post-transplant immune recovery for more effective anti-infective and antileukemic activities (82). Among these, αβ T-cell-depleted haplo-HSCT appears to be an effective platform for the treatment of HR-AML. In αβ T-cell-depleted haplo-HSCT the graft is manipulated to eliminate T cells that express αβ T cell receptor and which are demonstrated to be the main T cell population responsible for alloreactions that cause GvHD. In the studies by *Shelikhova L et al.* (71) and *Maschan M et al.* (72), children with primary refractory or relapsed AML who underwent αβ T-cell-depleted haplo-HSCT reached hematologic complete remission, despite 9/22 of them carried adverse-risk cytogenetics. However, the relapse rate and OS at 2 years after allo-HSCT were 42 and 52%, respectively. In different studies by *Locatelli F et al.* (73) and *Bertaina A et al.* (74), αβ T-cell and B-cell-depleted haplo-HSCT proved to be a safe and suitable approach in high-risk acute leukemias (HR-AL) in children. Indeed, it achieved a 5-year probability of chronic GvHD-free/relapse-free (GRFS) survival of 71% in HR-AL patients (73). A novel TCD haplo-HCT platform employs grafts that have been selectively depleted of naive T-cells. Indeed, depletion of naïve T cells ($T_N$) from PBSC preserves hematopoietic engraftment and allows for the transfer of donor-derived memory T cells, that can confer immunity against pathogens with low risk of GvHD (75). This approach has demonstrated to improve outcomes of HR-AL patients (the 2-year relapse rate was 21% and the 2-year RFS was 70%) in a single-arm trial (75). Thus, such approaches are promising, but relapse rates still reduce outcomes of HR-AL patients.

### Haploidentical HSCT Combined With Regulatory and Conventional T-Cells Adoptive Immunotherapy

We have recently demonstrated that haplo-HSCT combined with regulatory and conventional T-cells adoptive immunotherapy (Treg-Tcon haplo-HSCT) is able to overcome disease-intrinsic chemoresistance (18, 76). We enrolled 50 AML patients in the study; 40% of them (20/50) had HR-AML. An "age-adapted" myeloablative conditioning based on total body irradiation (TBI) for patients up to the age of 50 years and total marrow/total lymphoid irradiation (TMLI) for patients aged 51–65 years was followed by thiotepa, fludarabine, and cyclophosphamide. No pharmacological GvHD prophylaxis was given. Two millions/kg donor regulatory T cells were given at day −4 to allow for their alloantigen-specific *in vivo* expansion. One million/kg conventional T cells were given at day −1 and were followed by the infusion of a "megadose" of purified CD34+ hematopoietic progenitor cells at day 0. Fifteen/50 patients developed grade ≥2 acute GvHD (aGvHD). Moderate/severe cGvHD occurred in only one patient. Only two patients relapsed (4%). Consequently, at a median follow-up of 29 months, the probability of moderate/severe cGvHD/relapse-free survival was 75% (18, 76). TMLI allowed to safely extend the powerful effect of a myeloablative conditioning to older (>60 years old) patients. Further, when looking at the different genetic signatures of the enrolled AML patients, we found that HR-AML did not have a higher risk of relapse in comparison to more favorable subgroups. Indeed 17 of the 20 HR-AML patients are alive and leukemia-free despite many of them had detectable disease at transplant. Such results demonstrate HR-AML to be sensible to immune-mediated killing. Indeed, the absence of pharmacologic immune suppression in Treg/Tcon haplo-HSCT could have favored a potent GvL effect that was exerted across all the AML subsets and that was not limited by disease burden and previous refractoriness to chemotherapeutic agents. ELN AML genetic risk stratification is considered to retain outcome prediction after allo-HSCT (83). However, our study showed that effect was lost after Treg/Tcon haplo-HSCT in a single series of 50 AML patients. While larger multicentric studies are needed to support such conclusion, the potent GvL activity of Treg/Tcon haplo-HSCT appears to be an effective tool for the treatment of such unfavorable AML.

## DISCUSSION

HR-AMLs are usually characterized by a very poor response to conventional treatments and to conventional allo-HSCT. Indeed, relapse rates are high (often above 50%) and result in very low survival (often below 10–20%). Thus, novel effective strategies are needed. Recent studies on new adoptive cell strategies (CAR-T cells, CAR-NK cells, CIKs, activated NK cells) bring new hopes for the treatment of such unfavorable diseases. Indeed, immune-cell-based therapies may represent a powerful tool to successfully treat chemoresistant HR-AML. NK cell adoptive immunotherapies are a promising therapeutic, but their efficacy is still limited and fine-tuning of the approach is still

required for larger clinical use (32–35). The more recently introduced CAR-T- and CAR-NK-cell-based treatments demonstrated high potency in pilot studies and hold great promise (40–44). The growing body of clinical studies and broader use of these agents in different settings and against novel targets will provide key information on their ability to eradicate HR-AML. Furthermore, we have recently demonstrated that Treg-Tcon haplo-HSCT is able to overcome HR-AML intrinsic chemoresistance, prevent relapse, and improve survival (18, 76). This study strongly suggests that HR-AMLs are sensitive to antileukemic immunity. The introduction of new immune therapeutics that strengthen immune activity against leukemia and the development of transplantation approaches that favor unopposed GvL might help to develop powerful tools for an effective treatment of HR-AML.

## DATA AVAILABILITY STATEMENT

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

## ETHICS STATEMENT

All procedures were in accordance with the ethical standards of the institutional research committee. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

RL and AMar contributed equally to the figures and writing. AMan, SB, and EH reviewed the manuscript. AV provided guidance, and AP wrote the manuscript and provided critical review. SH and LR provided critical review. All authors contributed to the article and approved the submitted version.

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# Allogeneic Vγ9Vδ2 T-Cell Therapy Promotes Pulmonary Lesion Repair: An Open-Label, Single-Arm Pilot Study in Patients With Multidrug-Resistant Tuberculosis

*Juan Liang[1,2,3†], Liang Fu[2,4†], Man Li[3,5], Yuyuan Chen[3], Yi Wang[2], Yi Lin[2], Hailin Zhang[2], Yan Xu[3], Linxiu Qin[2], Juncai Liu[2], Weiyu Wang[2], Jianlei Hao[3], Shuyan Liu[2], Peize Zhang[2], Li Lin[5], Mohammed Alnaggar[6], Jie Zhou[7], Lin Zhou[8], Huixin Guo[8], Zhaoqin Wang[2], Lei Liu[2], Guofang Deng[2*], Guoliang Zhang[2*], Yangzhe Wu[1,3*] and Zhinan Yin[1,3*]*

[1] Zhuhai Institute of Translational Medicine, Zhuhai People's Hospital (Zhuhai Hospital Affiliated with Jinan University), Jinan University, Zhuhai, China, [2] National Clinical Research Center for Infectious Diseases, Guangdong Key Laboratory of Emerging Infectious Diseases, Shenzhen Third People's Hospital, Southern University of Science and Technology, Shenzhen, China, [3] The Biomedical Translational Research Institute, Faculty of Medical Science, Jinan University, Guangzhou, China, [4] Beijing Chest Hospital, Capital Medical University, Beijing, China, [5] Department for gdT Clinical Research and Development, Guangdong GD Kongming Biotech Ltd., Guangzhou, China, [6] Tongji Chibi Hospital, Tongji Medical College, Huazhong University of Science and Technology, Chibi, China, [7] Department for Tuberculosis Control, Foshan Fourth People's Hospital, Foshan, China, [8] Department for Tuberculosis Control, Centre for Tuberculosis Control of Guangdong Province, Guangzhou, China

The WHO's "Global tuberculosis report 2020" lists tuberculosis (TB) as one of the leading causes of death globally. Existing anti-TB therapy strategies are far from adequate to meet the End TB Strategy goals set for 2035. Therefore, novel anti-TB therapy protocols are urgently needed. Here, we proposed an allogeneic Vγ9Vδ2 T-cell-based immunotherapy strategy and clinically evaluated its safety and efficacy in patients with multidrug-resistant TB (MDR-TB). Eight patients with MDR-TB were recruited in this open-label, single-arm pilot clinical study. Seven of these patients received allogeneic Vγ9Vδ2 T-cell therapy adjunct with anti-TB drugs in all therapy courses. Cells ($1 \times 10^8$) were infused per treatment every 2 weeks, with 12 courses of cell therapy conducted for each patient, who were then followed up for 6 months to evaluate the safety and efficacy of cell therapy. The eighth patient initially received four courses of cell infusions, followed by eight courses of cell therapy plus anti-MDR-TB drugs. Clinical examinations, including clinical response, routine blood tests and biochemical indicators, chest CT imaging, immune cell surface markers, body weight, and sputum *Mycobacterium tuberculosis* testing, were conducted. Our study revealed that allogeneic Vγ9Vδ2 T cells are clinically safe for TB therapy. These cells exhibited clinical efficacy in multiple aspects, including promoting the repair of pulmonary lesions, partially improving host immunity, and alleviating *M. tuberculosis* load *in vivo*, regardless of their application in the presence or absence of anti-TB drugs.

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Federico Simonetta,
Geneva University Hospitals (HUG), Switzerland

**Reviewed by:**
Maite Alvarez,
University of Navarra, Spain
Patrizia Comoli,
San Matteo Hospital Foundation (IRCCS), Italy

***Correspondence:**
Zhinan Yin
tzhinan@jnu.edu.cn
Yangzhe Wu
tyzwu@jnu.edu.cn
Guoliang Zhang
szdsyy@aliyun.com
Guofang Deng
jxxk1035@yeah.net

[†]These authors share first authorship

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This pilot study opens a new avenue for anti-TB treatment and exhibits allogeneic Vγ9Vδ2 T cells as promising candidates for developing a novel cell drug for TB immunotherapy.

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## INTRODUCTION

Tuberculosis (TB), a major chronic infectious disease of the lungs caused by *Mycobacterium tuberculosis*, has become a top killer among infectious diseases due to an epidemic of coinfections and drug resistance (1, 2). According to the World Health Organization's "Global tuberculosis report 2020," in 2019, there were approximately two billion individuals infected with *M. tuberculosis* worldwide. These included an estimated 10 million new TB patients and half a million rifampicin-resistant patients, of which 78% are multidrug-resistant (MDR-TB). The treatment of patients with MDR-TB is challenging (3, 4), because of the requirement for long treatment cycles, low efficacy and side effects of anti-TB drugs, poor compliance of patients, easy recurrence, and a susceptibility for development of extensive drug resistance (XDR). The cases of TB drug resistance have greatly postponed the accomplishment of the End TB Strategy set for 2035. Therefore, in addition to traditional anti-TB chemical drugs, breakthroughs in new TB therapies and prevention strategies, such as immune cell-based biotherapies or vaccines, are urgently needed to accelerate the annual decline in the global TB incidence rate by approximately 17% in 2025 and per year afterward. Accomplishing these goals would then hopefully control the TB epidemic.

In the past, cell therapies, including stem cells (5, 6), αβ T cells (7, 8), NK cells (9), and γδ T cells (10), have been extensively utilized in the treatment of tumor- or autoimmune-related diseases, demonstrating sound clinical efficacy. Nonetheless, research on immune cell therapy in the field of infectious diseases, specifically *M. tuberculosis* infection, has been very limited (11, 12). As the pathogenesis of TB has been closely correlated with suppressive immune functions of the host, immune cell therapy should be a new promising direction for controlling TB. In particular, γδ T cells, a subset of T lymphocytes that bridges innate and adaptive immunity (13), account for less than 10% of T lymphocytes, with the Vγ9Vδ2 cell subset being dominant in peripheral blood.

Using a non-human primate model, the group of Chen proposed Vγ9Vδ2 T-cell-based cell therapy for the treatment of TB (11, 12). This groundbreaking study exhibited that adoptive cell transfer therapy of pre-expanded autologous Vγ9Vδ2 T cells from peripheral blood mononuclear cells (PBMCs) before *M. tuberculosis* infection could significantly decrease the postinfectious *M. tuberculosis* burden in the liver, kidneys, and spleen of macaques. Importantly, the study showed the remarkable alleviation of lesions in the lung tissues of infected animals, indicating the clinical prospect of Vγ9Vδ2 T cells for the treatment of patients with TB. However, the clinical efficacy of Vγ9Vδ2 T cells against chronic *M. tuberculosis* infections of patients with TB remains to be addressed. Furthermore, autologous Vγ9Vδ2 T cells of patients with TB are functionally impaired and therefore are quite difficult to be expanded *ex vivo*. In this context, allogeneic Vγ9Vδ2 T-cell-based immunotherapy (10, 14) should be an optimal strategy for the treatment of patients with TB and even MDR-TB.

In the present study, we utilized allogeneic Vγ9Vδ2 T cells for the treatment of patients with MDR-TB who had significantly limited options in effective anti-TB drugs. We enrolled eight patients with MDR-TB to receive allogeneic Vγ9Vδ2 T-cell therapy, and conducted a total of 12 cell transfer infusions for each patient. We found that allogeneic Vγ9Vδ2 T cells exhibited promising safety in patients with MDR-TB. Most importantly, we observed a significant relief in pulmonary lesions in all eight patients receiving either allogeneic Vγ9Vδ2 T-cell treatment alone or in combination with anti-MDR-TB drugs. This is a clear evidence of the promising efficacy of the therapy. Collectively, allogeneic Vγ9Vδ2 T-cell-based immunotherapy may provide a novel therapeutic strategy for patients with MDR-TB, and future extensive clinical application of allogeneic Vγ9Vδ2 T cells may be beneficial for controlling TB epidemics worldwide.

## MATERIALS AND METHODS

### Enrollment of Patients With MDR-TB and Ethics

Between July and December 2018, eight patients with MDR-TB diagnosed using *M. tuberculosis* sputum smear microscopic examination, culture, and drug sensitivity testing were recruited in our study after signing an informed consent. The age of the patients ranged between 18 and 50 years old. Patients with other conditions, including HIV, hepatitis B, diabetes, tumor, hypertension, coronary heart disease, endocrine system disease, mental disease, neurological disease, vascular circulation system disease, or other disease, were excluded from our study.

The Regional Ethics Committee of Shenzhen Third People's Hospital approved the study protocol (Approval ID SZLY2018017). All participants provided signed written informed consents in accordance with the Declaration of Helsinki. This study has been registered in the Clinical Trials website (ClinicalTrials.gov, ID: NCT03575299).

## *Ex Vivo* Selective Expansion of Vγ9Vδ2 T Cells From PBMCs of Healthy Donors

First, we obtained 100 ml peripheral blood from a healthy donor who had received and passed the screening of infectious diseases, including hepatitis B, hepatitis C, syphilis, and HIV. Human PBMCs were isolated using Ficoll–Paque-based density gradient centrifugation. Next, we induced and extended the population of Vγ9Vδ2 T cells using our patented formula (Patent No. ZL201811580040.2). PBMCs at a density of $3 \times 10^6$ cells/ml were cultured in RPMI 1640 (Gibco) containing 10% FBS and 1% penicillin–streptomycin and supplemented with 50 μM zoledronic acid (Sigma), 100 IU/ml recombinant human interleukin (IL)-2 (Beijing Four Rings Bio-Pharm Co.), 100 IU/ml recombinant human IL-15 (Peprotech), and 70 μM L-ascorbic acid (Sigma) on day 0. Afterwards, fresh medium and cytokines were replaced every 2–3 days. After being cultured for approximately 10 days, the viability of Vγ9Vδ2 T cells was determined using flow cytometry utilizing the PE-annexin V apoptosis kit (BD Biosciences, San Jose, CA, USA). In addition, Vγ9Vδ2 T cells were stained using anti-human CD3-APC-H7 (clone SK7; BD Biosciences), anti-human TCR Vδ2-Percp (clone B6; Biolegend), and anti-human CD314 (NKG2D)-PerCP/Cy5.5 (clone 1D11; BD Biosciences) antibodies to identify the cell phenotypes.

## Adoptive Transfer Therapy of MDR-TB Using Allogeneic Vγ9Vδ2 T Cells

During the process of cell expansion, cells were tested twice for pathogens, including bacteria, fungi, mycoplasma, and endotoxins. Vγ9Vδ2 T cells adhering to the quality criteria, including contamination-free, proportion higher than 90%, and activity greater than 90%, were used for adoptive transfer to patients. On the day of cell infusion, cells were washed three times with normal saline solution. Next, $1 \times 10^8$ cells were placed in 100 ml normal saline solution containing 1% human serum albumin. Subsequently, cells were infused in patients within half an hour. Each of the eight patients received each infusion of Vγ9Vδ2 T cells from a different donor.

## Immunophenotype Analysis for the Evaluation of Alterations in Peripheral Immune Cell Functions

We collected 5 ml of peripheral blood from patients before Vγ9Vδ2 T-cell infusion. The routine protocols of our lab were used for the isolation of PBMCs from the blood. The phenotype of immune cells, including T cells, NK cells, γδ T cells, B cells, and their subsets, was analyzed using flow cytometry (FACSanto™ II; BD Biosciences). The antibodies used included the following: PerCP-CY5.5-conjugated anti-CD3, anti-CD8, anti-CD19, and anti-CD94; APC-H7-conjugated anti-CD3, anti-CD4, and anti-CD45; FITC-conjugated anti-CD45RA and anti-CD8; BV510-conjugated anti-CD8 and anti-CD196; PE-conjugated anti-HLA-DR, anti-KIR, and anti-Vδ2; PE-CY7-conjugated anti-CD4 and anti-NKG2D; BV421-conjugated anti-CD56, anti-27, anti-CD194, anti-TCRγδ, and anti-P30; BV510-conjugated anti-CD8 and anti-CD196; Alexa Fluor 647-conjugated anti-CCR7, anti-CXCR5, anti-PD-1, and anti-CD186; and Alexa Fluor 484-conjugated anti-CD183. All antibodies were purchased from BD Biosciences.

## Clinical Evaluation of the Therapeutic Efficacy of Allogeneic Vγ9Vδ2 T Cells

To evaluate the therapeutic efficacy, all patients underwent chest CT examination before Vγ9Vδ2 T-cell infusion and every 2 months afterwards. The body weight of patients was also recorded before each Vγ9Vδ2 T-cell infusion and again in each follow-up. Routine blood and biochemical examinations were also performed before each Vγ9Vδ2 T-cell infusion. One of the gold standards in the treatment of TB is to change positive *M. tuberculosis* sputum to negative. Therefore, we conducted sputum tuberculosis examinations, including sputum acid-fast staining and sputum *M. tuberculosis* culture throughout this clinical study.

Furthermore, we performed plasma metabolite detection using plasma samples from patient #8 who only received allogeneic Vγ9Vδ2 T-cell therapy (first 4 times). To accomplish this, 5 ml of peripheral blood was collected from patient #8 before each Vγ9Vδ2 T-cell infusion, and metabolites were detected in the plasma using mass spectrometry (Guangdong Longsee Biomedical Co. Ltd.).

Two stool samples were collected from patient #8, one before and one after receiving allogeneic Vγ9Vδ2 T-cell therapy using the Fecal Microbial Genome Protective Kit (LS-R-P-003, Longsee). Microbial DNA was extracted using the Stool Microbial Genomic DNA Extraction Kit (LS-R-N-015, Longsee) in accordance with the instructions of the manufacturer. The V3–V4 highly variable region of 16S rRNA was amplified using PCR with primers 338F: 5′-ACTCCTACGGGAGGCAGCA-3′ and 806R: 5′-GGACTACHVGGGTWTCTAAT-3′. PCR amplification products were detected using 2% agarose gel electrophoresis. Subsequently, target fragments were cut and recovered using the AxyPrep DNA Gel Recovery Kit (AP-GX-50G, Axygen). Fluorescence quantification of the recovered products was performed using PCR amplification with the Quant-iT PicoGreen dsDNA assay kit (P11496, Invitrogen) in a FLx800 microplate reader (BioTek). After serially diluting each qualified sequencing library (index sequence was not repeatable), they were mixed in corresponding proportion according to the required sequencing volume and then denatured to a single strand by NaOH for sequencing. The MiSeq Reagent Kit V3 (600 cycles; MS-102-3003, Illumina) was used for sequencing. The MiSeq-PE250 sequencer was used for $2 \times 300$ bp paired-end sequencing on an Illumina platform according to standard protocols.

## Statistical Analysis

Data were analyzed using *t*-test with GraphPad Prism 7.0 software. *P*-values of $<0.05$ were considered statistically significant.

# RESULTS

## Research Cohort and Clinical Immunotherapy Protocol

Between July and December 2018, we enrolled eight patients with MDR-TB in our study. The information of these eight patients as well as drug treatment protocols is shown in **Table 1**. All enrolled patients were diagnosed with MDR-TB. Rather than a fixed drug recipe, the drug protocol for the treatment of TB was determined by a group of physicians according to the clinical symptoms of individual patients. Each patient received 12 cycles of adoptive transfer cell therapy. Therefore, the eight patients with MDR-TB received a total of 96 cell infusions in this clinical study. In particular, patient #8 only received allogeneic Vγ9Vδ2 T-cell therapy in the first 4 of the 12 total courses according to the requirement of the patient and continued with anti-TB drugs. The medical record of patient #8, which clearly illustrates the developmental timeline from TB to MDR-TB, is shown in **Supplementary Table 1**. The brief therapeutic protocol of this patient is shown in **Supplementary Figure 1**.

Regarding the therapy protocol (**Figure 1**), we *ex vivo* expanded cells in the peripheral blood collected from healthy donors using our previously established methodology (10, 14). We adoptively infused the expanded cells into the enrolled patients after they passed quality control requirements, which included purity (≥90%), viability (≥90%), and lack of pathogens (**Figure 1A**). In our protocol, patients received a single treatment of $1 \times 10^8$ Vγ9Vδ2 T cells every half month with a total of 12 courses completed in 6 months, followed by a 6-month follow-up (**Figure 1B**). Notably, all examinations including routine blood and biochemical assays, immune function phenotype, and chest CT were performed at the scheduled time points.

## Infusions of Allogeneic Vγ9Vδ2 T Cells Were Safe and Did Not Reduce the Side Effects of Anti-MDR-TB Drugs

Given that anti-TB drugs have various side effects, we explored whether these adverse effects were minimized under allogeneic Vγ9Vδ2 T-cell treatment. We statistically compared the alterations in 12 immunological/biochemical parameters, namely, hemoglobin, neutrophils, lymphocytes, leukocytes, alanine aminotransferase, aspartate aminotransferase, bilirubin, γ-glutamytransferase, uric acid, total protein, albumin, and creatinine. All of the biochemical markers are routinely and adversely changed by anti-TB drugs (**Figure 2**), before and after the 12 courses of cell therapy. Adverse effects were defined according to the Common Terminology Criteria for Adverse Events version 4.0. We observed that allogeneic Vγ9Vδ2 T-cell therapy did not significantly reduce these 12 types of adverse effects. Moreover, this finding also indicated that allogeneic Vγ9Vδ2 T-cell therapy does not exert additional unexpected side effects, thereby demonstrating its promising clinical safety, as supported by the 96 total courses of cell treatment in this trial study (**Table 1**).

Because patient #8 only received cell infusion exclusively in the first 4 out of the 12 total courses, we conducted intensive analyses to compare the biochemical alterations before and after the Vγ9Vδ2 T-cell therapy. We assessed various serological parameters, including total protein, albumin, total bilirubin, direct bilirubin, alanine transaminase, aspartate transaminase, creatinine, and uric acid. We found that all these biochemical markers were maintained at a normal level following Vγ9Vδ2 T-cell infusion (**Figure 3**). This suggests that allogeneic Vγ9Vδ2 T-cell therapy did not cause any impairments in liver and kidney functions, further indicating the clinical safety of allogeneic Vγ9Vδ2 T cells.

## Allogeneic Vγ9Vδ2 T-Cell Therapy Effectively Improved Pulmonary Lesions

All patients in our study received Vγ9Vδ2 T-cell infusions and concurrent anti-MDR-TB medications. We observed that lung lesions were reduced in all patients after the end of treatment (**Figure 4**). In particular, we noticed that the lung lesions were significantly decreased at the end of treatment compared with those before treatment in patients #2 through #6 (**Figure 4A**). Due to the simultaneous administration of Vγ9Vδ2 T cells and anti-TB drugs, it was difficult to estimate the efficacy of Vγ9Vδ2 T cells. The observed reduction in the lung lesions of patients might be attributed either to the anti-TB drugs, Vγ9Vδ2 T cells, or the synergism of both. However, we speculated that Vγ9Vδ2 T cells might have promoted the improvement of lung lesions.

Next, we evaluated the clinical status of two other patients to confirm our speculation. Patient #1 who received anti-TB treatment before Vγ9Vδ2 T-cell infusions showed progressive

**TABLE 1** | Preliminary information of the eight patients.

| | Gender | Age, years | Diagnosis | Case definition | Drug regimen | Times of cell therapy | Cell dosage |
|---|---|---|---|---|---|---|---|
| #1 | Female | 23 | MDR-TB | Retreatment | PZA, Mfx, Cs, Pto, Am | 12 | ~$1 \times 10^8$ |
| #2 | Male | 42 | MDR-TB | Retreatment | Lzd, Mfx, Cs, Cfz | 12 | ~$1 \times 10^8$ |
| #3 | Male | 47 | MDR-TB | Retreatment | Lzd, Mfx, Cs, Cfz, Pto | 12 | ~$1 \times 10^8$ |
| #4 | Male | 39 | MDR-TB | Retreatment | Mfx, Cs, Cfz, PAS | 12 | ~$1 \times 10^8$ |
| #5 | Female | 24 | MDR-TB | Retreatment | Lzd, Cs, Mfx, PZA, Am | 12 | ~$1 \times 10^8$ |
| #6 | Male | 50 | MDR-TB | Retreatment | Lzd, Cs, Mfx, PZA, Am | 12 | ~$1 \times 10^8$ |
| #7 | Male | 34 | MDR-TB | Retreatment | Lfx, Am, INH, PZA, EMB | 12 | ~$1 \times 10^8$ |
| #8 | Male | 41 | MDR-TB | Retreatment | * | 12 | ~$1 \times 10^8$ |

*MDR-TB, multidrug-resistant tuberculosis; Am, amikacin; Cfz, clofazimine; Cs, cycloserine; EMB, ethambutol; INH, isoniazid; Lzd, linezolid; Mfx, moxifloxacin; PAS, p-aminosalicylic acid; PZA, pyrazinamide; Pto, protionamide.*

**Only Vγ9Vδ2 T cells were used for treatment in four courses of therapy according to the requirement of the patient. Thereafter, anti-TB drugs (Lzd, Mfx, Cs, Cfz, propionyl isoniazid) were used.*

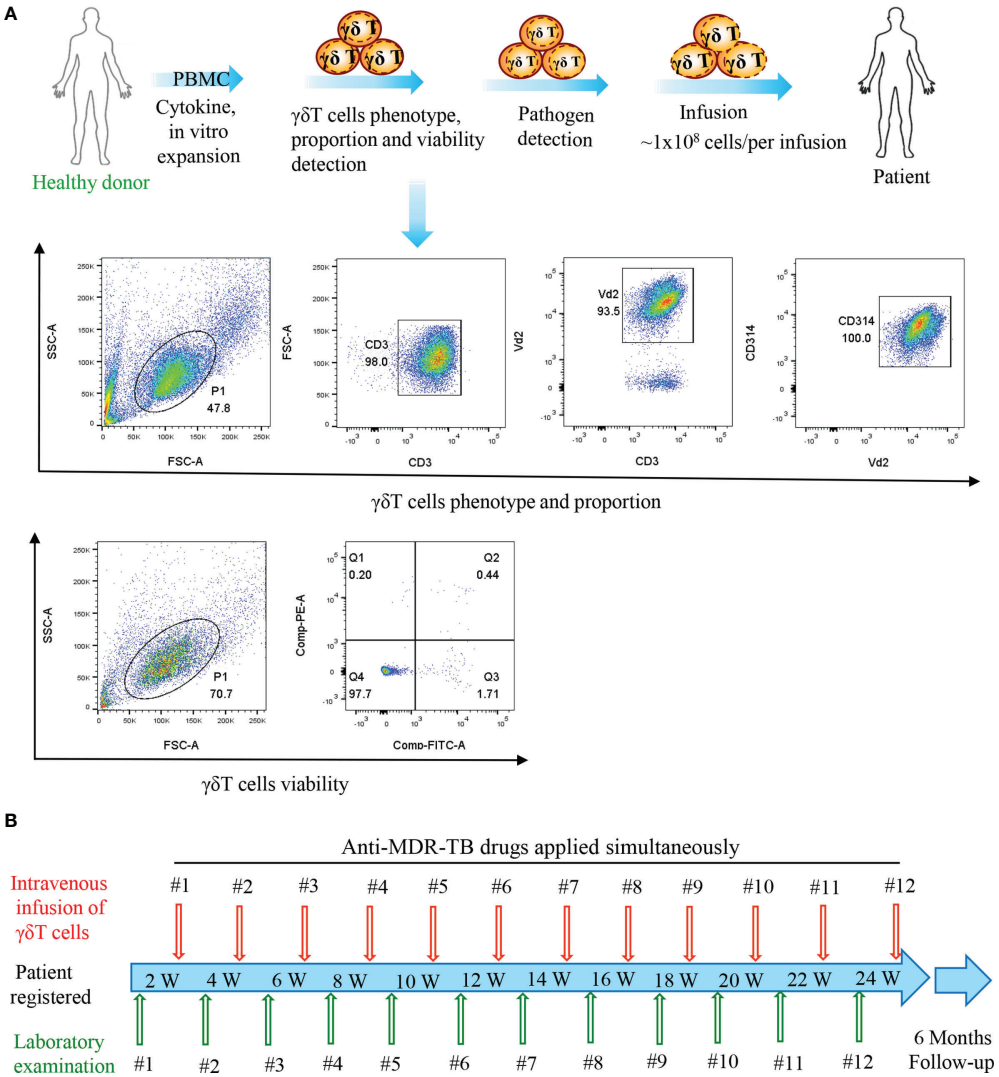


**FIGURE 1** | Flow diagram of the treatment of patients. **(A)** Vγ9Vδ2 T-cell expansion *in vitro*, quality control, and infusion. **(B)** Time points of Vγ9Vδ2 T-cell infusion and laboratory examination.

deterioration in lesion locations, as indicated by the red arrows in **Figure 4B** (first and second columns). We specifically found that the administration of allogeneic Vγ9Vδ2 T cells inhibited the progress of lesion locations during the process of the 12 courses of therapy, as indicated in **Figure 4B** (third through fifth columns). This finding endorsed the promising clinical efficacy of allogeneic Vγ9Vδ2 T-cell-based immunotherapy for MDR-TB and was further supported by the more convincing therapeutic effect seen in patient #8. According to contrast-enhanced axial CT images of the chest, patient #8 had severe cavitary pulmonary lesions, as indicated by the red arrows in **Figure 4C**. Cavitary lesions have a rough and discontinuous inner margin, implying the active state of *M. tuberculosis* that induced them. After three and four courses of cell therapy, we noticed that the size of the cavitary lesions was gradually and significantly reduced (second and third columns; **Figure 4C**), suggesting an observable therapeutic efficacy of allogeneic Vγ9Vδ2 T cells. Consecutively, both Vγ9Vδ2 T cells and anti-MDR-TB drugs were simultaneously administered in enrolled patients. Accordingly, we noticed the synergistic effects on the improvement of cavitary lesion or lesion locations of such a combined therapy strategy (fourth and fifth columns; **Figure 4C**).

## Allogeneic Vγ9Vδ2 T Cells Enhanced Some Immune Functions of Patients With MDR-TB

Because previously Vγ9Vδ2 T cells have been demonstrated to regulate the immune function of αβ T cells, NK cells, B cells, and other cells *in vivo* (10, 14, 15), it was crucial to analyze the immune phenotypes of all patients before and after the infusion

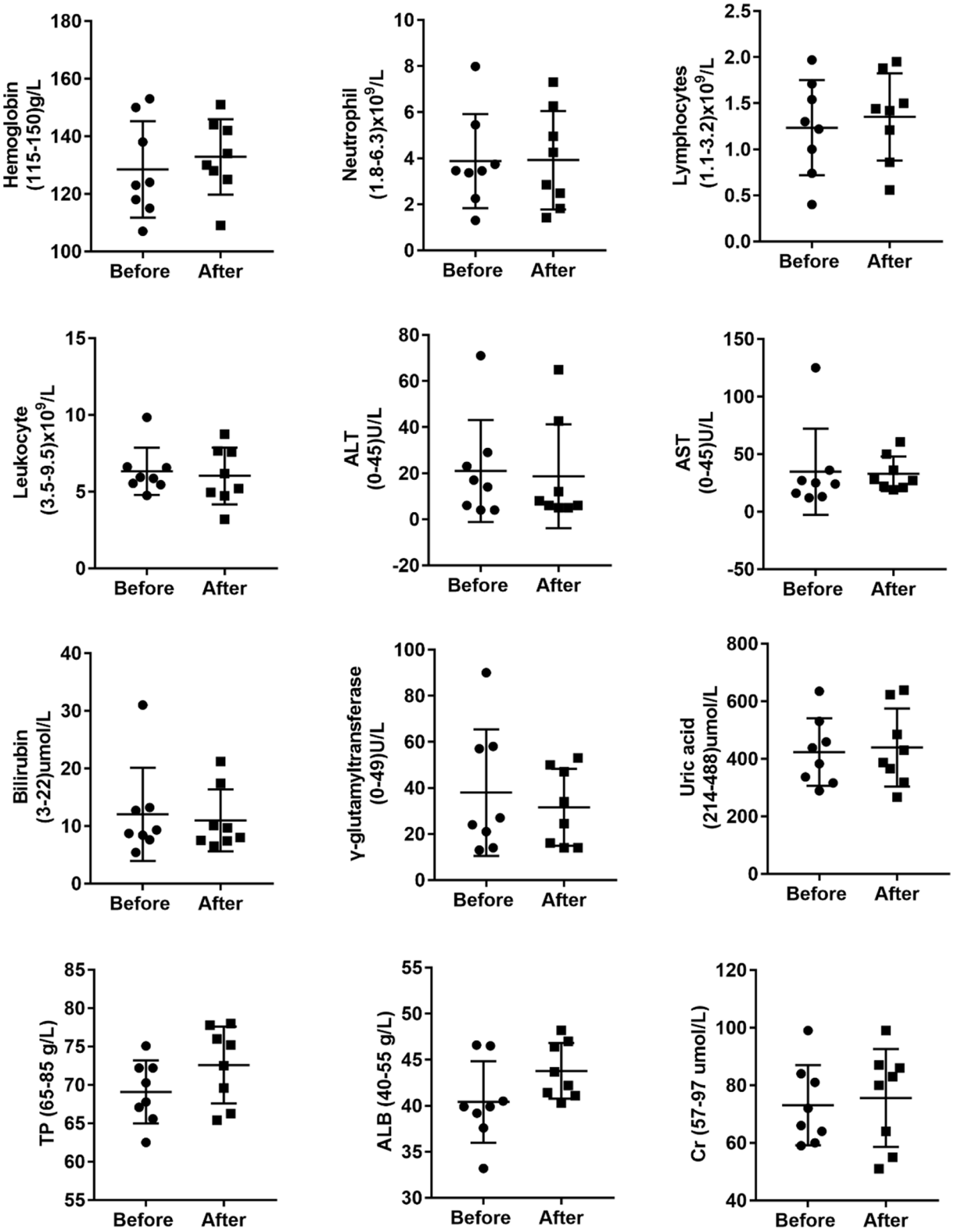


**FIGURE 2** | Allogeneic Vγ9Vδ2 T-cell therapy did not statistically reduce the clinical side effects induced by the application of anti-MDR-TB drugs. Side effects investigated before and after cell therapy included changes in hemoglobin, neutrophils, lymphocytes, leukocytes, alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin, γ-glutamytransferase, uric acid, total protein (TP), albumin (ALB), and creatinine (Cr) levels.

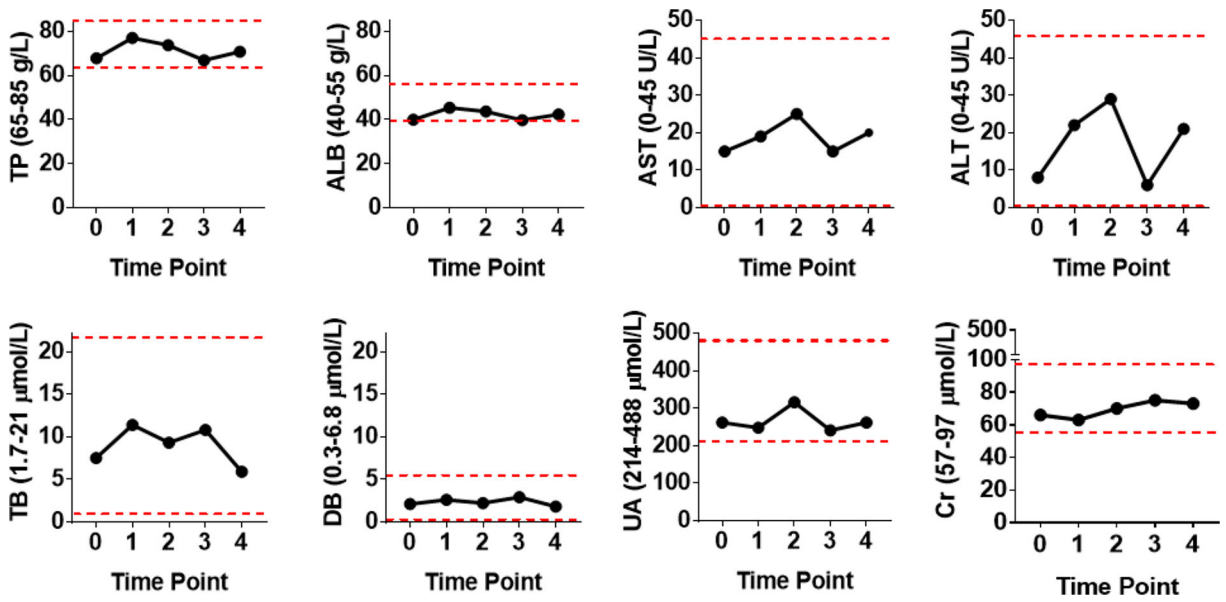


**FIGURE 3** | Blood biochemical examinations of patient #8 before and after receiving four courses of Vγ9Vδ2 T-cell immunotherapy. Assayed biochemical markers included total protein (TP), albumin (ALB), total bilirubin (TB), direct bilirubin (DB), alanine transaminase (ALT), aspartate transaminase (AST), uric acid (UA), and creatinine (Cr) levels. Red dashed lines stand for the normal reference range widely used in the clinical setting.

of Vγ9Vδ2 T cells (**Figure 5**). We found that the percentages of $CD3^+$, $CD4^+$, $CD8^+$, γδ T cells, B cells, and some cell subsets were not statistically different at the end of treatment compared with those before treatment. Conversely, the proportion of immature NK cells that were $CD3^-CD56^+$hi was significantly reduced with a concomitant significant increase in the proportion of mature NK cells that were $CD3^-CD56^+$lo. The data suggested that allogeneic Vγ9Vδ2 T-cell therapy partially enhanced host immune functions by regulating the proportion of mature NK cells.

We also analyzed the immune phenotypes of patient #8 before and after his limited Vγ9Vδ2 T-cell therapy (4 courses instead of 12; **Figure 6**). We found that although the percentage of $CD3^+$, $CD4^+$, $CD8^+$ T cells, total B cells, and naive B cells ($CD3^+CD19^+CD27^-IgD^+$) varied, it remained relatively stable during the courses of cell therapy. By contrast, we observed that the proportion of effective memory $CD4^+$ T cells ($CD3^+CD4^+CD45RA^-CCR7^-$), effective memory $CD8^+$ T cells ($CD3^+CD8^+CD45RA^-CCR7^-$), helper T-cell subsets ($CD3^+CD4^+CXCR5^-CD183^-CD194^-CD196^+$), cytotoxic $CD8^+$ T cells ($CD3^+CD8^+CXCR5^-CD183^-CD194^-CD196^+$), homing memory $CD8^+$ T cells ($CD3^+CD8^+HLADR^+$), and NK cells ($CD3^-CD56^+CD94^-KIR^+$ and $CD3^-CD56^+NKP30^+$) was increased. Notably, the proportion of PD-$1^+CD8^+$ T cells ($CD3^+CD8^+PD\text{-}1^+$) was significantly decreased after cell therapy. As for γδ T cells, we noticed that the proportion of both total and $V\delta2^+$ γδ T cells was maintained at a relatively high level following the γδ T-cell infusions. These results indicated that allogeneic Vγ9Vδ2 T-cell therapy enhanced some immune functions by regulating the proportion of αβ T cells and NK cells.

## Allogeneic Vγ9Vδ2 T-Cell Therapy Potentially Benefited Body Weight Gain and Gut Microbiota Regulation

Loss of body weight is one of the clinical manifestations in patients with TB. Accordingly, we evaluated the changes in the body weight of all patients before and after cell therapy (**Figure 7**). We specifically observed that 50% of patients had gained weight, whereas 50% exhibited weight loss. Because both the drugging regimen and nutritional status varied across patients, it was difficult to estimate the specific effect of Vγ9Vδ2 T cells in the changes in the overall body weight of patients. Interestingly, we observed a body weight increase from 46 to 49 kg in patient #8 after only four courses of Vγ9Vδ2 T-cell therapy (**Supplementary Figure 2A**). This suggested that cell therapy might improve the overall physical condition of patients. In addition, the metabolite assay in patient #8 revealed that the levels of three carnitine components in blood plasma, which are associated with fatty acid metabolism, namely, decanoyl L-carnitine (2.6–3.8-fold), octanoyl carnitine (1.9–3.2-fold), and dodecanoyl carnitine (1.7–2.6-fold), were greatly reduced (**Supplementary Figures 2B–D**). These results implied that cell therapy might increase the body weight of patients by altering carnitine metabolism.

In the past, gut microbiota have been increasingly associated with host recovery, prevention of *M. tuberculosis* infection, and resistance or clinical efficacy of anti-TB drugs (16–20). Therefore, we investigated whether the limited and only Vγ9Vδ2 T-cell therapy in patient #8 would alter the composition of gut microbiota. We collected two stool samples from patient #8 before and after cell therapy and analyzed them

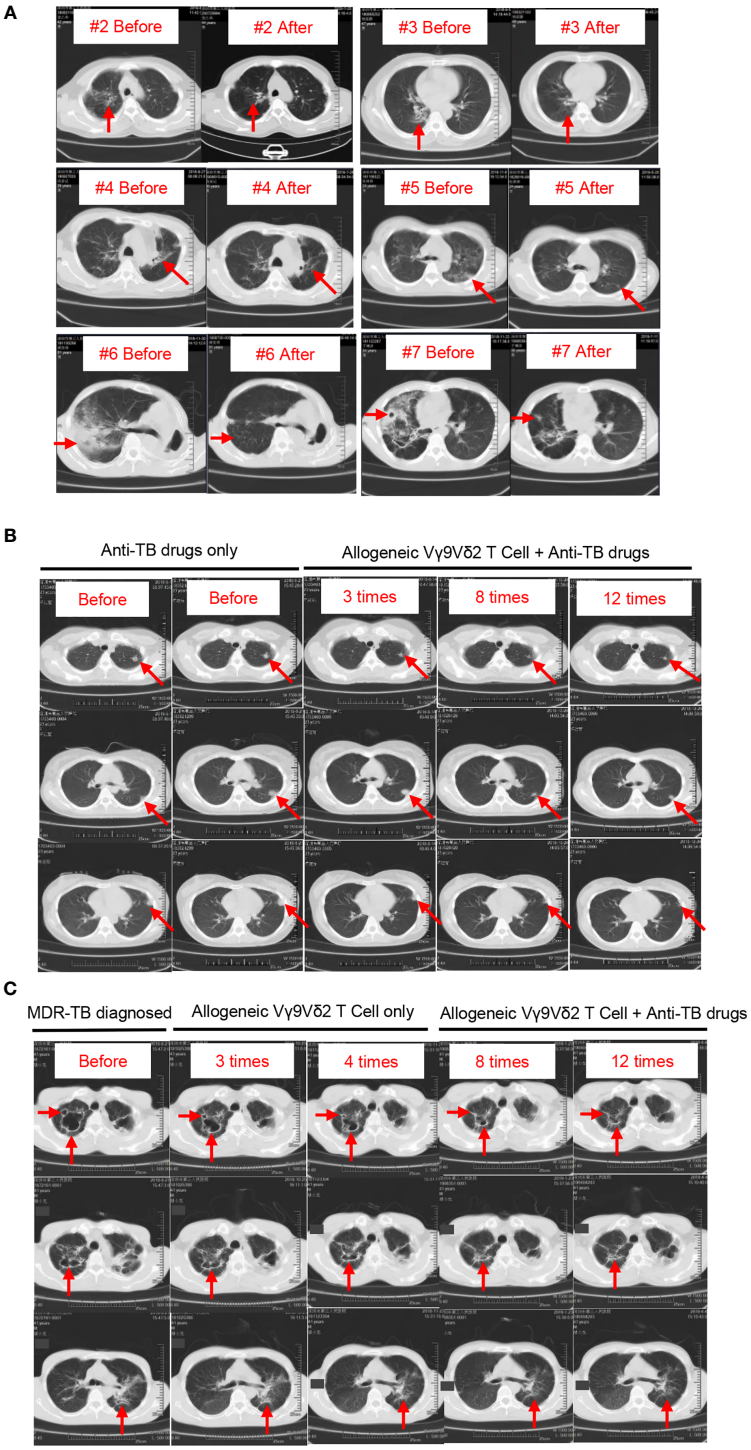


**FIGURE 4** | Chest CT images of the eight patients with MDR-TB before and after treatment. Pathological sites are indicated by red arrows. **(A)** Chest CT images of patients #2 through #6. After enrollment, these patients received Vγ9Vδ2 T-cell infusions and treatment with anti-TB drugs. CT images of two time points, one before and one after treatment. **(B)** Chest CT images of patient #2. This patient received anti-TB drugs before enrollment, and CT images show the progressive deterioration in lesion locations (first and second columns). After enrollment, this patient continued to receive anti-TB drugs (drug recipe not changed) plus Vγ9Vδ2 T-cell infusions. The size of pathological sites was gradually reduced with the progress of cell therapy (third through fifth columns). **(C)** Chest CT images of patient #8. The progressive repair of the cavitary lesion in pulmonary pathological sites after four courses of cell therapy alone (first through third columns) is shown. In the case of combined treatments with Vγ9Vδ2 T cells plus anti-MDR-TB drugs, pulmonary lesions continued to decrease (fourth and fifth columns).

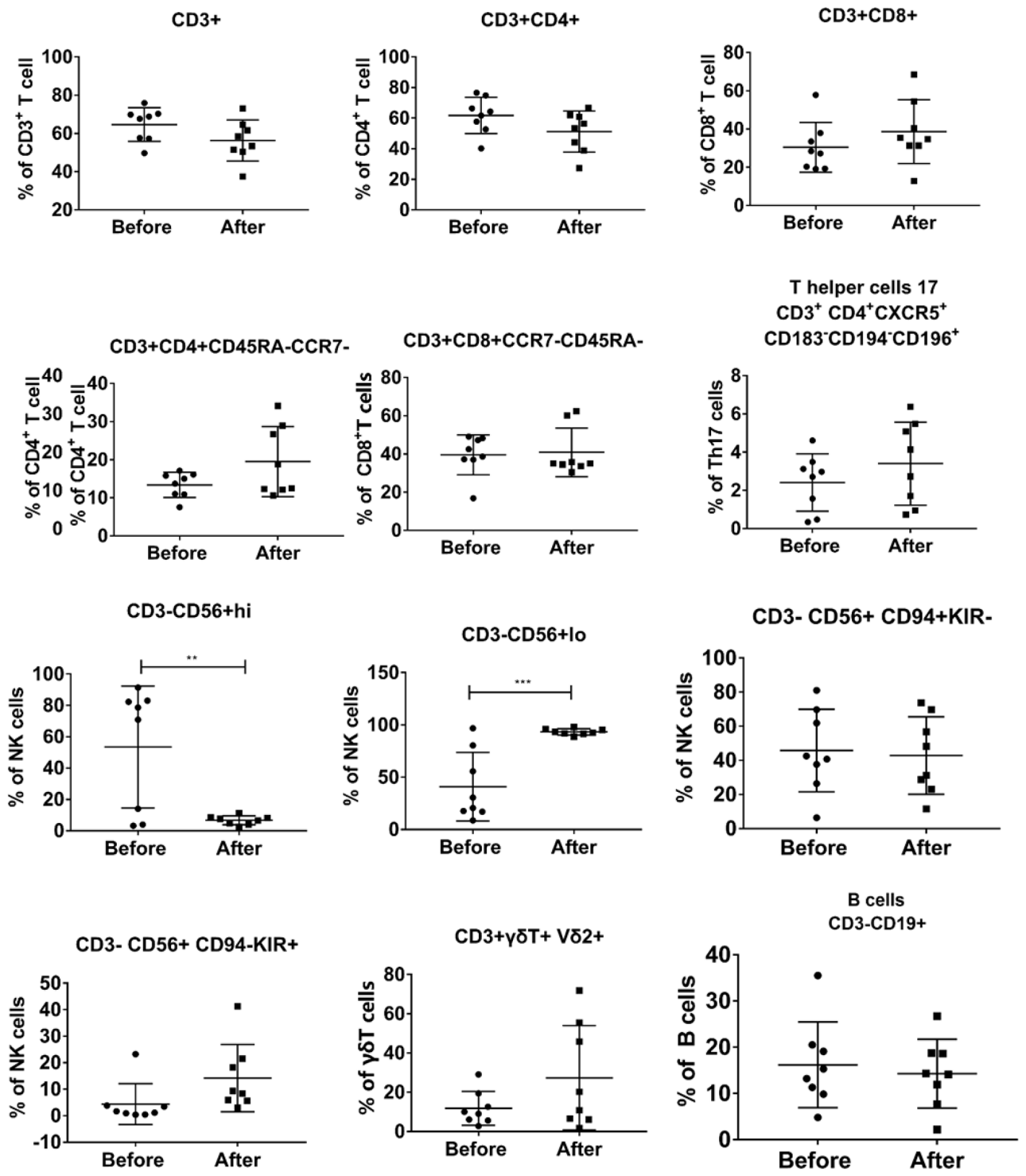


**FIGURE 5** | Evaluation of the changes in the phenotype of peripheral blood immune cells before and after Vγ9Vδ2 T-cell immunotherapy in the eight patients. PBMCs were collected from all patients before and after Vγ9Vδ2 T-cell infusions. Immunological phenotypes, which were analyzed using flow cytometry, included $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, NK cells, γδ T cells, B cells, and their subsets.

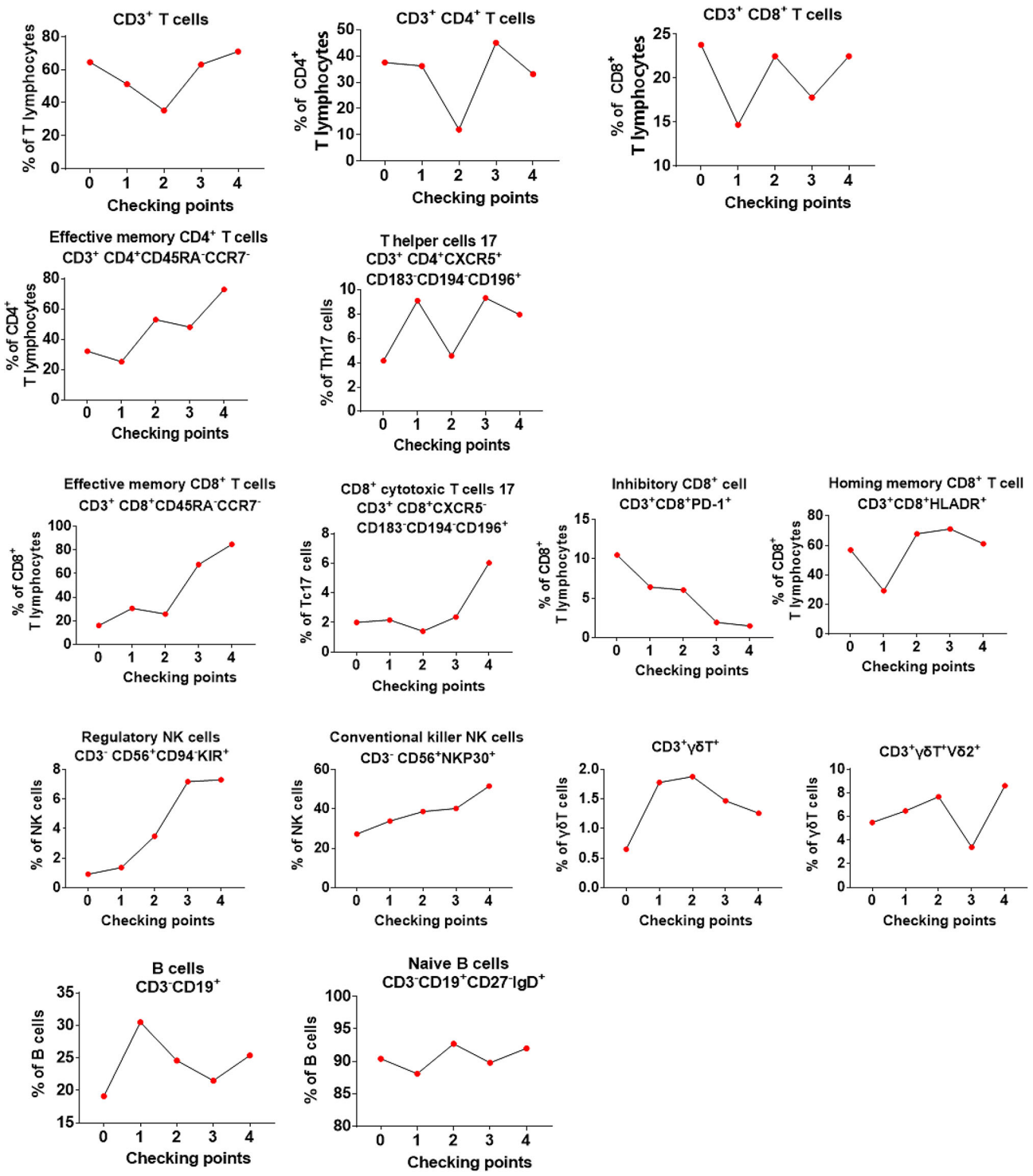


**FIGURE 6** | Evaluation of the changes in the phenotype of peripheral blood immune cells for the first 4 courses of Vγ9Vδ2 T-cell therapy in patient #8. In the graphs, point "0" indicates the immunophenotype before Vγ9Vδ2 T-cell treatment, whereas "1–4" indicates the immunophenotype from the first time to the fourth time of cell treatment.

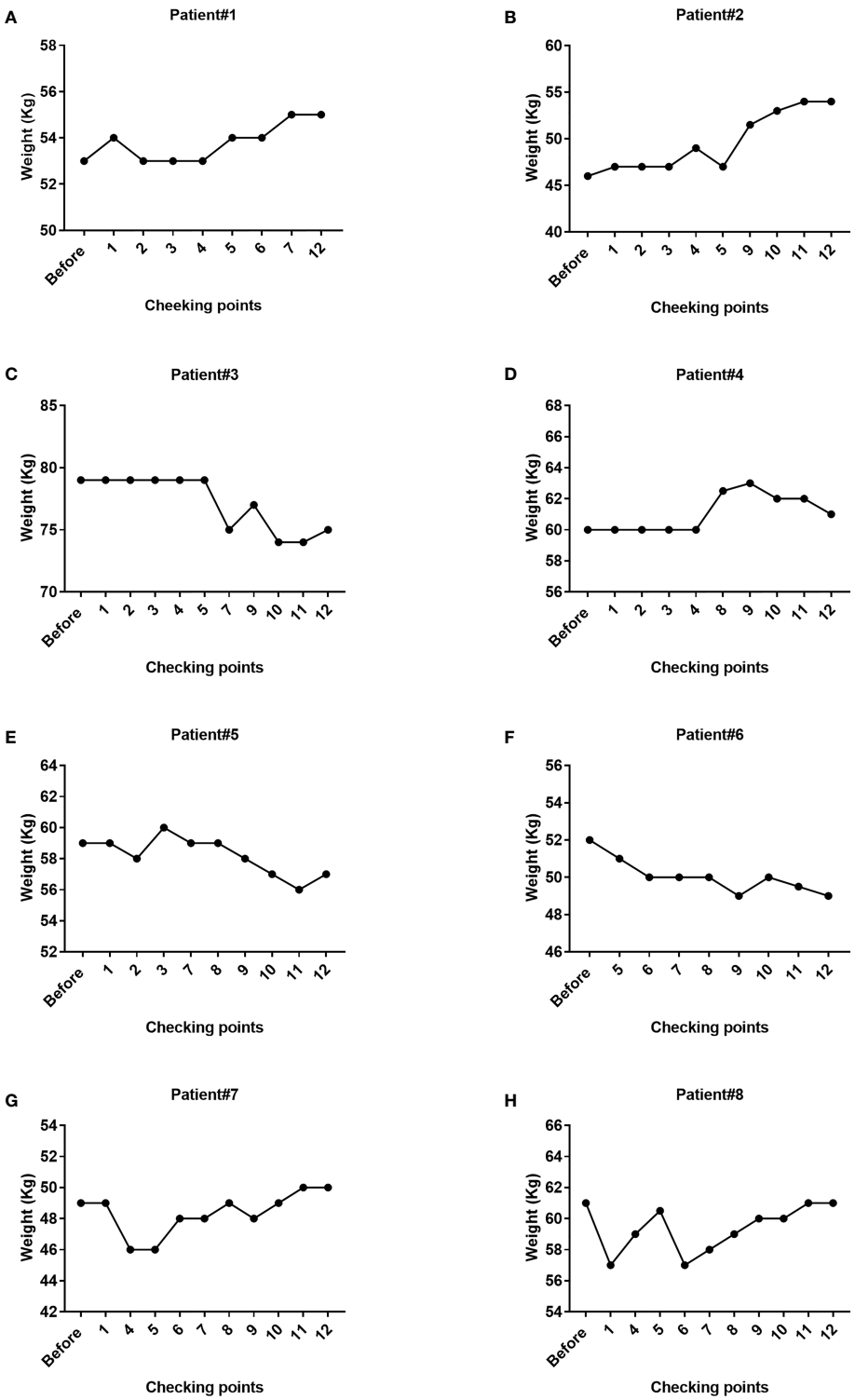


**FIGURE 7** | Changes in the body weight of the eight patients during Vγ9Vδ2 T-cell immunotherapy. The weight of patient #1, patient #2, patient #7, and patient #8 steadily increased, whereas the weight of patient #3, patient #5, and patient #6 decreased. The overall trend in patient #4 was a tendency for an increase in body weight. The weight of partial patients was not available at the designated checking points due to missing weight assessments.

**TABLE 2** | Sputum *Mycobacterium tuberculosis* examination of all patients before and after Vγ9Vδ2 T-cell immunotherapy.

| | | #1 | #2 | #3 | #4 | #5 | #6 | #7 | #8 |
|---|---|---|---|---|---|---|---|---|---|
| Sputum smear (acid-fast bacillus) | Before | – | + | – | + | – | + | + | + |
| Sputum *M. tuberculosis* culture | Before | + | – | + | + | + | + | + | + |
| Sputum smear (acid-fast bacillus) | After | – | – | – | – | – | – | – | – |
| Sputum *M. tuberculosis* culture | After | – | + | – | – | – | – | – | – |

*For sputum acid-fast bacillus smear examination, "–" means M. tuberculosis was not detected in 300 consecutive fields under the microscope (×1,000), whereas "+" means 3 to 9 M. tuberculosis were found in 100 consecutive fields under the microscope (×1,000). Regarding the sputum culture method, "+" indicates the presence of M. tuberculosis in the sputum.*

using 16S rRNA gene sequencing. We found increases in 10 types of commensal bacteria or probiotics, whereas the population of seven types of pathogenic bacteria was decreased (**Supplementary Figure 3**). This finding implied that allogeneic Vγ9Vδ2 T-cell therapy might also regulate gut immunity by altering the composition of intestinal microbiota, eventually benefiting the therapeutic efficacy of cell therapy in the patient.

## Allogeneic Vγ9Vδ2 T-Cell Therapy Contributes in Alleviating the *Mycobacterium tuberculosis* Load *In Vivo*

As the *M. tuberculosis* load is a key indicator for evaluating therapeutic efficacy, we performed sputum *M. tuberculosis* detection in all patients (**Table 2**). We observed that *M. tuberculosis* was detected in the sputum of patients regardless of the methods used (sputum smear acid-fast bacillus testing or sputum culture testing) before Vγ9Vδ2 T-cell therapy. After all patients had finished therapy, patient #2, who had severe MDR-TB, still had a *M. tuberculosis*-positive sputum culture. However, the lesions of patient #2 were significantly decreased. The sputum *M. tuberculosis* turned to be negative 3 times consecutively in the remaining seven patients. According to the criteria of the World Health Organization, a 3 times consecutive negative detection of sputum *M. tuberculosis* indicates complete clinical control of *M. tuberculosis* infection. Although we could not determine which component (Vγ9Vδ2 T cells or drugs) played the critical role against *M. tuberculosis*, the cure prevalence proportions were up to 87.5% when patients were treated with Vγ9Vδ2 T cells plus anti-MDR-TB drugs. According to the "Global tuberculosis report 2020" of the World Health Organization, the cure prevalence proportions were 54% when patients were treated with only anti-MDR-TB drugs. Obviously, the results demonstrate that allogeneic Vγ9Vδ2 T-cell therapy contribute in alleviating the *M. tuberculosis* load *in vivo*.

# DISCUSSION

At present, drug resistance is the most serious challenge for the treatment of tuberculosis. To address this issue, the development of new antituberculosis drugs is urgently needed. Cellular immunotherapy is a new and promising clinical treatment approach. Currently, almost all types of immune cells, such as $CD4^+$ T cells, $CD8^+$ T cells, NK cells, B cells, myeloid cells, and γδ T cells, have been used as a cellular platform for the development of immunotherapies for various diseases. Among these immune cell candidates, γδ T cells have been increasingly recognized as a new strategic tool for immunotherapy of both tumor and infectious diseases (10, 14, 15, 21–23).

Of note, γδ T cells, specifically the Vγ9Vδ2 subset explored in this study, are unconventional innate immune T cells, which play a significant role in controlling diverse diseases, including bacterial and viral infections, as well as tumorigenesis. Regarding tuberculosis, several published studies have demonstrated a significant reduction in the population of Vγ9Vδ2 T cells in the peripheral blood and alveolar lavage fluid of patients with active pulmonary tuberculosis, indicating that Vγ9Vδ2 T cells play a protective role in tuberculosis (24, 25). Previous studies have also shown that Vγ9Vδ2 T cells recognize *M. tuberculosis*-derived phosphoantigens and 6-O-methylglucose-containing lipopolysaccharides, which, in turn, activate and expand the population of Vγ9Vδ2 T cells (26–29). Activated Vγ9Vδ2 T cells contribute to both innate and adaptive immune responses *via* the secretion of cytokines and the release of cytolytic effectors. In general, Vγ9Vδ2 T cells produce cytokines such as interferon-γ, tumor necrosis factor-α, and IL-17 to enhance the protection against tuberculosis (30–36). Moreover, Vγ9Vδ2 T cells also produce cytolytic effector molecules such as perforin, granzyme B, and granulysin to help kill or inhibit intracellular and extracellular *M. tuberculosis* (12, 27, 32, 37–39). In the *M. tuberculosis*-infected macaque model, the histopathologic changes in pulmonary and extrapulmonary tissues were significantly attenuated by the adoptive transfer of autologous Vγ2Vδ2 T cells (Vγ9Vδ2 T cells) (11). A recent study reported that Vγ2Vδ2 T cells that were selectively immunized with HMBPP-producing attenuated *Listeria monocytogenes* (Lm ΔactA prfA*) in a rhesus TB model triggered rapid responses and long-lasting memory-like responses while amplifying the responses of other T-cell subsets (12). The result was a significant decrease in lung pathological indications and *M. tuberculosis* extrapulmonary dissemination.

Tuberculosis is a chronic infectious disease that usually does not show obvious symptoms until the total compromise of host immune homeostasis. Therefore, targeting immunoregulatory pathways might be a new strategy of anti-TB treatment. Given the reduction in the function of autologous Vγ9Vδ2 T cells and their unique property of targeting infected cells through MHC-unrestricted mechanisms without inducing graft-versus-host disease (40), we innovatively used allogenic Vγ9Vδ2 T cells to treat patients with MDR-TB in our study based on their previously demonstrated clinical safety (10, 14). According to our clinical observations, a total of 96 courses of allogenic Vγ9Vδ2 T-cell infusions in eight patients with MDR-TB exhibited no serious adverse events. In particular, four courses

of allogenic Vγ9Vδ2 T cells alone were sufficient to treat patient #8 without causing any liver or kidney function impairment. These results indicated the clinical safety of allogenic Vγ9Vδ2 T cells in patients with TB. The results of the present study along with those of previously published studies (10, 14) have collectively demonstrated that allogenic Vγ9Vδ2 T cells are safe and could be efficiently used in clinical practice.

Tuberculosis is usually accompanied by distinct inflammatory characteristics, which induce the formation of pulmonary lesions. According to previous reports, adoptive transfer of Vγ2Vδ2 T cells in a *M. tuberculosis*-infected macaque model significantly attenuated TB lesions (11), suggesting the efficacy of Vγ2Vδ2 T-cell transfer in controlling inflammation. The inhibitory effect of γδ T cells on inflammation was also reported in a mouse model (41). Our clinical observations of all eight patients with MDR-TB suggested the inhibitory effect of Vγ9Vδ2 T cells on *M. tuberculosis*-induced pulmonary inflammation/lesions regardless of being combined with anti-TB drugs or administered alone. Accordingly, we demonstrated that allogeneic Vγ9Vδ2 T-cell-based immunotherapy was efficient in relieving pulmonary lesions.

Given that the immune function of patients with TB is generally impaired, and CD4 and CD8 T cells have an important role in controlling TB infection (42, 43), an evaluation of the functional phenotypes of circulating immune cells is needed in the context of Vγ9Vδ2 T-cell therapy. By analyzing the functional phenotypes of immune cell data in eight patients before and after Vγ9Vδ2 T-cell therapy, we discovered that the proportion of immature NK cells was significantly reduced, and the proportion of mature NK cells was significantly increased. There was no visible change in the proportion of other cells. The results implied that allogeneic Vγ9Vδ2 T cells enhanced NK cell functions of patients with MDR-TB.

In parallel, we also analyzed the functional phenotypes in the immune cell data of patient #8 who only received limited courses (4) of allogeneic Vγ9Vδ2 T-cell therapy. We discovered that the populations of functional subsets of CD4 and CD8 T cells, such as effective memory $CD4^+$ T cells, effective memory $CD8^+$ T cells, helper T cells, and killer T cells, were increased after adoptive transfer of allogeneic Vγ9Vδ2 T cells. In addition, the PD-1 expression of CD8 T cells was significantly reduced, further endorsing our assumption that PD-$1^+CD8^+$ T cells are favorable factors for *M. tuberculosis* infection (44). Intriguingly, we also found that the NK cell subsets were dramatically increased after adoptive transfer of allogeneic Vγ9Vδ2 T cells, further indicating the beneficial effect of cell therapy for patients with TB, as patients with pulmonary tuberculosis show a reduced frequency of NK cell subsets (45). There has always been some controversy on the role of humoral immunity in TB. The contribution of B cells and antibodies in the protection against *M. tuberculosis* infection remains unclear (46). Here, we also observed no great changes in the populations of B cells before and after cell therapy. Altogether, our study indicated a certain regulatory effect of Vγ9Vδ2 T cells on cellular immunity.

Body weight was also used as an observational indicator though not a significant indicator during treatment of TB. We noticed that the body weight of half of the patients steadily increased, whereas the other half lost weight. Due to the low number of patients and the variation in their drugging regimens and nutritional status, we could not determine whether these changes in body weight were associated with Vγ9Vδ2 T-cell therapy. An increasing number of studies have shown that host lipid metabolism is involved in antituberculosis immunoregulation. For example, *M. tuberculosis* has been shown to impair mitochondrial fatty acid oxidation through the miR-33 host miRNA (47). A previous study in *M. tuberculosis*-infected macaques noted that adoptive transfer therapy of Vγ2Vδ2 T cells stabilized the body weight of macaques (11), implying that γδ T-cell-based immunotherapy regulated the host metabolism. Interestingly, we noted that the body weight of patient #8, who received four courses of allogeneic Vγ9Vδ2 T-cell therapy alone, was increased in a stepwise manner. Moreover, we also found that plasma carnitine concentration was decreased. Carnitine is known to not only promote lipid metabolism but also reduce the levels of triglycerides, total cholesterol, and LDL-cholesterol (48). Although these data were derived from only a single patient, it suggested that allogenic Vγ9Vδ2 T cells might favor antituberculosis treatment through the regulation of lipid metabolism. More studies will be needed in the future to support this observation.

Microbiota have been found to be closely related to the occurrence and development of many diseases (49–52). For instance, dysbiosis of intestinal microbiota aggravated pulmonary *M. tuberculosis* infection in a mouse model, resulting in increased *M. tuberculosis* extrapulmonary transmission (19). In this study, we only analyzed one precell therapy sample and one postcell therapy sample from patient #8 using 16S rRNA gene sequencing. We found that the population of commensal bacteria or probiotics of the patient was increased, whereas the population of pathogenic bacteria was decreased. This result suggested that allogeneic Vγ9Vδ2 T-cell therapy alters the constitution of intestinal microbiota in patients with MDR-TB. However, these findings need further investigation in future clinical trials.

*Mycobacterium tuberculosis* load is an important determinant for the evaluation of therapeutic efficacy. We also monitored changes in *M. tuberculosis* sputum using both sputum smear acid-fast bacillus and sputum culture testing. At the completion of the treatment period, the sputum *M. tuberculosis* of seven patients was detected to be negative in three consecutive readings. However, the sputum *M. tuberculosis* of patient #2, whose disease was very severe, remained positive at the end of Vγ9Vδ2 T-cell treatment. Clinically, the cure prevalence proportions were 54% for MDR-TB patients. Strikingly, in our study cohort, the cure prevalence proportions were 87.5% for MDR-TB patients who were treated with Vγ9Vδ2 T cells plus anti-MDR-TB drugs. In contrast, the therapeutic efficacy of Vγ9Vδ2 T cells plus anti-MDR-TB drugs was notably higher

than anti-MDR-TB drugs only. We considered that using Vγ9Vδ2 T-cell therapy could alleviate the *M. tuberculosis* load *in vivo*.

In conclusion, this clinical trial creatively applied allogenic Vγ9Vδ2 T cells for the treatment of patients with MDR-TB and clearly demonstrated that allogenic Vγ9Vδ2 T-cell infusion is safe in the clinical setting. Based on the results of the CT images of the patient, the assessment of peripheral immune cell function, and sputum *M. tuberculosis* detection, we believe that Vγ9Vδ2 T-cell therapy is effective. Taken together, this trial provided a new direction for biotherapy of MDR-TB and other hard-to-treat infectious diseases, such as TB/HIV and TB/HBV infections. However, due to the limitations of single-arm studies, the number of patients enrolled was limited. In the future, we aim to design a rigorous and standardized randomized controlled clinical trial to systematically and multidimensionally evaluate the therapeutic efficacy of Vγ9Vδ2 T cells.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI BioProject and PRJNA776411.

## ETHICS STATEMENT

The Regional Ethics Committee of Shenzhen Third People's Hospital approved the study protocol (Approval ID SZLY2018017). The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

## AUTHOR CONTRIBUTIONS

Protocol design: ZY, YZW, GZ, and GD. Cell expansion and quality control: JLiang, ML, and YZW. Clinical therapy for patients: GD and LF. Immuno-function testing and statistics analysis: JLiang, LF, YZW, ZY and GZ. Patient recruitment: GD, JLiang, YZW, LF, ZY, SL, PZ, ZW, and LLiu. Patient follow-up: JLiang, YZW, ML, FL, GD and GZ. Manuscript writing and revision: YZW, JL, ZY. Other co-authors contributed to either scientific inputs/comments or patient follow-up for this long-term clinical trial project.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.756495/full#supplementary-material

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# Prevalence of Pure Red Cell Aplasia Following Major ABO-Incompatible Hematopoietic Stem Cell Transplantation

*Panpan Zhu*[1,2,3,4], *Yibo Wu*[1,2,3,4], *Dawei Cui*[5], *Jimin Shi*[1,2,3,4], *Jian Yu*[1,2,3,4], *Yanmin Zhao*[1,2,3,4], *Xiaoyu Lai*[1,2,3,4], *Lizhen Liu*[1,2,3,4], *Jue Xie*[5], *He Huang*[1,2,3,4] *and Yi Luo*[1,2,3,4]*

[1] Bone Marrow Transplantation Center, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China, [2] Liangzhu Laboratory, Zhejiang University Medical Center, Hangzhou, China, [3] Institute of Hematology, Zhejiang University, Hangzhou, China, [4] Zhejiang Province Engineering Laboratory for Stem Cell and Immunity Therapy, Hangzhou, China, [5] Department of Blood Transfusion, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China

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*Alice Bertaina,*
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*Xiangyu Zhao,*
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*St. Jude Children's Research Hospital, United States*

***Correspondence:**
*Yi Luo*
*luoyijr@zju.edu.cn*

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**Background:** Pure red cell aplasia (PRCA) is one of the important complications in major ABO-incompatible allogeneic hematopoietic stem cell transplantation (HSCT). The established pathogenic factor of PRCA is the persistence of high anti-donor isohemagglutinins. As previously verified, the conditioning regimen and donor type were the factors associated with the development of PRCA in the small-sized studies. Currently, the prevalence, risk factors, and prognosis of PRCA are still worth studying to provide evidence.

**Methods:** We conducted a prospective nested case-control study to determine the prevalence, donor-related factors, and the outcomes of PRCA following major ABO-incompatible transplantation. A total of 469 patients who underwent ABO-incompatible grafts were observed.

**Results:** None of the patients were diagnosed with PRCA with minor or bidirectional ABO-incompatible HSCT. Thirteen of the187 patients (7%; 95% confidence interval [CI], 3.9%–11.9%) developed PRCA following major ABO-incompatible HSCT. Eleven of the 13 patients with PRCA recovered entirely. Donor type was an independent factor associated with post-HSCT PRCA (odds ratio [OR]=0.030; 95% CI, 0.003–0.321; *P*=0.004). The cumulative incidence rates of post-HSCT PRCA in the context of major ABO-incompatible HSCT were 0.8%, 13.1%, and 27.2% for the haploidentical donor (HID), unrelated donor, and matched related donor, respectively. No significant influence of PRCA on transplantation outcomes was observed.In conclusion, post-HSCT PRCA is a rare and less threatening complication in major ABO-incompatible HSCT. The majority of patients with PRCA could recover. Additionally, HIDs for recipients may have a low risk of post-HSCT PRCA. This trial was registered at www.chictr.org.cn (#ChiCTR2000041412).

**Keywords: pure red cell aplasia, major ABO-incompatible transplantation, haploidentical donor, allogeneic hematologic stem cell transplantation, isohemagglutinin**

## INTRODUCTION

ABO-blood group incompatibility occurs in 25% to 50% of human leukocyte antigen-matched hematopoietic stem cell transplantation (HSCT) (1). Although ABO incompatibility has no effect on transplantation survival and relapse of underlying disease (2–4), hemolysis, the delayed red blood cell engraftment, and pure red cell aplasia (PRCA) have potential clinical consequences following ABO-incompatible transplantation (5, 6).

PRCA is a critical complication in patients undergoing major ABO-incompatible HSCT, which may result in significant iron overload and increase non-relapse mortality after HSCT (7). The incidence rate of PRCA after ABO-mismatched transplantation ranged from 7% to 30% (7–9). The mechanism of post-HSCT PRCA was not clear enough until now. It was reported that the persistence of anti-donor isohemagglutinins (ISO) produced by recipient plasma cells may contribute to post-HSCT PRCA (6, 10). However, a few patients with a low pre-HSCT isohemagglutinin titer are likely to develop post-HSCT PRCA (11), which may be attributed to the rebound of anti-donor ISO and the post-HSCT transfusion of recipient-type RBC (12–14).

To the best of our knowledge, patients with type O blood who received the grafts from the donors with type A blood were at high risk for post-HSCT PRCA (8, 15, 16). Compared to type B blood antigen, there is more intensive type A blood group antigen on red blood cell (RBC) membrane, which leads to an increased complement-fixing capacity of red-cell-bound anti-A under the circumstance of a high level of anti-donor isohemagglutinins (17–19). Besides, delayed donor erythropoiesis was more common in reduced-intensity HSCT, which was associated with prolonged persistence of host anti-donor isohemagglutinins (6). In an ABO-incompatible HSCT study using 296 matched related donors (MSDs) and 420 matched unrelated donors (URDs), it was demonstrated that the graft-versus-plasma cell effect plays an essential role in the disappearance rate of anti-donor isohemagglutinins (20). However, this large study lacked of the information on post-HSCT PRCA. A previous study on post-HSCT PRCA was restricted to small sample size (21). Hence, the prevalence, risk factors, and prognosis of PRCA are still worth studying to provide evidence.

Here, a prospective nested case-control study was conducted to determine the prevalence, donor-related factors, and the outcomes of PRCA following major ABO-incompatible transplantation.

## METHODS

### Patient

Patients participated in this prospective observational study at the First Affiliated Hospital of Zhejiang University School of Medicine between August 1, 2014, and June 30, 2020. The final day of the last follow-up for all the surviving patients was October 31, 2020. Patients who underwent ABO-incompatible HSCT consecutively during this period were observed, and the study cohort comprised patients who received major ABO-incompatible grafts, as shown in **Figure 1**. Patients aged 15 years below, diagnosed with aplastic anemia before HSCT, and those who died within 2 months post-HSCT were excluded. For each PRCA case, four matched controls without PRCA were randomly selected from the same cohort at the onset of PRCA and were matched according to the patient's age (± 5years) and patient's sex. All patients provided their written informed consent in accordance with the Declaration of Helsinki. This study was approved by the Ethics Review Committee of the First Affiliated Hospital of Zhejiang University School of Medicine.

### Transplantation Procedure

Patients received myeloablative conditioning (MAC) or reduced-intensity conditioning (RIC). MAC consisted of busulfan and cyclophosphamide (BUCY) or modified BUCY according to the disease features and transplantation patterns described previously (22). RIC consisted of fludarabine and busulfan or fludarabine and cyclophosphamide. Graft-versus-host disease (GVHD) prophylaxis consisting of cyclosporin A, methotrexate, and low-dose mycophenolate mofetil was administered to patients. Rabbit anti-thymocyte globulin (ATG; Thymoglobulin, Genzyme, Cambridge, MA, USA) or anti-T-lymphocyte globulin (ATG-F; Fresenius, Bad Homburg, Germany) was administered. Grafts derived from peripheral blood were mobilized with recombinant human granulocyte colony-stimulating factor (5–7.5 μg/kg/d; Filgrastim; Kirin, Japan) for 5 to 6 consecutive days from day –4. All patients received unmanipulated grafts.

### ABO Blood Group and Anti-Donor Isohemagglutinins

The whole blood collected in ethylenediaminetetraacetic acid was used to determine the ABO forward/reverse typing. Anti-donor isohemagglutinins (ISO, anti-A IgG or IgM, anti-B IgG or IgM) were determined by incubating a 3% standard A and B erythrocyte suspension (RBC kits for human ABO reverse typing, Kinghaw, China) in saline with twofold serial dilutions of serum followed by centrifugation. Anti-donor isohemagglutinin titers were scored with a microscope for IgM and with a macroscope for IgG using an anti-human globulin test card (D.G. Gel Coombs, Diagnostic Grifols, S.A., Spain). Anti-donor isohemagglutinin titers were monitored at the time of stem cell transfusion (pre-HSCT ISO) and at 4 months post-transplantation (post-HSCT ISO) in 28 patients among this cohort, which comprised 7 patients with PRCA and 21 patients without PRCA.

### Definitions and Endpoints

Post-transplantation PRCA diagnosis was established if persistently severe normocytic anemia, reticulocytopenia, and absence of erythroblasts from otherwise normal bone marrow occurred for more than 60 days post-HSCT, which occurred in the absence of leukemia relapse, drug toxicity, or infection (11, 23). Primary post-HSCT PRCA was distinguished before the initial engraftment of red cells; otherwise, it was considered a secondary post-HSCT PRCA. The titer index was defined as the dilution time of the anti-donor isohemagglutinins. Neutrophil engraftment was defined as the first day of 3 consecutive days of an absolute neutrophil count $> 0.5 \times 10^9$/L. Platelet engraftment was defined as the first 7 consecutive days with a platelet count $> 20 \times 10^9$/L without transfusion support. Disease classification before transplantation was based on the established Refined

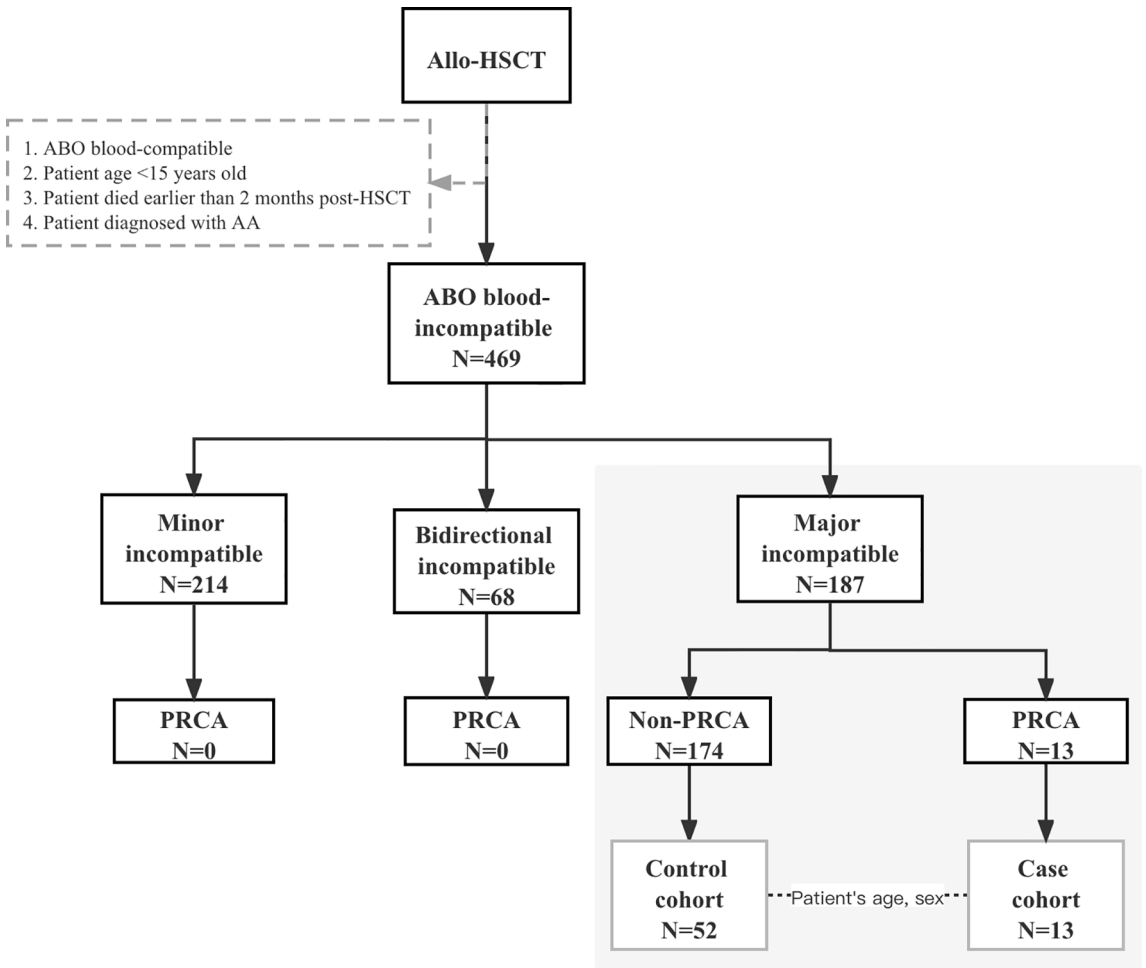


**FIGURE 1** | Diagram of patients with ABO-incompatible hematopoietic stem cell transplantation.

Disease Risk Index (DRI-R) (24). The primary endpoint of this study was the cumulative incidence of PRCA. Engraftment, red cell transfusion, acute GVHD and chronic GVHD, relapse rate, non-relapse mortality (NRM, death from any cause exclusive of leukemia), overall survival (OS), and disease-free survival (DFS) were also observed.

## Statistical Methods

Statistical analysis was performed using the Statistical Package for the Scoial Sciences (SPSS) version 26.0 software (SPSS, Chicago, IL, USA) and R language statistical software (http://www.r-project.org). Continuous variables are summarized as medians and ranges. Differences between cohorts normally distributed with homogeneity of variance were analyzed using Student's t-test. Otherwise, differences were tested using the Wilcoxon rank-sums test. Pearson's chi-squared test ($T \geq 5$ and $n \geq 40$), chi-squared test with continuity correction ($1 \leq T < 5$ and $n \geq 40$), and Fisher's exact test were used to compare categorical variables. The cumulative incidence of PRCA was estimated using the cumulative incidence function, with death as a competing event. Conditional logistic regression was used to examine the correlation between risk factors and PRCA occurrence. A forward likelihood ratio method was used. The Kaplan–Meier method was used to estimate the 2-year OS and disease-free survival by comparing patients with and without PRCA. Gray's competing risk method was used to compute acute GVHD, chronic GVHD, and the 2-year NRM cumulative incidence curve. Cox regression models were used to estimate the hazard ratio for transplantation survival. All tests were two-tailed, and $P$ values $<0.05$ were considered statistically significant.

# RESULTS

## Cohort Characteristic

A total of 469 patients underwent ABO-mismatched allogeneic HSCT, of which 187 patients received major ABO-incompatible grafts. In the major ABO-mismatched group, the median age was 35.5 (range, 15–67) years and 37 (range, 13–56) years for patients and donors, respectively. Patients with PRCA were only observed

in the major ABO-mismatched group, where 13 patients (7%; 95% confidence interval [CI], 3.9%–11.9%) were diagnosed with post-HSCT PRCA. The cohort characteristics and risk factors of PRCA in major ABO-incompatible transplantations are shown in **Table 1**. Fifty-two major ABO-incompatible transplantation recipients were selected as controls. No significant difference was observed between the two groups in terms of patient's age, patient's sex, disease, donor's sex, conditioning regimens, and the type of anti-donor isohemagglutinins. However, the DRI-R, donor type, donor's age, and GVHD prophylaxis were significantly different between the two groups.

## Occurrence of *Post*-HSCT PRCA

**Table 2** illustrated the detailed information of patients with post-HSCT PRCA. The post-HSCT PRCA group comprised seven patients with primary post-HSCT PRCA and six patients with secondary post-HSCT PRCA. A significant difference of underlying disease distribution was observed between the two groups, and there was no difference on patient age, patient sex, donor age, donor sex, donor type, and ATG usage between the primary and secondary PRCA groups (**Supplemental Table 1**). All patients with primary PRCA were initially identified the first month post-HSCT. The median occurrence of secondary PRCA was 60 (range, 45–114) days post-HSCT. In the primary post-HSCT PRCA subgroup, one patient had persistent pancytopenia, and died from transplantation-associated thrombotic microangiopathy and lung infection 73 days post-HSCT. The median recovery time of primary PRCA was 125.5 (range, 116–300) days post-HSCT in the remaining six patients with PRCA. In the subgroup of secondary post-HSCT PRCA, the median PRCA diagnosis time was 60 (range, 45–114) days post-HSCT. One patient with secondary PRCA was diagnosed 58 days post-HSCT and experienced leukemia relapse 90 days post-HSCT. The median interval from diagnosis to recovery of secondary PRCA was 158 (range, 66–777) days in the remaining five patients with PRCA. Up to the last follow-up, 11of the 13 patients with PRCA recovered from PRCA entirely and achieved HSCT success. Two patients with PRCA received blood transfusion support only, and three patients were treated with intravenous immunoglobulin (IVIG) and blood transfusion. Therapeutic plasma exchange (TPE) was provided for another six patients, of which two patients were provided additional interventions, such as eltrombopag, rituximab, and donor lymphocyte infusion. The immunosuppressive agent was tapered slowly once the patient was diagnosed with PRCA.

**TABLE 1** | Characteristics of the nest case-control cohort.

| Characteristic | PRCA (N = 13)* | Non-PRCA (N = 52)* | P-value |
|---|---|---|---|
| Patient sex, female/male, n | 9/4 | 36/16 | 1.000 |
| Patient age, median (range), years | 46 (27-55) | 45 (25-58) | 0.954 |
| Disease | | | 0.642 |
| AML/MDS | 7 | 34 | |
| ALL | 5 | 13 | |
| Other | 1 | 5 | |
| **DRI-R** | | | **0.018**[#] |
| Low/Int | 11 | 25 | |
| High/very high | 2 | 27 | |
| Donor sex, female/male, n | 6/7 | 17/35 | 0.559 |
| **Donor age, median (range), years** | 40 (14-53) | 26 (13-55) | **0.022**[#] |
| **Donor type** | | | **<0.001**[##] |
| MSD | 9 | 7 | |
| HID | 1 | 40 | |
| URD | 3 | 5 | |
| Intensity of conditioning regimen | | | 1.000 |
| MAC | 12 | 48 | |
| RIC | 1 | 4 | |
| **ATG for GVHD prophylaxis** | | | **<0.001**[##] |
| Yes | 6 | 48 | |
| No | 7 | 4 | |
| Donor-recipient blood type | | | 0.524 |
| A–O | 6 | 19 | |
| Other | 7 | 33 | |
| CD34+ cells, median (range), $\times 10^6$/kg | 5.72 (3.05-13.50) | 5.69 (2.03-15.09) | 0.973 |
| MNC, median (range), $\times 10^8$/kg | 13.48 (7.56-43.90) | 15.20 (5.65-45.76) | 0.820 |
| Red cell transfusion at d100, median (range), U | 6 (0-35) | 4.5 (0-33) | 0.451 |
| Acute GVHD at d100, n | | | |
| Grades II-IV | 2 | 14 | 0.614 |
| Grades III-IV | 0 | 5 | 0.561 |
| Moderate-severe chronic GVHD, n | 1 | 8 | 0.788 |
| Median follow-up (range), Mo | 24.6 (2.4-74.1) | 27.7 (3.5-65.4) | 0.825 |

*PRCA, pure red cell aplasia; AML, acute myelocytic leukemia; ALL, acute lymphoblastic leukemia; MDS, myelodysplastic syndrome; DRI-R, refined disease risk index; HID, haploidentical donor; MSD, matched sibling donor; URD, unrelated donor; GVHD, graft versus host disease; MAC, myeloablative conditioning; RIC, reduced intensity conditioning.*

**For each PRCA case, 4 controls were selected at random from the same cohort.*

*[#]the P-value < 0.05; [##]the P-value < 0.01.*

**TABLE 2** | Characteristics of patients with pure red cell aplasia.

| UPN | Disease /DRI-r | Donor type/ graft | Age P/D | Sex P/D | ABO blood type P/D | Conditioning regimen/GVHD prophylaxis | aGVHD at d100 | initial time (days) | PRCA Course (days) | Iso-titer (diagnosis) | Iso-titer (resolution) | Treatment of PRCA | Outcome |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | AML/Int | MSD/ PB | 46/ 51 | F/F | O/A | MAC CsA/MMF/MTX | Grade 2 | primary | 126 | IgG 1:128 IgM 0 | IgG 1:1 NA | IVIG/ transfusion | Alive, CR |
| **2** | AML/Int | MSD/ PB | 30/ 39 | M/F | O/B | MAC CsA/MMF/MTX | Absent | 62 | 66 | IgG 0 IgM 1:1 | IgG 0 IgM 0 | IVIG/ transfusion | Alive, CR |
| **3** | AML/Int | MSD/ PB | 50/ 53 | F/M | O/B | MAC CsA/MMF/MTX | Absent | 45 | 777 | IgG 1:512 IgM 1:8 | IgG 1:1 NA | IVIG/ transfusion | Alive, CR |
| **4** | AML/Int | MSD/ PB | 53/ 50 | M/M | O/B | MAC CsA/MMF/MTX | Absent | 58 | NA | IgG 1:512 IgM 1:128 | Relapse* | transfusion | AML relapse, demise |
| **5** | ALL/Int | MSD/ PB | 52/ 45 | F/M | O/B | MAC CsA/MMF/MTX | Absent | primary | 300 | IgG 1:1024 IgM 1:16 | IgG 1:64 IgM 1:8 | TPE/IVIG/ transfusion | Alive, CR |
| **6** | HLH | MSD/ PB | 37/ 27 | F/F | O/A | RIC CsA/MTX+ATG | Absent | 97 | 664 | IgG 1:64 IgM 1:2 | IgG 1:64 IgM 1:2 | TPE/RTX/ Eltrombopag/ transfusion | Alive, CR |
| **7** | ALL/Int | HID/PB | 51/ 14 | M/F | O/A | MAC CsA/MMF/MTX +ATG | Absent | primary | 116 | IgG 1:128 IgM 1:16 | IgG 1:64 IgM 1:4 | Transfusion | Alive, CR |
| **8** | MDS/Int | URD/ PB | 49/ 36 | F/F | O/B | MAC CsA/MMF/MTX +ATG | Grade 2 | primary | NA | IgG 1:1024 IgM 1:2 | NRM* | Transfusion | Viremia, demise |
| **9** | ALL/Int | MSD/ PB | 28/ 26 | F/M | O/A | MAC CsA/MTX | Absent | primary | 195 | IgG 1:128 IgM 1:32 | IgG <1:64 IgM 1:4 | TPE/DLI/ Eltrombopag/ transfusion | Alive, CR |
| **10** | ALL/Int | MSD/ PB | 41/ 50 | F/M | O/B | MAC CsA/MMF/MTX | Absent | primary | 123 | IgG 1:64 IgM 1:16 | IgG 1:64 IgM 1:1 | TPE/ transfusion | Alive, CR |
| **11** | AML/ High | URD/ PB | 40/ 43 | F/M | O/B | MAC CsA/MMF/MTX +ATG | Absent | 114 | 158 | IgG 1:64 IgM 1:2 | IgG 1:64 IgM 1:1 | Transfusion | Alive, CR |
| **12** | AML/ High | URD/ PB | 55/ 32 | M/M | O/A | MAC CsA/MMF/MTX +ATG | Absent | 55 | 82 | IgG 1:64 IgM 1:4 | IgG 1:8 IgM 1:1 | TPE/ transfusion | Alive, CR |
| **13** | ALL/Int | MSD/ PB | 38/ 40 | F/F | O/A | MAC CsA/MTX +ATG | Absent | primary | 125 | IgG 1:64 IgM 1:4 | IgG 1:64 IgM 1:1 | TPE/IVIG/ transfusion | Alive, CR |

**the data was unavailable because of leukemia relapse and non-relapse mortality (NRM).*

*AML, acute myelocytic leukemia; ALL, acute lymphoblastic leukemia; MDS, myelodysplastic syndrome; HLH, hemophagocytic lymphohistiocytosis; P, patient; D, donor; F, female; M, male; TPE, therapeutic plasma exchange; IVIG, intravenous immunoglobulin; RTX, rituximab; DRI-R, refined disease risk index; HID, haploidentical donor; MSD, matched sibling donor; URD, unrelated donor.*

## Pure Red Cell Aplasia (PRCA) Prevalence in the Entire Group

**Figure 2** illustrates the cumulative incidence rates of PRCA in the different subgroups of 187 patients with major ABO-incompatible HSCT. The cumulative incidence rates of post-HSCT PRCA were 0.8%, 13.1%, and 27.2% for HID, URD, and MSD, respectively ($P$<0.001). A significant difference in PRCA rate was found between patients who received ATG and those who did not receive ATG (3.7% versus 29.2%, $P$<0.001). Patients with high/very high disease status tended to have lower PRCA rates than those with low/Int disease status, but the difference ($P$=0.099) did not reach statistical significance. No difference in PRCA rate was observed between groups with respect to donor age, donor sex, and the anti-donor isohemagglutinins type.

## Risk Factors of PRCA

According to the cohort study of 65 patients receiving major ABO-incompatible peripheral blood grafts, the factors associated with post-HSCT PRCA were DRI-R, donor age, donor type, and ATG in the univariate analysis (**Table 3**). However, in the multivariate analysis using conditional logistic regression test, donor type was the only independent factor associated with post-HSCT PRCA (odds ratio=0.030; 95% CI, 0.003–0.321; $P$=0.004). Patients using MSDs or URDs had a significantly higher post-HSCT PRCA rate than those using HIDs (2.4% versus 50.0%, $P$<0.001; **Supplemental Figure 1A**). Patients with high/very high disease status tended to have a lower risk of post-HSCT PRCA occurrence than those with low/Int disease status (6.9% versus 30.6%, $P$=0.016; **Supplemental Figure 1B**), although the difference was not statistically significant ($P$=0.065).

Meanwhile, we performed the univariate and multivariate analysis for post-PRCA using Cox proportional-hazards model in the entire cohort of 187 patients with major ABO-mismatched HSCT. The baseline characteristics of the entire cohort was shown in **Supplemental Table 2**. As shown in **Table 4**, the patient age ($P$=0.022), ATG ($P$<0.001), and donor type ($P$<0.001) had an impact on the development of PRCA in the univariate analysis. Donor type was the only independent factors for post-HSCT PRCA in the multivariate analysis, which was consistent with the multivariate analysis in the nested cohort. We also performed a multivariate analysis in the subgroup of 33 patients with MSD-HSCT (9 patients with PRCA vs. 24 patients without PRCA), no significant difference of ATG for post-HSCT PRCA was observed (**Supplemental Table 3** and **Supplemental Table 4**).

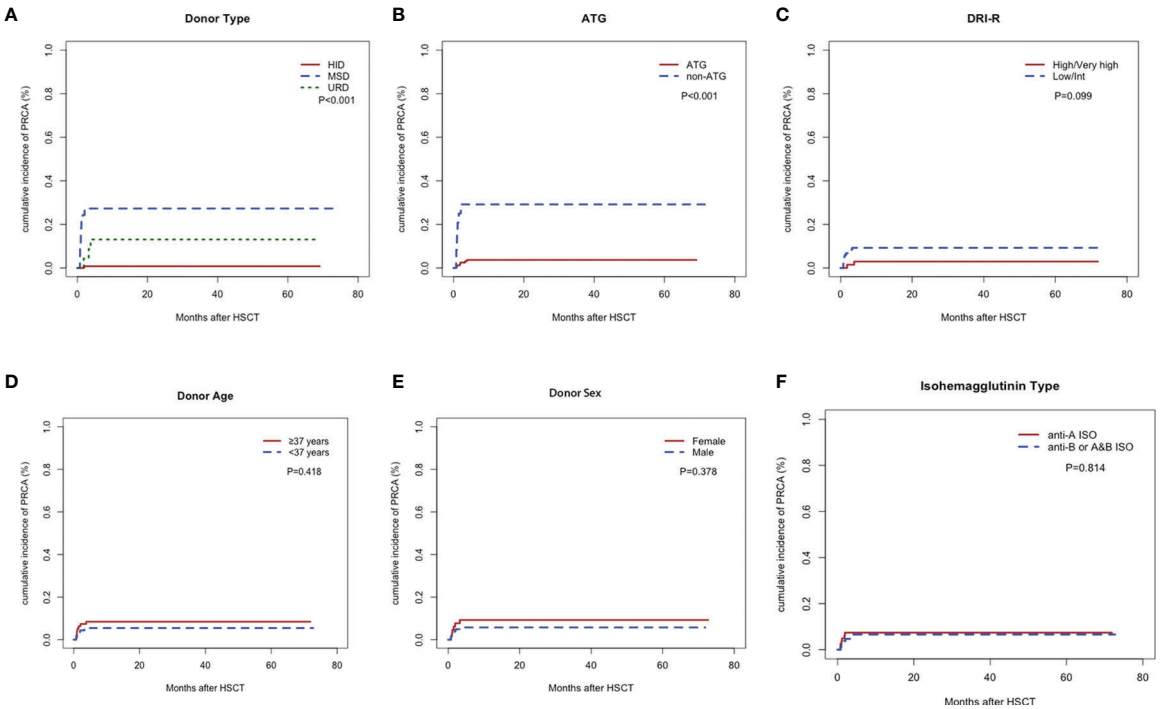


**FIGURE 2** | Cumulative incidence rates of pure red cell aplasia. Donor type **(A)**, anti-thymocyte globulin **(B)**, Refined Disease Risk Index **(C)**, donor age **(D)**, donor sex **(E)**, and anti-donor isohemagglutinins type **(F)**.

**TABLE 3** | Univariate and multivariate analyses of risk factors for pure red cell aplasia in the nested case control cohort.

| | Univariate analysis | | | Multivariate analysis | | |
|---|---|---|---|---|---|---|
| | **OR** | **95% CI** | ***P*-value** | **OR** | **95% CI** | ***P*-value** |
| DRI-R (High/very high vs Low/Int) | 0.167 | 0.033-0.829 | 0.029 | 0.100 | 0.009-1.151 | 0.065 |
| Donor age (≥37 vs <37) | 4.959 | 1.221-20.134 | 0.025 | NA | NA | 0.610 |
| Donor type (HID vs MSD/URD) | 0.037 | 0.005-0.295 | 0.002 | 0.030 | 0.003-0.321 | 0.004 |
| ATG (Yes vs No) | 0.085 | 0.017-0.415 | 0.002 | NA | NA | 0.464 |
| Donor-recipient blood type (A–O vs other) | NA | NA | 0.525 | – | – | – |
| Donor gender (Male vs female) | NA | NA | 0.332 | – | – | – |

*DRI-R, refined disease risk index; HID, haploidentical donor; MSD, matched sibling donor; URD, unrelated donor; NA, not available.*

## Anti-Donor Isohemagglutionins

At the time of PRCA diagnosis, the median level of IgG anti-donor isohemagglutinins (ISO) in the PRCA cohort was 1:128 (range, 1:1–1:1024). However, IgM anti-donor ISO in patient with PRCA was at a relatively low level (≤1:32). At the time of PRCA recovery, the IgG anti-donor ISO titer was less than 1:64, and the IgM anti-donor ISO titer was less than 1:8. The median decreases in the anti-donor ISO titer index were 0 (range, 0–4) and 2 (range, 0–4) for IgG and IgM, respectively.

Patients with PRCA had a higher IgG anti-donor ISO in the first 4 months post-HSCT than patients without PRCA (**Figure 3A**). Both post-HSCT IgM and post-HSCT IgG ISO were more elevated in patients with PRCA than in patients without PRCA ($P$=0.001, **Figures 3B, C**). The disappearance of IgM anti-donor ISO was more significant in patients without PRCA than in patients with PRCA ($P$=0.028, **Figure 3D**). Patients using HIDs had a lower post-HSCT IgM and IgG anti-donor ISO than those using MSDs or URDs (**Figures 3E, F**). IgM anti-donor ISO was observed to significantly decrease in HID-HSCT rather than MSD-HSCT or URD-HSCT ($P$=0.036, **Figure 3G**). No difference in IgG or IgM anti-donor ISO level was found between the groups on the donor blood type (**Supplemental Table 5**).

## Transplantation Outcome

The median red cell infusions 100 days post-HSCT were 6 (range, 0 to 35) U for patients with PRCA and 4.5 (range, 0 to 33) U for patients without PRCA, respectively. In patients with PRCA, neutrophils and platelets engrafted at a median of 12 (range, 10–53) days and 12 (range, 0–26) days, respectively. In patients

**TABLE 4 |** Univariate and multivariate analyses of risk factors for pure red cell aplasia in the entire cohort.

| | Univariate analysis | | | Multivariate analysis | | |
|---|---|---|---|---|---|---|
| **Univariate analysis** | **OR** | **95% CI** | **P-value** | **OR** | **95% CI** | **P-value** |
| Donor type (HID vs MSD/URD) | 0.178 | 0.064-0.494 | <0.001 | 0.032 | 0.004-0.244 | <0.001 |
| ATG (Yes vs No) | 0.324 | 0.187-0.559 | <0.001 | NA | NA | 0.182 |
| Patient age (≥35 vs <35) | 5.782 | 1.281-26.088 | 0.022 | NA | NA | 0.060 |
| DRI-R (High/very high vs Low/Int) | NA | NA | 0.120 | – | – | – |
| Donor age (≥37 vs <37) | NA | NA | 0.413 | – | – | – |
| Donor-recipient blood type (A–O vs other) | NA | NA | 0.814 | – | – | – |
| Donor gender (Male vs female) | NA | NA | 0.382 | – | – | – |
| Patient gender (Male vs female) | NA | NA | 0.138 | – | – | – |

*DRI-R, refined disease risk index; HID, haploidentical donor; MSD, matched sibling donor; URD, unrelated donor; NA, not available.*

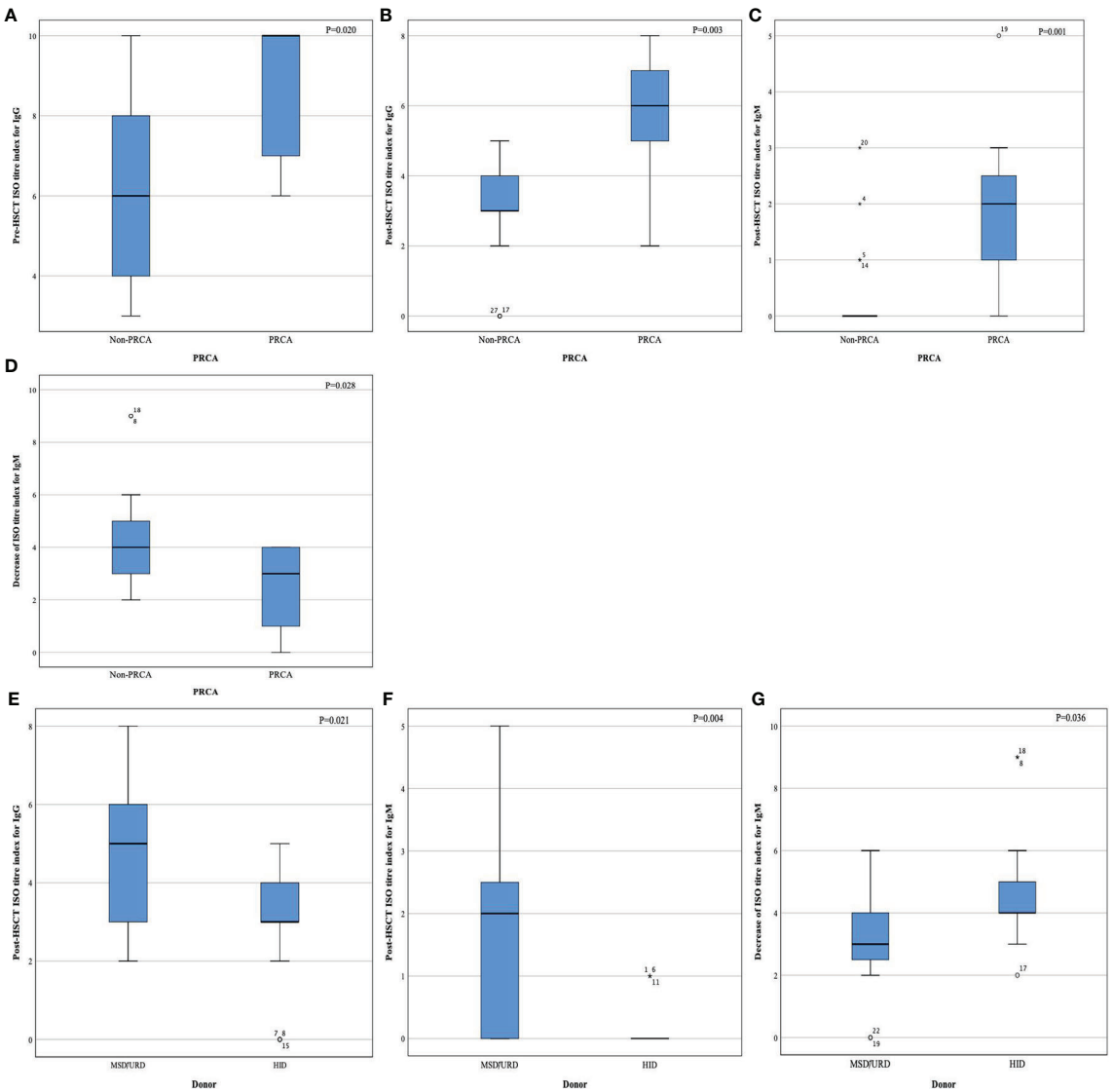


**FIGURE 3 |** Index of anti-donor isohemagglutinin titer. Pre- HSCT IgG **(A)**, post-HSCT IgG **(B)**, post-HSCT IgM **(C)**, decrease index of IgM after HSCT **(D)**, post-HSCT IgG **(E)**, post-HSCT IgM **(F)**, and decrease index of IgM after HSCT **(G)**.

without PRCA, neutrophils and platelets engrafted at a median of 12 (range, 7–20) days and 13 (0–33) days, respectively. No difference was observed between the two groups in red cell infusion, neutrophil and platelet engraftment, acute GVHD incidence, and chronic GVHD (**Table 1**). The median survival rates were 24.6 (2.4-74.1) months post-HSCT for patients with PRCA and 27.7 (3.5-65.4) months post-HSCT for patients without PRCA, respectively. **Figure 4** shows the 2-year OS, DFS, relapse rate, and NRM between the two groups, but no significant difference was found. The development of PRCA did not affect the transplantation outcomes in the multivariate analysis using the Cox regression model (**Supplemental Table 6**).

## DISCUSSION

The present study is a prospective nested case-control study aiming to determine the prevalence, donor-related factors, and outcome of PRCA following major ABO-incompatible transplantation. It is revealed for the first time that patients undergoing HID-HSCT may be at a lower risk for developing post-HSCT PRCA than those using MSDs or URDs in major ABO-incompatible transplantation. Meanwhile, the effect of post-HSCT PRCA on transplantation prognosis is not sufficiently significant.

This study revealed that patients with PRCA tend to have a higher level of pre-HSCT anti-donor ISO for IgG and post-HSCT anti-donor ISO for both IgG and IgM than patients without PRCA in the major ABO-incompatible transplantation method. In addition, half of the 13 patients with PRCA had a high IgG ISO level of 1:128 when diagnosed with PRCA initially. This phenomenon may suggest that the development of post-HSCT PRCA may depend on the persistence of anti-donor ISO for the first 4 months post-HSCT, especially for IgG anti-donor ISO. As reported, Bolan et al. revealed that the delayed onset of donor red blood cell (RBC) was associated with the time of anti-donor ISO disappearance, and the time of post-HSCT anti-donor ISO disappearance was significantly linked to pre-HSCT anti-donor ISO titer (6). On the other hand, Griffith et al. found that considerable proportion of residual plasma cells from recipients after transplantation may secrete sufficient antibodies to destroy nascent erythroblast precursors and prevent timely maturation (10).

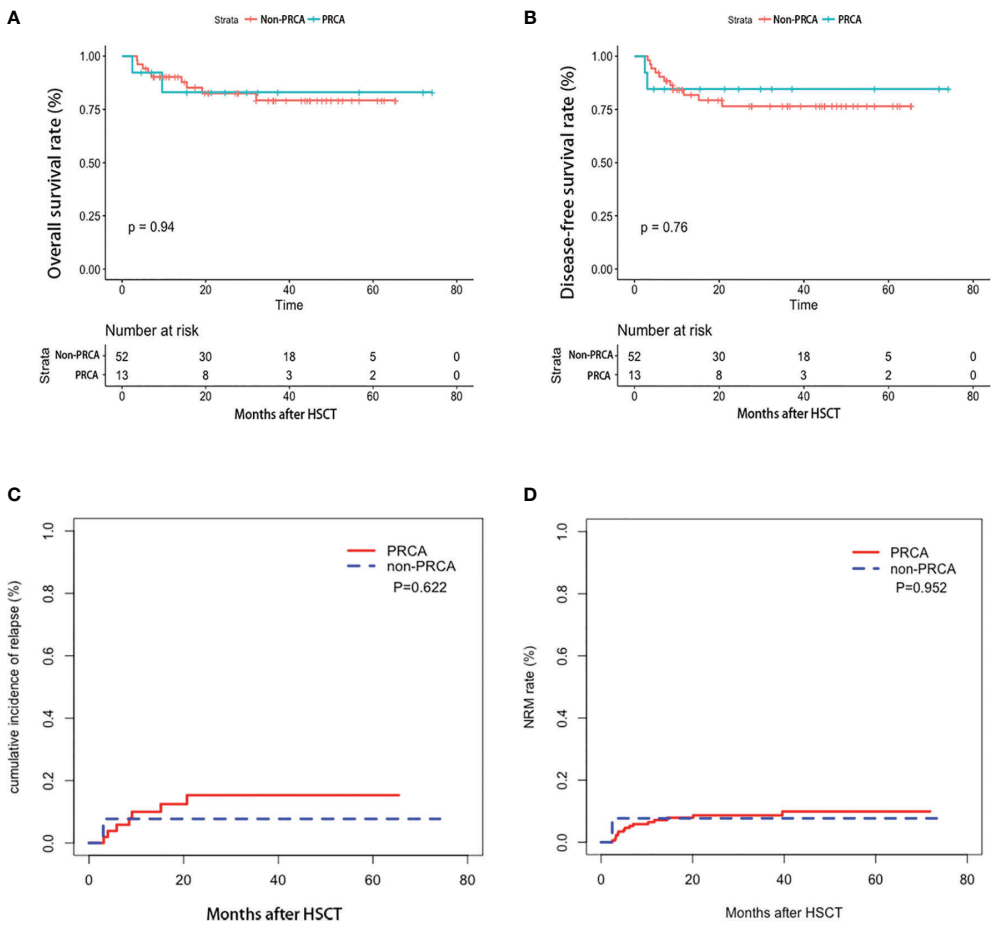


**FIGURE 4** | Transplantation outcome in the cohort. Overall survival **(A)**, disease-free survival **(B)**, relapse rate **(C)**, and non-relapse mortality **(D)**.

Similarly, a previous study by Gmür showed that donor RBCs engrafted following the decrease in isohemagglutinins titer to below 1:16.[22.] Another report by Longval et al. also demonstrated that a high level of pre-HSCT anti-donor ISO was associated with increased risk of PRCA (25). Taken together, the primary post-HSCT PRCA could be explained by the high level of IgG ISO at the time of transplantation to some extent. Interestingly, it was found that recipient-derived ISO was expected to disappear within 120 days after HSCT by a poster (26). Correspondingly, our study showed that the median recovery time of primary PRCA was 125.5 days post-HSCT. In addition, several studies found that the rebound in titers of anti-donor ISO during the first 4 to 10 weeks post-HSCT may account for the development of the secondary post-HSCT PRCA (12, 13, 27). This finding coincided with our study that patients with secondary PRCA were identified approximately 60 days post-HSCT. However, our research failed to find a cut-off value of anti-donor ISO to predict the occurrence of PRCA due to the small sample for anti-donor ISO monitoring.

Donor type was the only factor associated with the development of post-HSCT PRCA in our study. Patients using HIDs had a lower cumulative incidence rate of PRCA (0.8%) compared to those using URDs and MSDs ($P<0.001$). Additionally, we observed that patients undergoing haploidentical transplantation appeared to have a lower titer level of anti-donor ISO post-HSCT for both IgG and IgM and a higher disappearance rate of IgM-ISO. Hence, it could be hypothesized that HID-HSCT may be more effective in removing the ISO compared to other transplantation methods. Previous studies have illustrated that the disappearance of anti-donor ISO was more effective in URD-HSCT rather than in MSD-HSCT (49 versus 166 days, $P<0.001$) (13, 20), and the patients who underwent URD-HSCT showed a trend toward a lower incidence rate of post-HSCT PRCA compared to those who underwent MSD-HSCT (9). Consequently, transplantation using major ABO-incompatible grafts from different donor types is possible to have the additional potential of removing anti-donor ISO owing to the discrepancy of graft-versus-host plasma cells effect.

Remarkably, ATG was added to all patients in the haploidentical transplantation in our study, which may confuse the effect of donor type on the development of post-HSCT PRCA. However, ATG was not associated with PRCA in the multivariate analysis. Occasionally, some patients with post-HSCT PRCA could be treated effectively with ATG (28, 29). A recent case series revealed that ATG (equine-ATG, 40 mg/m$^2$ for 4 days) was a viable salvage approach for five patients with refractory PRCA associated with ABO-incompatible HSCT (28).

The standard treatment for post-HSCT PRCA remains to be determined. Whether the recovery of patients with PRCA results from self-limited remission or treatment is difficult to distinguish. Hirokawa et al. demonstrated that the intervals of reticulocyte recovery in patients with the additional intervention were similar to those without, which implied the failure to provide supportive evidence on the superiority of treatment (30). Notably, pre-HSCT plasma exchange was confirmed to reduce pre-HSCT ISO titer significantly efficiently, which led to a lower incidence rate of post-HSCT PRCA (3/98 versus 9/55) (9). Therapeutic plasma exchange (TPE) was an effective strategy for post-HSCT PRCA, as reported previously (31–34). Apart from five patients with PRCA receiving transfusion and IVIG only in our study, six patients were treated with TPE additionally and achieved definite treatment effect. In patients with refractory PRCA, daratumumab, eltrombopag, and rituximab may be effective strategies in previous cases (35–38).

This study has some limitations. The two factors we applied to match may not be the best choice in our nested case-control study. Additionally, we only monitored anti-donor ISO titers twice (pre-HSCT and the fourth month post-HSCT) in a small group of 28 patients. It was required that anti-donor ISO be monitored more intensively during the first 4 months post-HSCT and the time when patient received any therapy treatment. Due to the limited group number, it was of difficulty to determine a cut-off value of anti-donor ISO pre-HSCT or post-HSCT to predict the development of post-HSCT PRCA. A larger cohort should be included to observe dynamic changes in isohemagglutinins titers. Moreover, the changes in reticulocyte and donor myeloid chimerism should be monitored regularly, which restricts the ability to reveal the association between donor type and PRCA.

In summary, post-HSCT PRCA is a less threatening complication and is prevalent among a small group after major ABO-incompatible HSCT. Most patients with PRCA could recover within half a year after the diagnosis of the PRCA. In addition, haploidentical donors may help to achieve a relatively low risk of post-HSCT PRCA.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

PZ, analyzing data, writing of the original draft. YW, constructing the draft and re-editing of the revised manuscript. JS, YZ, XL, LL recruiting patients, data collecting, and discussing the results. DC and JX, offering the help of testing the anti-donor isohemagglutinins and the data collecting of red cell transfusion. HH, funding acquisition, and project administration. YL, funding acquisition, project administration, review, and validation. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.829670/full#supplementary-material

**Supplementary Table 1 |** Characteristics of patients with post-HSCT PRCA.

**Supplementary Table 2 |** Characteristics of the entire cohort.

**Supplementary Table 3 |** Characteristics of patients with MSD-HSCT.

**Supplementary Table 4 |** Univariate and multivariate analysis of risk factors for pure red cell aplasia in 33 patients with MSD-HSCT.

**Supplementary Table 5 |** The level of anti-donor isohemagglutinins.

**Supplementary Table 6 |** Multivariate analysis of risk for transplantation outcomes.

**Supplementary Figure 1 |** Cumulative incidence rates of pure red cell aplasia in the cohort. Donor type **(A)**, Refined Disease Risk Index **(B)**, Donor age **(C)**, and anti-thymocyte globulin **(D)**.

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