RECENT DEVELOPMENTS IN HAPLOIDENTICAL STEM CELL TRANSPLANTATION: THERAPY AND COMPLICATIONS

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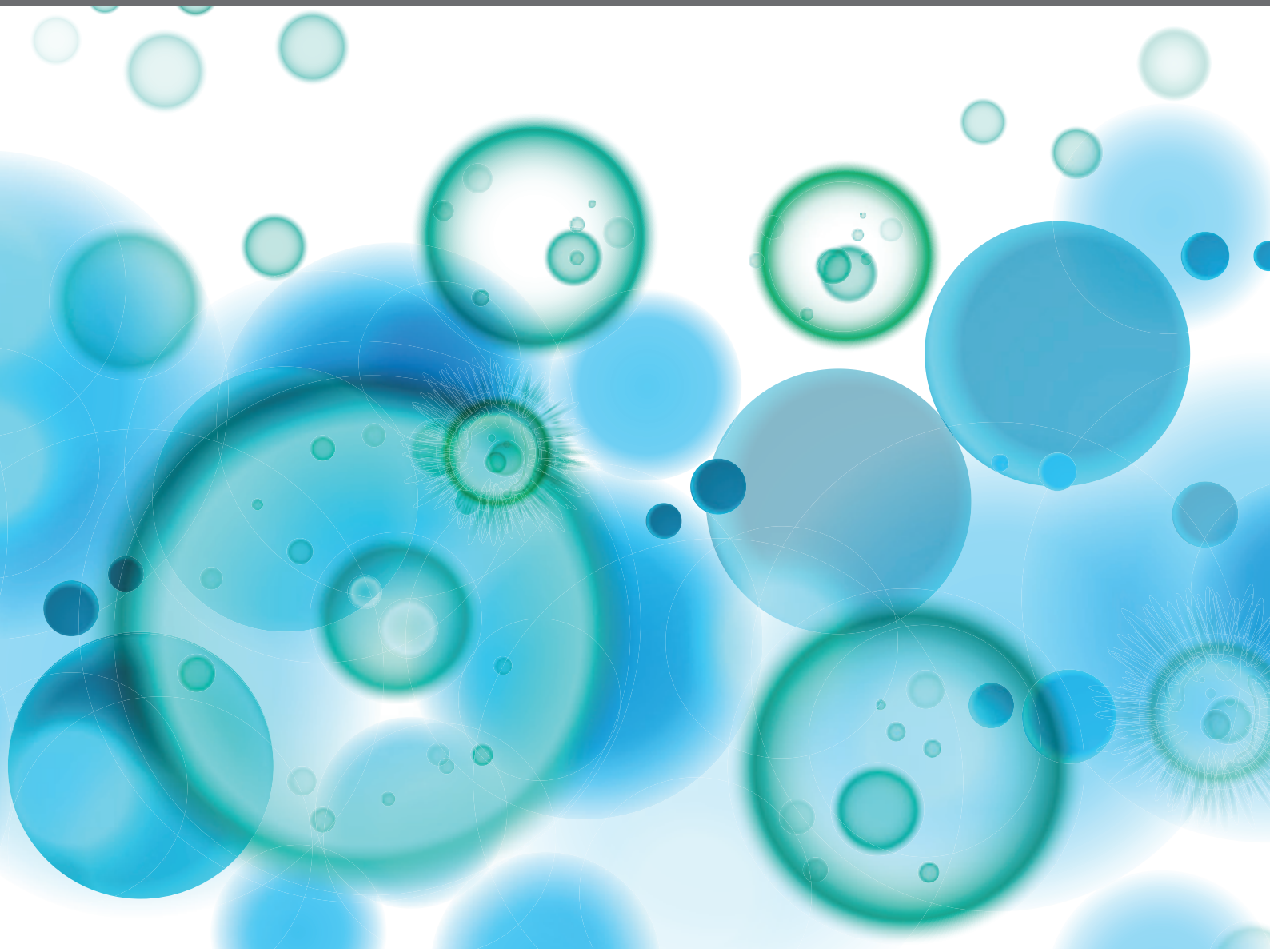

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RECENT DEVELOPMENTS IN HAPLOIDENTICAL STEM CELL TRANSPLANTATION: THERAPY AND COMPLICATIONS

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EDITORIAL
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Editorial: Recent Developments in Haploidentical Hematopoietic Cell Transplantation: Therapy and Complications

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Keywords: stem cell transplantation, graft-*versus*-host disease, graft failure, graft-*versus*-leukemia effect, car-t, infection

Editorial on the Research Topic

Recent Developments in Haploidentical Hematopoietic Cell Transplantation: Therapy and Complications

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The successful application of haploidentical hematopoietic stem cell transplantation (haplo-HSCT) worldwide has made it a reality that almost every allograft candidate has a donor. In the past two decades, significant advances had been achieved in the field of haplo-HSCT. Currently, the outcomes of haplo-HSCT are not inferior to those of other transplant modalities, including human leukocyte antigen (HLA)-matched sibling donor transplantation (MSDT), umbilical cord blood transplantation, and HLA-identical unrelated donor transplantation. Impressively, the numbers of haplo-HSCT increased rapidly in Asia, Europe, and United States of America in the past ten years, especially in China, where the cases of haploidentical allograft exceeded MSDT since 2013. However, complications after transplantation, such as graft failure (GF), leukemia relapse, and graft-versus-host disease (GVHD) are the main bottlenecks for further improving outcomes of haplo-HSCT. Therefore, there is an urgent need to understand the underlying mechanisms and to establish novel strategies for the prevention and treatment of the abovementioned complications in order to improve haploidentical allograft outcomes.

IMMUNE TOLERANCE

The successful clinical application of haplo-HSCT is determined by the donor and host T-cell alloreactivities, which lead to unacceptably high incidences of GF and GVHD. Strategies for crossing HLA barriers in the haplo-HSCT modalities include immune tolerance induced by either granulocyte-colony-stimulating factor primed grafts and antithymocyte globulin (ATG) or post-transplant cyclophosphamide as well as *ex vivo* T cell depletion. Further elucidating the underlying mechanisms of immune tolerance in the haplo-HSCT settings would contribute to clinical developments with respect to the lower incidence of GF and GVHD. Original research reported

by Weber et al. identified the interferon-γ pathway as the target for exploring therapeutic strategies against GF especially for patients who underwent haplo-HSCT. In the two review papers, Yang et al. summarized recent advances on T cell tolerance, discussing how regulatory T cells maintain self-tolerance either in early life or in allogeneic transplant settings. Hong et al. focused on the roles of antigen presenting cells (APCs), such as dendritic cells, macrophages, played in the pathophysiology of chronic GVHD. They discussed potential new therapeutic approaches targeting APCs for chronic GVHD. Overall, these primary and review papers delineate the mechanisms of GF, T cell tolerance, and chronic GVHD, which provide insights into the treatment for both GF and chronic GVHD.

CHIMERIC ANTIGEN RECEPTOR T-CELL

The use of chimeric antigen receptor T-Cell (CAR-T) therapy has changed the landscape for the treatment of relapsed or refractory acute lymphoblastic leukemia. Zhang and Huang not only discussed the complementary anti-leukemia mechanisms on combination of CAR-T cell therapy with allogeneic HSCT, but also provided evidence suggesting the role of CAR-T cell in post-transplant relapse and peri-transplant residual leukemia cell eradication. In addition, CAR technology could be incorporated into the strategy for GVHD treatment. The report from a multi-center retrospective study by Yan et al. demonstrated different characteristics and risk factors of cytokine release syndrome in different B-cell hematological malignancies, suggesting which should be treated individually. Both the aforementioned strategies could further improve transplant outcomes of patients with lymphoblastic malignancies.

LEUKEMIA RELAPSE AND VIRUS INFECTION

For patients who underwent allogeneic HSCT, particularly haplo-HSCT, relapse remain the main cause of death. Furthermore, viral infections is also an important cause of morbidity and mortality in those patients. Zhao et al. reported the association of decreased inhibitory killer immunoglobin-like receptor (iKIR) HLA C with transplant outcomes of patients with myeloid diseases, including higher relapse rate and inferior survival. The authors suggested that decreased iKIR-HLA C pair should be avoided in ATG based haplo-HSCT settings. In another original article, Zhou et al. identified that patients with CMV and EBV co-reactivation experienced higher incidence of viral pneumonitis, delayed $CD4^+CD25^+$ T cell reconstitution and poor survival. In allo-HSCT settings, Wu et al. highlighted mechanisms underlying increase in EBV viral load, risk factors and treatment for HBsAg-positive donors and recipients, which might allow the inclusion of HBsAg-positive individuals as donors or transplant candidates. Wang and Zhao reviewed the effects of IL-15 on natural killer cell development through activation of several downstream signaling pathways, such as Ras-MEK-MAPK, JAK-STAT5, and PI3K-ATK-mTOR pathways. All of these suggest the advances in factors associated with transplant complications and potential strategies for prevention and treatment of leukemia relapse and virus infection.

This Research Topic "Recent Developments in Haploidentical Stem Cell Transplantation: Therapy and Complications" provides some insights into the recent advances of haplo-HSCT. Moreover, this Research Topic may also contribute to the body of knowledge in haplo-HSCT for the prevention of GF, leukemia relapse, and virus infection as well as the enhancement of the graft-versus-leukemia (GVL) effect. However, challenges remain in the haplo-HSCT settings. For example, could the indications for haplo-HSCT be further expanded? Should pre-HSCT residual disease be eradicated to improve outcomes? Could we identify new subgroup patients who will benefit the strong GVL effect of haplo-HSCT? Could novel strategies for complication prevention or treatment be established through elucidating the underlying mechanisms of hematopoietic recovery and immune reconstitution? etc. Should these challenges be successfully dealt with, *we can teach young dog (haploidentical transplantation) new tricks*.

AUTHOR CONTRIBUTIONS

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

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

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REVIEW
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T cell Tolerance in Early Life

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T cell-mediated immune tolerance is a state of unresponsiveness of T cells towards specific self or non-self antigens. This is particularly essential during prenatal/neonatal period when T cells are exposed to dramatically changing environment and required to avoid rejection of maternal antigens, limit autoimmune responses, tolerate inert environmental and food antigens and antigens from non-harmful commensal microorganisms, promote maturation of mucosal barrier function, yet mount an appropriate response to pathogenic microorganisms. The cell-intrinsic and cell extrinsic mechanisms promote the generation of prenatal/neonatal T cells with distinct features to meet the complex and dynamic need of tolerance during this period. Reduced exposure or impaired tolerance in early life may have significant impact on allergic or autoimmune diseases in adult life. The uniqueness of conventional and regulatory T cells in human umbilical cord blood (UCB) may also provide certain advantages in UCB transplantation for hematological disorders.

Keywords: neonatal period, T cell tolerance, regulatory T cells, conventional T cells, allogeneic hematopoietic stem cell transplantation

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INTRODUCTION

Immune tolerance is a state of unresponsiveness of the immune cells towards specific self or non-self antigens. It is an essential mechanism to prevent unwanted or self-reactive immune responses. In allogeneic hematopoietic stem cell transplantation (HSCT), failure to develop immune tolerance to autoantigens and alloantigens results in chronic graft-*versus*-host disease (GVHD), a leading cause of non-relapse morbidity and mortality (1).

Immune tolerance was first discovered in neonatal dizygotic cattle twins with cellular chimerism that was due to naturally occurring placental anastomoses and exchange of non-self antigens (2). Anderson et al. then showed that skin grafts between these calves were well accepted (3). Since then, the concepts of neonatal immune tolerance and transplant tolerance were first described (4, 5).

T cells play an essential role in neonatal immune tolerance. Thymectomy at day 3 (d3Tx) after birth quickly leads to the development of an autoimmune wasting disease in mice which could be rescued by a thymus transplant (6, 7). At the neonatal period (from birth through the first month of life in human or the first 1–2 weeks in mice), T cells are exposed to a rapidly and dramatically changing environment, not only from the thymus to peripheral tissues with variable maturity, but also from a relatively pathogen-free and stable environment *in utero* to the diverse microbial environment in the outside world. During this period, T cells need to avoid rejection of the maternal host, limit autoimmune responses, tolerate inert environmental and food antigens and antigens from non-harmful commensal microorganisms,

promote maturation of mucosal barrier function, yet mount an appropriate response to pathogenic microorganisms (8). The clonal deletion of autoreactive T cells in the thymus (central tolerance) (9, 10) and the suppressive activity of regulatory T cells (Tregs) in the periphery (peripheral tolerance) (11–15) are both crucial in immune tolerance. But the mechanisms underlying the uniqueness of neonatal T cell tolerance and its adaptation to the adult state are just beginning to be understood after decades of comparison between neonatal and adult T cells. In this review, we will summarize current knowledge on T cell tolerance in early life and subsequent advantages of umbilical cord blood (UCB) T cells in tolerance development in allogeneic HSCT.

T CELL REPERTOIRE BEFORE THYMIC SELECTION IN EARLY LIFE

The stepwise T cell development, selection, and the generation of a functional T cell repertoire occur in the thymus (16). Compared to adult T cells, both human and murine neonatal conventional T (Tconv) cells and Treg cells have shorter T cell receptor (TCR) or shorter complementarity determining region (CDR)3α stretches, fewer N-region additions (more germ line-encoded clonotypes), and are less clonally expanded (17–27). Human UCB T cells also revealed higher percentage of nonfunctional TCRβ mRNAs, likely due to suppressed nonsense-mediated decay mechanism (26). The shorter TCRs in neonatal T cells do not limit TCR diversity. The results from deep sequencing and single cell sequencing demonstrate higher diversity of TCR repertoire in human neonatal Tconv and Tregs when compared to adult ones (28, 29). In addition, UCB Treg cells are also shown to have more clones with TCRs specific for autoantigens (28).

Terminal deoxynuceotidyl transferase (TdT) is responsible for template-independent nucleotide addition during the V(D)J rearrangement. It contributes to 90% of TCR$\alpha\beta$ diversity. The activity of TdT is believed to be low in the fetal period of both humans and mice. In particular, TdT expression could be only detected until 4–5 days after birth in mice and beyond 20th week of gestation in human. Such delayed TdT expression not only makes a significant contribution to short CDR3 length and less N-addition in TCRs of human and murine neonatal T cells (26, 30–32), but also leads to relatively high numbers of public clonotypes shared among human UCB samples (26).

In addition to different diversity, neonatal TCR repertoire is also biased toward TCRs with high affinity and high cross-reactivity. This is mainly based on the studies of *Tdt*-deficient mice but is confirmed later with other mouse models. T cells lacking *Tdt* showed increased affinity of TCR to the α helices of self-MHC (major histocompatibility complex) (33, 34). One of the surface markers that can report the TCR avidity for peptide/MHC complexes is CD5. Higher levels of CD5 (peaked at day 7 after birth) were found in wild type and several types of mutant murine neonatal Tconv and Tregs when compared to their adult counterparts (35). However, the high affinity between TCRs and self-peptide/MHC complexes did not increase the likelihood to generate autoreactive T cells during neonatal period or incidence of autoimmune pathologies (36–38), at least in a rodent model with the transplantation of NOD thymi to NOD.*scid* mice (39). Instead, it promotes Tregs' capability to undergo proliferation and likely, to modulate specific immune responses (40, 41). *Tdt*-deficient T cells also had an increased frequency for a given antigen, including self, commensal, and pathogenic ones (33, 34, 42). Such promiscuous peptide recognition is clearly an advantage to defend against a variety of environmental or infectious insults during neonatal period or during reconstitution after HSCT when the number of peripheral T cells is limited. Indeed, specific and competent CD8$^+$ T cell responses against a range of viral infections (Vesicular Stomatitis Virus, Vaccinia Virus, and Lymphocytic Choriomeningitis Virus) *in vivo* have been observed in murine *Tdt*-deficient or neonatal T cells (34, 43, 44). In human samples, T cells in UCB had higher level of CD5 expression and higher precursor frequency for certain tumor-associated antigens or pathogens than T cells in adults (**Table 1**) (28, 42, 45). Together with delayed TdT expression and similar TCR sequencing feature between human fetal T cells and mouse neonatal T cells, it is believed, although more evidence is needed, that human TCR repertoire also has high cross-reactivity.

THYMIC SELECTION IN EARLY LIFE

During thymocyte development, the stochastic V(D)J recombination of TCR α and β chains inevitably generates thymocyte clones with high potential for self-reactivity. These autoreactive clones will either be removed by negative selection or develop into self-reactive thymic Tregs (tTregs) by agonist selection (59, 60). Thymic epithelial cells in the medulla (mTECs) are essential in these thymic selections by displaying a broad spectrum of self-peptide called tissue-specific self antigens (TSAs) to developing T cells (61). The expression of these TSAs in mTECs is regulated, in a significant part, by the transcriptional modulator autoimmune regulator (AIRE). Other regulators include but not limited to the transcription factor forebrain embryonic zinc fingerlike protein 2 (Fezf2) and mTECs' autophagy machinery (62–64). Other cell types in the thymus, including cortical TECs, corneocyte-like mTECs (16), various types of dendritic cells (DC) (65–67), and B cells (68, 69), also contribute to negative selection of conventional T (Tconv) cells and agonist selection of tTregs. These different types of antigen presenting cells (APCs), with their different ways to sample and process self antigens, likely have non-redundant roles in thymic selection and in the determination of negative selection *versus* agonist selection (70, 71).

The uniqueness of thymic selection during neonatal period is not fully understood yet. Most of the evidence so far comes from murine studies. For instance, the interaction of developing thymocytes with medullary APCs may be limited due to small "islands" of thymic medulla in newborn animals in comparison with large and organized structure in adult ones (39). The spectrum of peptide presented by various thymic APCs is also different between neonatal and adult mice. Perinatal mTECs had a much lower ratio of HLA-DO : HLA-DM (non-classical MHC-II molecules that regulate peptide loading of MHC-II) and lower level of CD74/CLIP (MHC-II-associated invariant chain

TABLE 1 | Unique features of human Tconv and Treg cells in umbilical cord blood.

Human T cell types in UCB	Unique features (in comparison with adult counterparts)	Reference
Tconv	Higher CD5 expression in naïve $CD4^+$ cells	(42)
	Higher frequency of pathogen-specific and PR1-specific clonotypes with smaller average clonotype size	(26, 45)
	Higher TCR diversity	(28, 46)
	Lower numbers of randomly added nucleotides in TCRs without affecting the functional diversity	(26)
	Higher percentage of nonfunctional TCRβ mRNAs	(26)
	Higher numbers of public clones shared among samples	(26)
	More naïve $CD4^+$ and $CD8^+$ T cells	
	Upregulated Treg markers (*FOXP3 TIGIT* and *IKZF2*),, after 14-day expansion	(28)
	Higher expression of inhibitory receptors including CTLA-4 (in $CD4^+CD28^+$ cells), LAIR-1, CD31, and CD200 in all T cells	(47, 48),
	Higher expression of costimulatory molecules including ICOS and CD26 in all T cells; higher/lower IFN-γ production and cytotoxicity upon stimulation *in vitro*	(49–51)
	Enhanced rejection of HLA-mismatched B cell lymphoma in a xenogeneic mouse model	(52)
	Transcriptional features associated more with cell cycle and innate immune responses and chromatin architecture of $CD8^+$ T cells are similar to adult effector cells	(53, 54)
Treg	More diverse TCR repertoire	(28)
	Less effector-like cells	(28, 55)
	More clones with TCRs specific for autoantigens	(28)
	Higher integrin $\beta7$ expression and lower CLA expression	(55)
	Upon stimulation, Treg cells are more proliferative, have higher percentage of activated/effector cells, and perform better in the suppression assay	(27, 56–58)

peptide) expression when compared to adult mTECs, indicating that mTECs in young animals have higher efficiency in loading a diverse repertoires of TSA peptides in the antigen-binding grooves of MHC-II molecules (27). MHC-$II^{hi}CD8\alpha^+$ conventional DC (cDC) that can cross-present diverse TSAs to thymocytes, however, are less in perinatal than in adult thymi (27). The seeding of migratory DCs, including $B220^+$ plasmacytoid DCs and $Sirp\alpha^+CD11b^+$ cDCs, to induce negative selection against peripheral self- and non-self antigens in the thymus also takes time, in particular when the number of DCs and the expression levels of MHC-II, CD86, and IL-12p70 in DCs were low during neonatal period (72–75).

The impact of the unique antigen presentation in neonatal thymus was demonstrated recently. Tconv cells specific for islet β cells can be observed within 1 week after birth, and the appearance of Tconv and tTreg specific for Peptidyl arginine deiminase, type IV (Padi4) and Adducin 2 (Add2) was restricted to 1–3-week-old mice (39, 76). Beyond the above indicated period, β cell-, Padi4- or Add2-reactive CD4 single positive T cells or tTreg cells were depleted in the thymus. The coincidence of bone marrow (BM)-derived cells accumulating in the thymus beyond weaning age indicates the likelihood of migratory DCs in inducing a late stage negative selection of these autoreactive T cells (76). The second piece of evidence comes from Aire-related studies. Mathis's group found that the level of Aire expression and the repertories of Aire-dependent transcripts in mTECs were indistinguishable between <3-day-old and 5-week-old mice (27). However, thymectomy at day 3 after birth, turning off Aire expression before or shortly after birth, or tuning on Aire expression only after birth in the inducible *Aire* transgenic mice quickly led to the wasting disease and multiorgan autoimmune pathology (77), while turning off Aire expression beyond weaning age induced a different spectrum of pathologies (77–80). In addition, the multiorgan pathology in *Aire*-deficient mice could be ameliorated by the adoptive transfer of perinatal Tregs, but not adult Tregs (27). Collectively, these murine studies clearly demonstrate the differences in the antigen presentation machineries and post-selected repertoires between neonatal and adult thymi. Whether different selection machineries also exist in human thymi over the course of a lifespan is not clear. But infants who receive fully allogeneic thymi from unrelated infants generate Treg cells with diverse repertoires and Tconv being tolerant to self as well as the thymic transplant (81–83).

TREG CELLS IN EARLY LIFE

Treg cells are an essential mode of immune tolerance that can be transferred into naïve animals to prevent rejection of tissue/cell transplantation, development of autoimmune diseases and atopic disorders, such as allergies (11–13, 84–86). The importance of Treg cells specifically in fetal tolerance is realized by the onset of IPEX (immune dysregulation, polyendocrinopathy, entheropathy, X-linked)-related autoimmunity at second-trimester in humans that lack functional FOXP3 (87). Using a *Foxp3-DTR* transgenic mouse system, we and Yang et al. showed that Treg depletion during the day 0–10 or day 7–11 age-window quickly resulted in significant weight loss and autoimmune pathology (27, 41). When Treg cells were depleted beyond weaning age (35–45-day window), only scattered individual mouse developed mild autoimmune inflammation (27). Collectively, these data demonstrate an active and tight control of fetal/neonatal autoimmune responses by Treg cells

In addition to self antigens, Treg cell-mediated immune tolerance to commensal microbiota-derived antigens is also critical at barrier sites. Notably, the preferential barrier sites for neonatal Treg regulation are the intestine in humans but the skin in mouse. In humans, Treg cells with gut tropism (integrin $\beta7$ expression) and resting phenotype are found most abundant at birth and decreased with age, while the frequency of Treg cells with skin tropism (cutaneous lymphocyte antigen (CLA) expression) and activated phenotype is increased later in life (55) (**Table 1**). IL-2

and IL-7, but not retinoic acid, promote the expression of $\beta 7$ in Treg cells after thymic egress (55). Reduced tTreg cells in UCB were found to be associated with higher susceptibility to food allergies in infants (88). Thus, human neonatal tTreg cells may preferentially migrate to the gut and promote the establishment of mucosal immune tolerance (oral tolerance), in preparing for progressive exposure of microbial, diet, and environmental antigens after birth (89, 90). The reason for the delayed acquisition of skin homing potential in human neonatal Treg cells is not clear. But with impaired barrier function, such as in atopic dermatitis, late coming Tregs may increase the susceptibility to allergen sensitization through the skin (55).

In mouse, however, a unique neonatal Treg population was recently found to migrate to hair follicles and get activated at 1–2 weeks after birth, coinciding with the initial colonization of microbes to the skin (91, 92). Such rapid recruitment of Treg cells in neonatal skin depends on Ccl20–Ccr6 pathway stimulated by commensal bacteria and their surface molecules. Blocking Treg cell entry into hair follicles during neonatal window or colonization of bacteria during adult period all leads to increased antigen-specific effector T cells in the draining lymph nodes, demonstrating the importance of murine neonatal Tregs in promoting immune tolerance to skin commensal microbiota. It further indicates that certain chronic tissue inflammation in adults may be closely associated with impaired tolerance to commensal microbiota established during the neonatal period. Whether murine Treg cells (93–96) accumulate in other barrier sites, including lung and gut, during a defined early developmental period is not as clearly studied as the ones in the mouse skin.

A second difference between human and murine Treg cells is the timing of appearance, with the former emerging at gestational week 13 (97, 98) while the latter being detected in the thymus 2–3 days after birth (27, 99, 100). The frequency of human Treg cells in $CD4^+$ T cells significantly increases during the second trimester then decreases during the third trimester. Within the first week after birth, Treg cell ratio rapidly increases again (56, 101, 102). Depletion of $CD25^+$ Treg cells enhanced fetal T cell activation against self and maternal cells, but not against unrelated donor cells (103). Loss of FOXP3 leads to the occurrence of autoimmune inflammation specifically at second-trimester. Thus, the early appearance of human Treg cells in fetus plays a unique but critical role in maintaining self-tolerance as well as feto-maternal tolerance (8, 103, 104).

Murine neonatal Tregs and human fetal Tregs also have common features. They are more proliferative, have higher percentage of activated/effector cells, and perform better in the suppression assay *in vitro* when compared to adult Treg cells (27, 56). The transcriptome of human neonatal/fetal Tregs is also different from that of adult Treg cells, supporting the enhanced cell division and suppressive functions (57, 58).

ORIGIN OF T CELLS IN EARLY LIFE

Although having different dynamics in T cell emergence, the origin of human and murine prenatal/perinatal T cells with distinct intrinsic properties, including short TCR, promiscuous antigen recognition, and high CD5 expression, is the same, *i.e.* both are derived from hematopoietic stem cells (HSCs) from fetal liver (53, 58, 105–108). High expression of *Lin28b* and high expression of let-7 microRNA mark the difference between fetal liver/thymus and adult BM/thymus, respectively. The detailed *in vivo* experiments in murine system further demonstrate that ectopic expression of *Lin28b* or loss of *Ezh2* in adult BM hematopoietic stem/progenitor cells (HSPCs) induces activation of fetal-specific genes (including let-7 target genes) in HSPCs and fetal-like lymphopoiesis, including the development of B-1 cells, marginal zone B cells, and $\gamma\delta$ T cells (106, 109).

Both human or mouse fetal/neonatal $CD4^+$ T cells preferentially differentiate into induced Tregs (iTregs) when compared to adult $CD4^+$ T cells (58, 103, 110, 111). Inhibiting *Lin28b* in human fetal $CD4^+$ T cells leads to let-7 upregulation and reduced Treg cell differentiation (112). Human fetal naïve T cells also express higher level of Helios, and deletion of Helios results in impaired Treg differentiation and regulatory function (113). These results demonstrate that fetal liver-derived T cells have unique intrinsic properties to promote Treg cell differentiation.

PERSISTENCE OF NEONATAL T CELLS IN ADULTHOOD

The uniqueness of neonatal T cells and their roles in immune tolerance are not restricted to early life. Using a fate-mapping model, Yang et al. found that the number and function of murine neonatal Tregs were stably maintained in adulthood (27). Thus, the adoptive transfer of the persisting neonate-derived Treg cells from adult mice suppressed the progression of multi-organ autoimmune pathology in *Aire*-deficient mice. Similarly, human fetal Treg cells specific for maternal antigens can be found more than a decade later, right into the teenage year (103). Therefore, Treg cells produced during a specific ontogenic window in early life are unique and essential in maintaining self-tolerance in adulthood.

Notably, the persistence of fetal T cells in young adults is not limited to Treg cells. The analysis of deep sequencing data of human TCR repertoire recently reveals that large numbers of naïve T cell clones without N-region addition (fetal origin) are public clones and also persist for decades (114). A better understanding of the impact of these persisting fetal/neonatal T cells on self-tolerance and immune responses against pathogen/tumor in adults will thus be important and may bring benefits in the development of vaccine and therapeutics.

EARLY-LIFE T CELL TOLERANCE AND UMBILICAL CORD BLOOD TRANSPLANTATION

Allogeneic HSCT from an HLA-matched related or unrelated donor has been more and more widely used to treat patients with

malignant or non-malignant hematological disorders (115). The HSCs used in the transplantation can be derived from BM, peripheral blood, or UCB. Multiple comparisons between the transplantation of UCB and BM/peripheral blood HSCs have shown that UCB grafts are associated with lower incidence of GVHD, and in some cases such as patients with pre-transplant persistent minimal residual disease, better long-term outcomes (116). When $CD34^+$ cells from a third-party HLA-haploidentical donor were transplanted together with unrelated UCB cells, an early haploidentical engraftment was frequently replaced by durable UCB engraftment (117, 118). The distinct features of fetal liver-derived HSCs and Tconv/Treg cells described above may build the basis for these advantages in UCB transplantation (UCBT). Whether T cells reconstituted from UCBT could provide further benefits, such as better self-tolerance and lower incidence of autoimmune diseases later in life, will be an interesting question to investigate.

CONCLUDING REMARKS

T cell-mediated immune tolerance is essential in preventing unwanted or self-reactive immune responses throughout life. The distinct features of prenatal/neonatal Tconv and Treg cells provide a unique layer of tolerance against maternal and self antigens, certain allergens, and commensal microbes-derived products. The in-depth investigation of these T cell populations in early life may shed light on a better understanding of the immune responses in infants, the early-life root of certain adult immune alterations, and the choice and prognosis of UCBT in treating hematological disorders.

AUTHOR CONTRIBUTIONS

LY and QG wrote the manuscript. DL and RJ gave critical comments and revision. All authors contributed to the article and approved the submitted version.

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REVIEW
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How to Combine the Two Landmark Treatment Methods—Allogeneic Hematopoietic Stem Cell Transplantation and Chimeric Antigen Receptor T Cell Therapy Together to Cure High-Risk B Cell Acute Lymphoblastic Leukemia?

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Allogeneic hematopoietic stem cell transplantation (allo-HSCT) has made tremendous progress in the last few decades and is increasingly being used worldwide. The success of haploidentical HSCT has made it possible to have "a donor for everyone". Patients who received transplantation in remission may have a favorable outcome, while those who were transplanted in advanced stages of disease have a poor prognosis. Although chimeric antigen receptor T (CAR-T) cell therapy is currently a milestone in the immunotherapy of relapsed or refractory (R/R) B cell acute lymphoblastic leukemia (B-ALL) and has demonstrated high remission rates in patients previously treated in multiple lines, the relatively high relapse rate remains a barrier to CAR-T cell therapy becoming an excellent cure option. Therefore, combining these two approaches (allo-HSCT and CAR-T cell therapy) is an attractive area of research to further improve the prognosis of R/R B-ALL. In this review, we will discuss the current clinical practices of combining allo-HSCT with CAR-T cell therapy based on available data, including CAR-T cells as a bridge to allo-HSCT for R/R B-ALL and CAR-T cell infusion for post-transplant relapse. We will further explore not only other possible ways to combine the two approaches, including CAR-T cell therapy to clear minimal residual disease peri-transplantation and incorporation of CAR technology to treat graft-*versus*-host disease, but also the potential of CAR-T cells as a part of allo-HSCT.

Keywords: chimeric antigen receptor, acute lymphoblastic leukemia, relapsed or refractory, graft *versus* host disease, minimal residual disease, stem cell transplant

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) has achieved great progress in the past few decades. Advances in graft-*versus*-host disease (GVHD) prophylaxis and supportive care have significantly improved the outcomes of allo-HSCT. The success of haploidentical hematopoietic stem cell transplantation (haplo-HSCT) has expanded the application of allo-HSCT, making it possible to have "a donor for everyone". In recent years, the results of haplo-HSCT have been comparable to HSCT with matched sibling donors and unrelated donors (1–4). As a result, there has been a dramatic increase in the number of haplo-HSCT worldwide (5–7).

However, only transplantation of patients in remission may obtain favorable outcomes, whereas the prognosis of transplantation of patients with advanced disease is poor, with a long-term survival rate of only about 20% (7). Therefore, the efficacy of salvage allo-HSCT for patients with relapsed or refractory (R/R) hematological malignancies is very limited. In addition, post-transplantation relapse still occurs frequently and is the main cause of death after allo-HSCT, yet there is no satisfactory salvage method (8, 9).

The advent of chimeric antigen receptor T (CAR-T) cell therapy offers hope for patients with R/R hematological malignancies. CAR-T cell therapy has shown a high remission rate in these patients with severe pre-treatments (10–19). However, the relatively high relapse rate remains a barrier to CAR-T cell therapy becoming a curable method (10, 11, 20, 21). The integration of allo-HSCT and CAR-T cell therapy becomes an attractive area of research to fully exploit each other's advantages and further improve the treatment of B-cell malignancies, especially high-risk B-cell acute lymphoblastic leukemia (B-ALL).

To sum up, we will explore the current clinical practices of combined allo-HSCT and CAR-T cell therapy including CAR-T cell therapy as a bridge to allo-HSCT for R/R B-ALL and CAR-T cell infusion for post-transplant relapse, based on available data. And we will also further explore other possible ways to combine the two methods, including the clearance of minimal residual disease (MRD) peri-transplantation by CAR-T cell therapy and the incorporation of CAR technology in the treatment of GVHD. Meanwhile, we will also focus on a number of preclinical or pilot clinical studies targeting for CAR-T cells as part of the graft or conditioning regimen in allo-HSCT.

IS CAR-T CELL THERAPY A BRIDGE TO ALLO-HSCT OR A DEFINITIVE TREATMENT?

The relapse rate of B-ALL after CAR-T cell therapy was 20–70% when the follow-up period was long enough (22). Therefore, it is still controversial whether CAR-T cell therapy is the definitive treatment or bridging therapy to allo-HSCT. Currently, the need for allo-HSCT after CAR-T cell therapy usually depends on the characteristics and persistence of CAR-T cells, the duration of B cell aplasia, institutional experience, and the patient's intent and general physical condition. For patients who intend to receive allo-HSCT after CAR-T cell therapy, haploidentical donors are an important source of donors due to the rapid donor preparation and the strong effect of graft *versus* leukemia (GVL) (1, 23). **Table 1** presents the results of current large clinical studies of patients requiring allo-HSCT after CAR-T cell therapy. We will discuss pediatric and adult patients separately.

For pediatric and young adult patients with R/R B-ALL, a phase 1/2a study involved 30 patients treated with CD19 CAR-T cell therapy. After CAR-T cell therapy, only 10% of patients underwent allo-HSCT. Despite the low percentage of subsequent allo-HSCT, the event-free survival (EFS) rate was 67%, and the overall survival (OS) rate was 78% at 6 months of continuous remission (17). Subsequently, a global phase 2 study of Tisagenlecleucel in 75 patients showed that only eight patients in remission underwent allo-HSCT (15). The EFS and OS rates at 12 months were 50 and 76%, and the median duration of remission was still not reached after a median follow-up of 13.1 months. In both studies, the persistence of CAR-T cells and the duration of B cell aplasia were long.

In contrast, a phase 1 study at Seattle Children's Hospital enrolled 45 children and adolescents with R/R B-ALL in CD19 CAR-T cell therapy. The MRD-negative complete remission (CR) rate was 93%, but the median expected duration of B cell aplasia was only 3 months. Of the 40 patients with MRD-negative CR, 11 (27.5%) underwent consolidative allo-HSCT, and only two (18%) patients experienced relapse after allo-HSCT. Of the 29 patients who did not undergo consolidative allo-HSCT, 16 patients (55%) relapsed with a median follow-up of 12.2 months (25). Another study from Pediatric Oncology Branch of the National Cancer Institute enrolled 20 children and young adults with R/R B-ALL who received a single infusion of CD28-containing anti-CD19 CAR-T cells (27). A total of 12 patients achieved MRD-negative CR. The persistence of CAR-T cells was relatively short, and no CAR-T cells were detected after day 68. Thus, a high proportion (83%) of patients who obtained MRD-negative CR underwent subsequent allo-HSCT. All 10 patients who underwent allo-HSCT remained disease-free, and no unexpected peri-transplant toxicity was observed. Two patients were judged ineligible to undergo allo-HSCT and both relapsed within a short time (27). In a recent large phase 1/2 study from China, a total of 110 patients with B-ALL were infused with CD19 CAR-T cells (30). The majority of patients were children. Morphologic CR was observed in 93% of patients, and 87% achieved MRD negativity. 75 patients (73.5%) subsequently received allo-HSCT and 50 patients received haplo-HSCT. Leukemia-free survival (LFS, 76.9 *vs* 11.6%, $P<0.0001$) and OS (79.1 *vs* 32.0%, $P < 0.0001$) were significantly better in patients who underwent allo-HSCT compared with those who received only CAR-T cell therapy. The authors speculated that in the majority of the patients, haplo-HSCT (67%) and a myeloablative conditioning regimen may play a role to reduce leukemia relapse.

For adults with R/R B-ALL, a phase 1 trial from MSKCC first reported the results of patients receiving 19-28z CAR-T cell

TABLE 1 | Summary of large clinical studies related to the need for allo-HSCT after CAR-T cell therapy in B-ALL.

Study	N	Costimulatory domain	Previous HSCT, %	CR/CRi rate, %	MRD- CR rate, %	Allo-HSCT in CR, %	Haplo-HSCT, %	Overall OS, %	Overall RFS/EFS/LFS, %	Allo-HSCT *vs* non- HSCT
Children and young adults										
Maude et al. Phase I/IIA (17)	30	4-1BB	60	90	79	10	NA	78 (at 6 mo)	67 (at 6 mo)	NA
Maude et al. (ELIANA) (15, 24)	79	4-1BB	61	82	81	10	NA	70 (at 18 mo)	66 (at 18 mo)	NA
Gardner et al. (25, 26)	45	4-1BB	62	93	93	28	NA	69 (at 12 mo)	51 (at 12 mo)	LFS, P = 0.057
Lee et al. (27–29)	51	CD28	35*	61	55	75	NA	52 (at 10 mo)*	49 (at 18 mo)	Relapse (9 *vs* 86%, P = 0.001); LFS, P = 0.006
Zhang et al. (30)	110 (65% children)	4-1BB (81%) CD28 (19%)	14	93	87	73	67	64 (at 12 mo)	58 (at 12 mo)	LFS (77 *vs* 11%, P < 0.0001); OS (79 *vs* 32%, P < 0.0001)
Adults										
Park et al. (10)	53	CD28	36	83	67	39	NA	50 (at 13 mo)	50 (at 6 mo)	EFS, P = 0.64; OS, P = 0.89
Jiang et al. (31)	58 (5 children)	4-1BB	5	88	81	45	62	61 (at 12 mo)	50 (at 7.3 m)	RFS, P = 0.001; OS, P = 0.099
Turtle et al. (32, 33)	53	4-1BB	43	85	85	40	0	50 (at 20 mo)†	50 (at 7.6 mo)†	EFS (HR = 0.39 P = 0.088)
Gu et al. (34)	56 (Ph+ ALL)	4-1BB	0	91	68	59	83	50 (at 16 mo)	50 (at 15 mo)	OS (59 *vs* 23%, P = 0.005); EFS (53 *vs* 19%, P < 0.001)
Zhao et al. (35)	122	4-1BB	20	100	100	45	100	NA	NA	LFS, P < 0.001; OS, P < 0.001

HSCT, hematopoietic stem cell transplantation; CR, complete remission; CRi, complete remission with incomplete count recovery; MRD, minimal residual disease; Allo-HSCT, allogeneic HSCT; Haplo-HSCT, Haploidentical HSCT; OS, overall survival; RFS, relapse-free survival; EFS, event-free survival; LFS, leukemia-free survival.

**Results were reported from the first 21 patients.*

†The authors reported survival rates in patients achieving MRD negative CR after CAR-T cell therapy.

therapy (10). A total of 53 adults were enrolled and 44 (83%) patients achieved CR. Among the 44 patients with CR, 17 (39%) patients proceeded to allo-HSCT. There was no significant difference in EFS and OS between MRD-negative patients who underwent allo-HSCT and those who did not. A clinical trial from China included 53 adults and five pediatric R/R B-ALL patients who received CD19 CAR-T cell therapy (31). Of the 47 patients with MRD-negative remission, 21 were bridged to allo-HSCT. Overall, no difference was found in OS between patients who received allo-HSCT and those who did not. However, the trial further identified subgroups of patients with high (≥5%) pre-infusion bone marrow MRD or poor prognostic markers and found that only this subgroup benefited from allo-HSCT with significantly prolonged EFS.

On the contrary, in a phase 1/2 clinical trial from Fred Hutchinson Cancer Research Center, 45 (85%) of the 53 patients who received CD19 CAR T-cell therapy achieved MRD-negative CR. Eighteen (40%) patients in MRD-negative CR underwent allo-HSCT. Multivariable stepwise modeling demonstrated that allo-HSCT after CAR-T cell therapy may achieve a better EFS (32, 33). Gu B et al. reported a study of adults with R/R Philadelphia-chromosome positive ALL receiving humanized CD19 CAR-T cell therapy. Fifty-one/56 (91.1%) patients achieved CR or CR with inadequate count recovery (CRi). Subsequently, 30/51 CR/CRi patients received consolidative allo-HSCT. Patients with allo-HSCT had better 2-year OS and LFS than those without allo-HSCT. Multivariable analysis revealed that allo-HSCT and MRD-negative remission were independent prognostic factors of OS and LFS (34). Recently, we conducted a multicenter retrospective study to assess whether patients can benefit from haplo-HSCT after CAR-T cell therapy or not (35). A total of 122 patients were enrolled, including 55 patients with subsequent haplo-HSCT and 67 patients without subsequent transplantation. Compared to the non-transplant group, patients who received subsequent haplo-HSCT had higher 2-year OS (77.0 *vs* 36.4%, $P < 0.001$) and LFS (65.6 *vs* 32.8%, $P < 0.001$). In addition, MRD-negativity before transplantation predicts a favorable outcome of CAR-T cell therapy followed by haplo-HSCT.

From the above findings, the need to bridge allo-HSCT after R/R B-ALL remission with CAR-T cell therapy is still a controversial topic. **Table 2** lists the ongoing clinical trials of CAR-T cell therapy bridging to allo-HSCT in the treatment of B cell malignancies. Bridging allo-HSCT, while reducing relapse rates, is associated with transplant-related mortality. The most critical factor for the future will be the identification of risk factors for relapse after CAR-T cell therapy and selective bridging of allo-HSCT in high-risk patients. For patients with a low risk of relapse after CAR-T cell therapy, close monitoring is all that needed.

CAR-T CELL THERAPY TO TREAT POST-TRANSPLANT RELAPSE WITH LOW INCIDENCE OF GVHD

Relapse is the leading cause of death after allo-HSCT (36). The prognosis of relapse after allo-HSCT is very dismal, with low remission rates and poor long-term survival (37, 38). The median survival after relapse is 5.5 months. The estimated survival rates at 1-, 2- and 5-year after relapse are 30, 16, and 8%, respectively (9). Despite the development of allo-HSCT for the decades, the treatment of relapse after allo-HSCT remains a major challenge. Augmentation of the GVL effect through donor lymphocyte infusion (DLI) is one of the major salvage interventions for post-transplant relapse (39–43).

However, DLI has a limited effect on ALL relapse after allo-HSCT, with a CR rate of only 27% (44). Moreover, the application of DLI is limited by the development of acute or chronic GVHD (40–60%) (45, 46). Therefore, new therapeutic strategies are urgently needed to improve the prognosis of ALL relapsed after allo-HSCT. CAR-T cell therapy has brought revolutionary progress in the treatment of R/R hematological malignancies. At present, CAR-T cells still show great potential in the treatment of post-transplant relapse. T cells harvested for CAR-T preparation may come from donors or recipients (**Table 3**).

TABLE 2 | Ongoing clinical trials of CAR-T cell therapy bridging to allo-HSCT in the treatment of B cell malignancies.

Trial ID	Phase	Disease	Disease status	Target	Estimated enrollment	Conductor
NCT03366324	1/2	B-cell Malignancies	MRD positive	CD19	20	Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, China
NCT03366350	1/2	B-cell Malignancies	R/R	CD19	50	Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, China
NCT04626726	1/2	B-ALL	R/R	CD19/ CD22	50	No.2 Hospital of Hebei Medical University, China
NCT02846584	2	B-cell Malignancies	R/R	CD19/ CD20	100	Southwest Hospital of Third Military Medical University, China
NCT03110640	1	B-cell Leukemia/ Lymphoma	R/R	CD19	20	The First Affiliated Hospital of Wenzhou Medical University, China
NCT02431988	1	Diffuse Large B Cell Lymphoma	R/R	CD19	10	University College London Hospital, London, United Kingdom

B-ALL, B cell acute lymphoblastic leukemia; MRD, minimal residual disease; R/R, relapsed or refractory.

TABLE 3 | Clinical outcomes of CAR-T cell therapy for post-transplant relapse.

Study	N	Costimulatory domain	CR/CRi rate, %	Acute GVHD, %	Chronic GVHD, %
Donor derived allogeneic CAR-T cells					
Kochenderfer et al. (47, 48)	20	CD28	80*	0	10
Cruz et al. (49)	8	CD28	50†	0	0
Dai et al. (50)	2	4-1BB	50	100 (grade 2 to 3)	0
Hu et al. (51)	3	4-1BB	67	33.3 (grade 3)	NA
Recipient derived allogeneic CAR-T cells					
Park et al. (10)	19	CD28	84	0	0
Maude et al. (17)	18	4-1BB	NA	0	0
Lee et al. (27)	7	CD28	57	0	0
Zhang et al. (30)	16	4-1BB‡	94	12.5 (grades 1 and 3)	12.5
Hu et al. (51)	11	4-1BB	100	18.2 (grade 2)	NA
Turtle et al. (32)	11	4-1BB	93	0	9
Gardner et al. (25)	27	4-1BB	93	3.7 (grade 3)	0

CR, complete remission; CRi, complete remission with incomplete count recovery; GVHD, graft versus host disease.
**CR rate was calculated from five ALL patients.*
†CR rate was calculated from two relapsed ALL patients.
‡81% of 110 enrolled patients received 4-1BB costimulatory CAR-T cells.

For the first time, Kochenderfer et al. infused donor-derived allogeneic CD19 CAR-T cells into patients with malignancies that persisted after allo-HSCT and standard DLI (47, 48). CAR-T cells were infused without previous chemotherapy or lymphocyte depletion conditioning. Eight of 20 patients with B-cell malignancies obtained remission, which included six CRs and two partial remissions. B-ALL had the highest response rate, with four of five patients achieving MRD-negative CRs. In another study, Cruz et al. reported a phase one study in which donor-derived virus-specific T cells were engineered to express CD19 CAR. CR was achieved in one of two patients with B-ALL relapsing after allo-HSCT (49). In our report, two of three patients (66.7%) with relapsed B-ALL post-transplantation obtained CR after receiving donor-derived CD19 CAR-T cell therapy (51).

In addition to donor-derived T cells, CAR-T cells can also be manufactured from T cells harvested from the recipients. In several studies described in the previous chapters (10, 17, 27), patients with R/R B-ALL who relapsed after allo-HSCT were also included. The reported CR rates after CAR-T cell therapy ranged from 57 to 84%. In our study (51), we included 11 patients who received recipient-derived CAR-T cell therapy for post-transplant relapse. All patients (100%) achieved CR after CAR-T cell therapy. In another study from China, efficacy of CD19 CAR-T cell in high-risk B-ALL was evaluated (30). Sixteen patients had allo-HSCT prior to CAR-T cell therapy, and 11 (68.8%) had at least one DLI. After CAR-T cell therapy, 15 (93.8%) patients achieved CR. No statistically significant difference was observed in the rate of CR in patients who received allogeneic or autologous CAR-T cell therapy.

From the above data, CAR-T cell therapy has good efficacy in the treatment of post-transplant relapse. In addition to the routine complications such as cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS), allogeneic CAR-T cells infusion brings concerns about GVHD induction. In the study from Kochenderfer et al. (47, 48), a total of 14 patients had a history of GVHD, but none developed new-onset acute GVHD after CAR-T cell infusion. One patient developed mild chronic ocular GVHD 2 years later, and another patient had chronic GVHD at study entry, but the disease slowly and progressively worsened. In the study by Cruz et al. (49), no GVHD was observed after donor-derived CAR-T cell therapy, whereas we observed that acute GVHD in one of three patients following donor-derived CAR-T cell infusion. This patient was diagnosed with grade 3 gastrointestinal GVHD with secretory diarrhea more than 10 times per day. Symptoms improved after combination therapy with steroids, cyclosporin, mycophenolate, and ruxolitinib (51).

For recipient-derived CAR-T cell therapy, Park et al. (10), Maude et al. (17), and Lee et al. (27) reported a total of 43 cases but no GVHD was observed. Two studies from China showed that a small proportion of patients experienced GVHD after CAR-T cell infusion. One study showed that out of 16 patients, two (12.5%) patients developed acute GVHD (grade 1 and grade 3), and two (12.5%) patients developed extensive chronic GVHD (30). In our report, two of 11 patients (18.2%) developed grade 2 acute skin GVHD after infusion of recipient-derived CAR-T cells (51).

For GVHD caused by allogeneic CAR-T infusion, it is unclear whether treatment of GVHD affects the persistence and effectiveness of CAR-T cells. In a pilot study, two B-ALL patients received donor-derived 4-1BB costimulatory CAR-T cell therapy after allo-HSCT and developed grades 2–3 acute GVHD 3–4 weeks after cell infusion. Symptoms of GVHD were easily relieved with short-term use of steroids and/or cyclosporin A. However, after anti-GVHD therapy, one patient with moderately reduced blasts in bone marrow rapidly progressed and died, and another patient with hematologic CR achieved CD19 positive relapse (50). Nevertheless, a recent case report presented that allogeneic donor-derived 4-1BB based CAR-T cells were persistent up to 6 months after infusion under therapeutic levels of cyclosporine A (52).

In contrast to the aforementioned studies using CAR-T cells prepared from unselected T cells, two studies engineered 4-1BB containing CAR-T cell products, which consisted of a defined 1:1 ratio of CD4+: CD8+ CAR-T cells (25, 32). This highly

defined CD19 CAR T-cell product was remarkably potent, with over 90% of patients achieving CR after CAR-T cell therapy in both studies. Turtle et al. reported that 27 (93%) of 29 patients with R/R B-ALL achieved bone marrow remission after CAR-T cell therapy. Patients who received lymphodepletion with fludarabine and cyclophosphamide before CAR-T cell therapy achieved a 1-year DFS rate greater than 60%. Eleven patients with prior allo-HSCT received infusions of CAR-T cells manufactured from recipients. None of the 11 patients developed acute GVHD after CAR-T cell therapy. One patient who had grade 1 acute skin GVHD before study enrollment developed chronic GVHD at 3 months after CAR-T cell infusion and required corticosteroid therapy (32). In another study of 45 patients with R/R B-ALL, the MRD negative remission rate after CAR-T cell therapy was 93%. The estimated 12-month EFS of the infused patients was 50.8%, the estimated 12-month OS was 69.5%, and the median follow-up time was 9.6 months. Twenty-seven patients in this study had undergone prior allo-HSCT. One patient had a history of GVHD, which had been phased off GVHD medication for more than 1 year prior to CAR-T cell therapy, and developed grade 3 acute skin GVHD (25).

Compared with DLI, CAR-T cell therapy has a higher remission rate for post-transplant relapse and the incidence of GVHD associated with CAR-T cells infusion seems to be relatively low. To date, a summary of all data on CAR-T cell therapy for post-transplant relapse showed that the incidence of GVHD was less than 10%. The risk factors for allogeneic CAR-T cell-associated GVHD have not been fully defined. But from the current data, it may be related to the source of T cells (donor- or recipient-derived), CAR structure (53–56), CAR-T cell subpopulation, the history of GVHD after allo-HSCT, which needs to be further clarified by larger data support.

CAR-T CELL THERAPY TO CLEAR PERI-TRANSPLANTATION MRD

CAR-T cell therapy improves the outcomes of R/R ALL strikingly, but has potentially life-threatening complications, including CRS and ICANS, especially in patients with high disease burdens. Although most patients make a full recovery after treatment, patients with grades 3 to 4 CRS or ICANS are recommended to be transferred to the intensive care unit, and a small percentage of patients still die because of serious complications. Therefore, CAR-T cell therapy could be used more safely to clear MRD with morphological remission, which is suggested to accompany mild complications. In addition, MRD is a powerful prognostic factor in the treatment of ALL (57–63). For ALL patients receiving allo-HSCT, peri-transplantation MRD levels have been confirmed to be significantly associated with post-transplant relapse and long-term survival. Thus, for B-ALL patients undergoing allo-HSCT, the application of CAR-T cell therapy to clear peri-transplantation MRD is an effective and safe way to improve the prognosis. Previous studies on CAR-T cell therapy included patients with MRD-positive remission and patients with elevated MRD after transplantation.

Park et al. included 15 patients who had MRD with bone marrow blasts rates ranging from 0.01 to <5% and six patients with MRD-negative remission (10). Results showed that when compared with higher disease burden (≥5% bone marrow blasts), lower disease burden (<5% bone marrow blasts) was associated with a lower risk in severe CRS (41 *vs* 5%, P = 0.004) and neurotoxic effects (59 *vs* 14%, P = 0.002). Moreover, patients with lower disease burden had significantly longer EFS (10.6 *vs* 5.3 months, P = 0.01) and OS (20.1 *vs* 12.4 months, P = 0.02) than patients with higher disease burden. But there was no significant difference in survival between patients with lower disease burden who underwent transplantation and those who did not.

Another study included six patients with marrow blasts less than or equal to 5%, two of whom were MRD-positive after transplantation (27). Patients with higher disease burden were significantly more likely to have grades 3 or 4 CRS than patients with lower disease burdens (P = 0.039). After CAR-T cell therapy, all six patients obtained MRD-negative remission. Five of them underwent subsequent allo-HSCT after MRD clearance and remained disease-free with no unexpected peri-transplant toxicities. One patient with previous allo-HSCT was ineligible to receive a second allo-HSCT and relapsed with CD19-negative leukemia 3 months later.

In a study of 110 high-risk ALL patients treated with CAR-T cell therapy, 42 patients with MRD-positive remission were included (30). CAR-T cell therapy successfully cleared MRD in all 42 patients with a significantly lower incidence of grades 3 to 4 CRS and grades 2 to 3 neurotoxicity compared with patients who had morphologic relapse. The majority of patients (73.5%) in this study received subsequent allo-HSCT and achieved an LFS of 76.9% at 1 year. Notably, among the 75 patients who received allo-HSCT, only seven (10.1%) of 69 MRD-negative patients relapsed after transplantation, while three (50%) of six MRD-positive patients relapsed after transplantation. This reflected the importance of clearing MRD before transplantation to reduce post-transplant relapse.

Kebriaei et al. conducted a phase 1 trial in 17 B-ALL patients who received allogeneic CD19 CAR-T cells infusion to target MRD at a median of 64 days after allo-HSCT (64). CAR T cells were administered without additional lymphodepletion. GVHD prophylaxis was tapered and discontinued by 6 months after allo-HSCT. No unexpected acute infusion or delayed toxicities were noted. Three patients developed GVHD, one patient with grade one acute skin GVHD and one patient with chronic skin GVHD who responded to steroids. One patient with a prior history of drug-induced hepatotoxicity died from hepatic GVHD. Following allo-HSCT, 1-year PFS and OS were 53 and 63%, respectively. When the subset of patients who received haplo-HSCT was analyzed, the respective1-year rates were 75 and 100%, respectively. In a similar study, Zhang C et al. reported that two high-risk ALL patients who received haplo-HSCT were prophylactically infused with donor CAR-T cells on day 60 without CRS and GVHD. Two patients survived with disease-free for 1 year and 6 months, respectively (65).

From the above results of the studies, CAR-T cell therapy is an effective and safe method to clear peri-transplantation MRD. At present, there are an increasing number of clinical studies in this field. As more studies confirm the results, the clearance of MRD will greatly expand the application of CAR-T cell therapy. In addition, whether prophylactic CAR-T cells infusion for high-risk ALL with MRD-negative remission can prevent relapse is another interesting topic.

INCORPORATION OF CAR TECHNOLOGY INTO THE TREATMENT OF GVHD

GVHD is the most frequent complication after allo-HSCT (66, 67). Despite improvements in post-transplant immunosuppression, 20–60% of recipients still develop GVHD, which is the leading cause of non-relapse mortality following allo-HSCT (7). Alloreactive T cells mediated immune injury to the host organ is a key process in GVHD. Therefore, negative regulation of T cells to induce immune tolerance is the main method to prevent and treat GVHD. In recent decades, the commonly used immunosuppressive agents for GVHD include steroids, calcineurin inhibitors, and mycophenolate mofetil, *etc*. However, due to the lack of specificity of these drugs and the requirement of long-term maintenance, they can lead to loss of T cell immune function, weaken the anti-infection and anti-leukemic effects of T cells after allo-HSCT, and increase the risk of infection and relapse.

In recent years, an increasing subpopulation of immune cell have been considered to play a role in GVHD (68). Adoptive transfusion of immune cells in GVHD has attracted increasing attention. Previous studies have shown that regulatory T cells (Tregs) infusion can prevent and treat GVHD effectively and have little influence on GVL effects (69–73). Other immune cell subsets, such as NK cells, NKT cells, myeloid derived suppressor cells and type II innate lymphocytes, have also been proved to reduce the incidence of GVHD in a series of preclinical and clinical studies, while the GVL effect remains (74–79).

However, a large number of polyclonal Tregs infusion without antigen specificity leads to widespread, non-specific immunosuppression. Compared with polyclonal Tregs, antigen-specific Tregs have the advantage of migrating to target antigen, persisting in local tissues and mediating local immunosuppression (80, 81). Thus, a relatively small number of antigen-specific Tregs will be sufficient to produce immunosuppression (80, 82). Antigen-specific Tregs can be enriched from alloreactive T cells following stimulation with allogeneic antigen-presenting cells *in vitro*. The expansion efficiency *in vitro* is relatively low, which can limit the number of cells and their universal application in patients. In addition, the extensive expansion of antigen-specific Tregs by antigen-presenting cells stimulation will lead to loss of FOXP3 (83) and decreased survival *in vivo* (84).

The emergence of CAR technology enables T cells to specifically recognize, bind and clear targeted cells in a non-MHC restricted manner. These characteristics of CAR technology have opened new ideas for conferring Treg cell specificity, or CAR-Tregs. CAR-Tregs have a stable phenotype and function without MHC restriction and are less dependent on IL-2. It preferentially migrates to target sites and has stronger specific immunosuppressive effects (85). In animal models, CAR-Treg has shown great potential in the treatment of various diseases, especially autoimmune diseases (86–90).

MHC class I molecules are constitutively expressed on the surface of almost all nucleated cells and are major determinants of allo-HSCT compatibility. Therefore, MHC class I molecules are potential target antigens for CAR-Tregs to induce immune tolerance after allo-HSCT. In 2016, a group created HLA-A2–specific CAR and its application in generating antigen-specific Tregs (91). *In vitro*, A2-CAR-Tregs maintained their expected phenotype and inhibitory function before, during, and after A2-CAR-mediated stimulation and did not have cytolytic activity. In a mouse model of xenogeneic GVHD transplanted from human PBMCs to NSG mice, human A2-CAR-Tregs were superior to Tregs expressing unrelated CAR in preventing xenogeneic GVHD caused by HLA-A2+ T cells. Two other groups also established A2-CAR-Tregs and demonstrated their enhanced inhibitory function in a human skin xenograft transplant model (92, 93). More recently, Dawson et al. developed a panel of humanized A2-CARs and tested them in Tregs. Adoptive transfer of humanized A2-CAR Tregs *in vivo* showed that humanized A2-CAR Tregs migrate rapidly and persist in A2-expressing allografts, suppress HLA-A2+ cell-mediated xenogeneic GVHD, and diminish rejection of human HLA-A2 + skin allografts (94).

Besides cell-based immunosuppression, another strategy to control GVHD is to target important cells or molecules in the process of GVHD. CD83 is an important marker to define activated human dendritic cells. CD83 is also expressed on activated human T lymphocytes, but not on natural Treg (95). Previous studies have shown that monoclonal antibodies targeting CD83 can reduce GVHD in mice without affecting GVL and antiviral activity (96). Therefore, CD83 may be a potential target for CAR-T cells for the prevention and treatment of GVHD. As mentioned above, CAR-T cells have the property of recognizing, binding, and clearing cells carrying target antigens and infusion of donor-derived CAR-T cells after allo-HSCT is less likely to elicit GVHD. Based on these characteristics of CAR-T cells, human CD83-targeted CAR-T cells have been developed for the prevention of GVHD (97). Human CD83 CAR-T cells can eradicate pathogenic CD83+ target cells, substantially increase the ratio of Tregs to allo-activated conventional CD4+ T cells, and have preventive and therapeutic effects on xenogeneic GVHD.

ALLOGENEIC CAR-T CELLS AS PART OF HAPLO-HSCT

For patients with high leukemia burden, it is difficult to collect enough autologous T cells in CAR-T cell production. There are also cases where the autologous T cells fail to produce CAR-T cells due to T cell dysfunction and the effects of previous

chemotherapy. Allogeneic CAR-T cells may solve this problem. However, allogeneic CAR-T cells will be quickly eliminated by the patient's immune system without additional gene editing or long-term lymphodepletion.

Two groups from China developed a new method to co-infuse allogeneic CAR-T cells with allogeneic hematopoietic stem cells from haploidentical donor into R/R B-ALL patients (98–100). After re-induction of chemotherapy or a reduced-intensity conditioning regimen, haploidentical donor-derived CD19-CAR-T cells were infused in incremental numbers for 4 days. Haploidentical hematopoietic stem cells were infused after CAR-T cells infusion. The infusion of CAR-T cells as part of the conditioning regimen eradicated leukemia cells and the patients' normal B cells, and may improve hematopoietic stem cells engraftment. In turn, engraftment of allogeneic hematopoietic stem cells can further enhance the amplification and persistence of allogeneic CAR-T cells. A total of 4 patients with R/R B-ALL were reportedly treated with this protocol. An MRD-negative remission was achieved and complete donor cell engraftment was established. One patient did not have GVHD because of GVHD prophylaxis, but had a short duration of CAR-T cells persistence. The remaining three patients without GVHD prophylaxis developed varying degrees of GVHD, but the CAR-T cells persist relatively longer with the longest persistence up to 20 months. Two patients died from severe infections and two patients survived for 100 days and 20 months with disease-free, respectively.

Recently, Wiebking et al. designed an intriguing approach which combined both allo-HSCT and CAR-T cell therapy with complementary anti-leukemia mechanisms: the HLA-dependent activity of GVL effect and the HLA-independent mechanism of CAR-T cell (101). In this setting, a TCR$\alpha\beta$/CD19-depleted haplo-HSCT platform was employed, which was associated with very low transplantation-related mortality and GVHD incidence (102–105). CAR-T cells were manufactured from depleted $\alpha\beta$ T cells by genome editing to express CD19-specific CARs, while simultaneously inactivating the T cell receptor and rejoining the graft of haplo-HSCT. *In vivo*, the

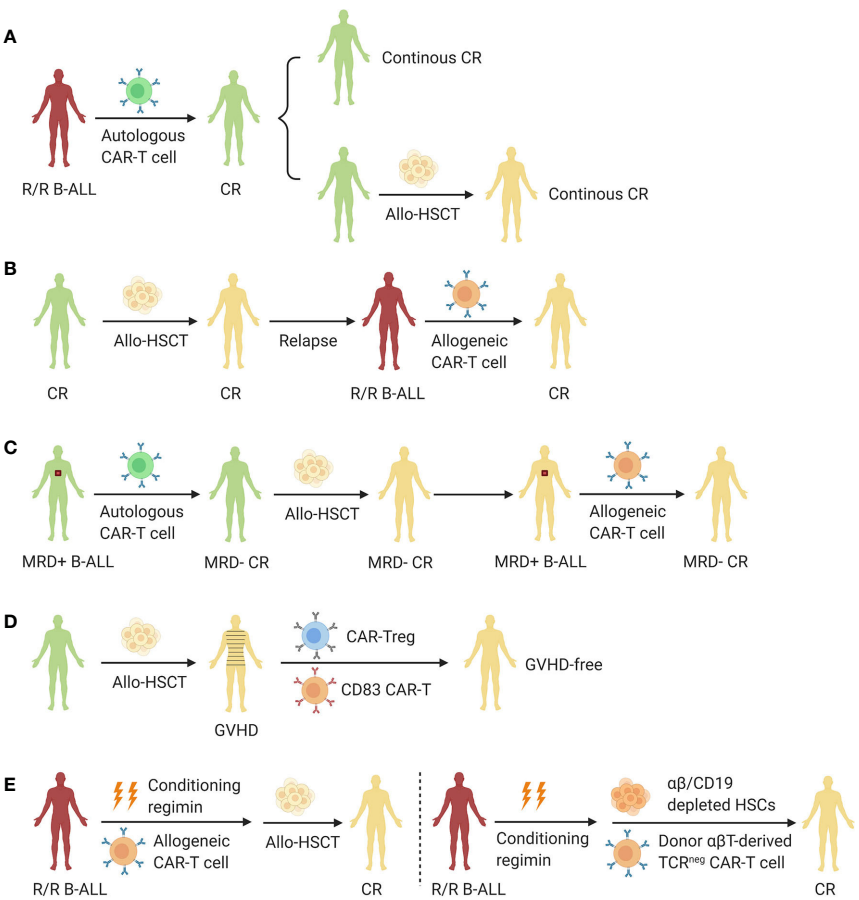

FIGURE 1 | Allo-HSCT in combination with CAR-T cell therapy aiming to improve the prognosis of B-ALL. **(A)** CAR-T cell therapy as a definitive treatment or a bridge to allo-HSCT for R/R B-ALL. **(B)** Infusion of allogeneic CAR-T cells to treat post-transplant relapse. **(C)** Clearance of minimal residual disease peri-transplantation by CAR-T cell therapy. **(D)** Incorporation of CAR technology into the treatment of GVHD. **(E)** CAR-T cells as part of the conditioning regimen or graft in allo-HSCT. R/R B-ALL, relapsed or refractory B cell acute lymphoblastic leukemia; CR, complete remission; Allo-HSCT, allogeneic hematopoietic stem cell transplantation; CAR, chimeric antigen receptor; MRD, minimal residual disease; GVHD, graft-versus-host disease; Treg, regulatory T cell.

$\alpha\beta$TCR-CD19 CAR-T cells eliminated leukemia without causing GVHD in a preclinical xenograft model. This appealing program needs to be further verified in the clinical setting.

CONCLUSIONS

The treatment of high-risk ALL remains a challenging. Especially for adult ALL, the outcomes of receiving chemotherapy alone are still poor (106). The establishment of the haplo-HSCT system, which allows almost all patients to have a donor, has greatly improved the prognosis of ALL. The emergence of CAR-T cell therapy has further brought an amazing breakthrough in the treatment of R/R B-ALL. At present, the two therapeutic approaches (allo-HSCT and CAR-T cell therapy) have their own indications and mechanisms, which are difficult to be completely replaced. Combining the two approaches to establish a complete B-ALL treatment system will become an important development area at present and in the future, so as to further improve the prognosis of B-ALL and approach the goal of curing B-ALL (**Figure 1**). According to the available data, CAR-T cell therapy can obtain a high remission rate in R/R B-ALL patients. After remission, some patients can obtain long-term CAR-T cells persistence and disease-free survival, which makes CAR-T cell therapy a definitive method, while other patients need subsequent allo-HSCT to further reduce relapse rates. For B-ALL patients with post-transplant relapse, infusion of allogeneic CAR-T cells also achieves high remission rates with low incidence of GVHD. It is not clear whether secondary transplantation is necessary or not according to the small number of cases. Haplo-HSCT is suggested to be associated with higher incidence of GVHD compared with allo-HSCT from matched sibling donors. CAR technology is a good strategy for the treatment of GVHD. The results from preclinical studies are encouraging and its clinical application is worth expectation in the future. In addition, CAR-T cells are also being explored as a part of haplo-HSCT, such as conditioning regimen or graft, and the complementary mechanism of the two methods are expected to bring better therapeutic effect.

AUTHOR CONTRIBUTIONS

HH and MZ designed the structure of the paper. MZ wrote this paper. All authors contributed to the article and approved the submitted version.

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Identification of New Soluble Factors Correlated With the Development of Graft Failure After Haploidentical Hematopoietic Stem Cell Transplantation

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Graft failure is a severe complication of allogeneic hematopoietic stem cell transplantation (HSCT). The mechanisms involved in this phenomenon are still not completely understood; data available suggest that recipient T lymphocytes surviving the conditioning regimen are the main mediators of immune-mediated graft failure. So far, no predictive marker or early detection method is available. In order to identify a non-invasive and efficient strategy to diagnose this complication, as well as to find possible targets to prevent/treat it, we performed a detailed analysis of serum of eight patients experiencing graft failure after T-cell depleted HLA-haploidentical HSCT. In this study, we confirm data describing graft failure to be a complex phenomenon involving different components of the immune system, mainly driven by the IFNγ pathway. We observed a significant modulation of IL7, IL8, IL18, IL27, CCL2, CCL5 (Rantes), CCL7, CCL20 (MIP3a), CCL24 (Eotaxin2), and CXCL11 in patients experiencing graft failure, as compared to matched patients not developing this complication. For some of these factors, the difference was already present at the time of infusion of the graft, thus allowing early risk stratification. Moreover, these cytokines/chemokines could represent possible targets, providing the rationale for exploring new therapeutic/preventive strategies.

Keywords: graft failure, cytokines, chemokines, inflammation, Th1 T cells, macrophage activation, hemophagocytic lymphohistiocytosis

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INTRODUCTION

One of the main complications occurring after allogeneic hematopoietic stem cell transplantation (HSCT) is represented by graft failure (GF). It is a complex and multifactorial syndrome characterized by hypocellular bone marrow (BM) associated with severe pancytopenia in peripheral blood (PB). GF can be defined based either on the pathophysiology mechanisms or on the timing of the event. Primary GF is characterized by lack of initial engraftment of donor cells,

while secondary GF by the progressive loss of donor cells after initial engraftment. From a pathophysiological point of view, immune-mediated GF is caused by the attack of the donor cells by host immune cells, mainly T and Natural Killer (NK) cells surviving the conditioning regimen. Several factors have been reported to be associated with GF, including HLA disparity in the donor/recipient pair, presence of anti-HLA antibodies in the recipient, underlying disease, viral infections, type of conditioning regimen (particularly reduced-intensity conditioning and non-myeloablative conditioning), T-cell depletion of the graft (TCD) and stem cell source (1–4).

Our group has recently focused on a deep characterization of this phenomenon, analyzing a cytokine/chemokine asset in PB, (*i.e.*, IFNγ, sIL2Rα, CXCL9, CXCL10, TNFα, IL6, IL10, and sCD163), as well as the cellular features in BM biopsies of patients experiencing GF. From this analysis, we confirmed i) the *in vivo* role of the IFNγ-pathway in the development of immune-mediated GF; ii) that the sole inhibition of this pathway by an anti-IFNγ monoclonal antibody (mAb) was able to prevent GF. Finally, after observing a strong similarity between immune-mediated GF and hemophagocytic lymphohistiocytosis (HLH), we treated with Emapalumab, an anti-IFNγ mAb (5), on a compassionate use basis, three patients with primary HLH who, after having experienced GF, underwent a second successful HSCT.

In the present study, we tested other 44 cytokines/chemokines in the PB of the previously reported patients experiencing GF (5) with the aim of: i) further characterizing the GF signature; ii) identifying new possible targets to prevent/treat GF; iii) developing strategies capable to target a single pathway/molecule or a combination of them in order to prevent the occurrence of GF in patients at high-risk of developing this complication.

MATERIALS AND METHODS

Patients and Controls

Children aged 0.3 to 21 years, given an allograft from any type of donor/stem cell source [including matched family donor (MFD), matched unrelated donor (MUD), unrelated cord blood unit (UCB), haploidentical family donor], between January 1, 2016, and August 31, 2017, at IRCCS Bambino Gesù Children's Hospital in Rome, were considered eligible for the study. Patients or legal guardians provided written informed consent, and research was conducted under institutional review board approved protocols, in accordance with the Declaration of Helsinki. The Bambino Gesù Children's Hospital Institutional Review Board approved the study.

After completing the main study (5), we performed further analyses on the remaining samples of 8 out of 15 patients experiencing GF after TCD haplo-HSCT and compared them with those of eight controls, matched for transplant characteristics, who had been transplanted reaching sustained donor engraftment during the same period.

Cytokine Profile

Serum derived from patients experiencing GF and from a control group were analyzed by immunoassays incorporating magnetic microsphere technology (Merck, Darmstadt, Germany), according to the manufacturer's instructions, as previously described (6). Plates were read on MAGPIX® and analyzed using xPONENT® software (Luminex, Austin, Texas, USA). The following cytokines and chemokines were analyzed: CCL1, CCL2, CCL3, CCL5 (Rantes), CCL7, CCL19, CCL20 (MIP3α), CCL24 (Eotaxin-2), CXCL11, CX3CL1, PDGFαα, CD40L, G-CSF, GM-CSF, FLT3-L, IL1α, IL1β, IL2, IL4, IL5, IL7, IL8 (CXCL8), IL9, IL11, IL12p40, IL12p70, IL13, IL15, IL17A, IL17E, IL17F, IL18, IL21, IL22, IL23, IL27, IL28A, IL31, IL33, SCF, and TNFβ.

Statistical Analyses

Data are summarized as mean ± standard error of mean (SEM) and expressed as pg/ml. Student *t*-test (two-sided) was used to determine statistically significant differences between samples. When multiple comparison analyses were required, statistical significance was evaluated by a repeated measures ANOVA followed by a Log-rank (Mantel-Cox) test for multiple comparisons. P-values were reported in detail if statistically significant, i.e., <0.05 (*), <0.01 (**) and <0.001 (***). Graph generation and statistical analyses were performed using Prism version 7 software (GraphPad, La Jolla, CA). Interactome analysis on identified cytokines and chemokines modulated during GF was performed using STRING software (https://string-db.org) with a high interaction score (0.7).

RESULTS AND DISCUSSION

The samples of eight patients experiencing GF after receiving TCRαβ/CD19-depleted haploidentical HSCT (7) were compared to those of eight patients who did not develop this complication (during the study period we performed 115 haploidentical HSCT and 15 patients developed GF, the GF rate being 13%). Patient and control characteristics are detailed in **Table 1**. Main transplant characteristics were comparable between the two groups (except for a trend for a lower age in the GF group).

We found a significant modulation of IL7, IL8, IL18, IL27, CCL2, CCL5 (Rantes), CCL7, CCL20 (MIP3a), CCL24 (Eotaxin2), and CXCL11 in patients experiencing GF (see **Figure 1**).

Interestingly, several of these molecules (IL7, IL8, IL18, CCL5, CCL7, CCL20, and CCL24) were significantly different from the control group already at the time of graft infusion (IL7: 47.8 ± 9.2 pg/ml vs. 24.2 ± 2.5; IL8: 127.5 ± 18.7 vs. 68.7 ± 10.3; IL18: 4334.6 ± 2993 vs. 468.8 ± 53.9; CCL5: 2188.3 ± 721.8 vs. 4148.8 ± 590.1; CCL7: 169.8 ± 19.2 vs 94.9 ± 11.3; CCL20: 108.1 ± 13.9 vs. 42.1 ± 8.2; and CCL24: 652.7 ± 217.8 vs 1426.5 ± 406.7). These findings suggest possible effects related to the conditioning regimen.

It is well known that the conditioning regimen can cause mild to severe tissue damage, which induces a production of several

TABLE 1 | Characteristics of patients and controls.

	GF	CTRL	p
Total	8	8	
Gender			0.99
Female	3	4	
Male	5	4	
Age at transplant, years (median and range)	2.4 (0.2–9.6)	7.0 (1.1–19.8)	0.08
Disease			0.37
PID	2§	1ç	
AL	1	4	
Hbpathies/IBMFS	2	2	
Others	3*	1#	
Type of transplant			0.2
T-cell depleted haploidentical	8	5	
MUD	0	3	
Source of stem cells			0.2
PBSC	8	5	
BM	0	3	
Conditioning regimen			0.43
TBI-based	0	1	
Busulfan-based	7	5	
Treosulfan-based	1	2	
Sex mismatch			0.99
Yes	2	3	
No	6	5	

§One case each of combined immunodeficiency and HLH.
çOne case of autosomal recessive hyper-IgE syndrome.
**One case each of metachromatic leukodystrophy, mucopolysaccharidosis type 1 and osteopetrosis.*
#One case of adrenoleukodystrophy.
PID, primary immunodeficiencies; AL, acute leukaemia; IBMFS, inherited bone marrow failure syndromes; MUD, matched unrelated donor: PBSC, peripheral blood stem cells; BM, bone marrow.

pro-inflammatory cytokines and chemokines from both hematopoietic cells, as well as by damaged endothelium and epithelia, increased expression of adhesion molecules, major histocompatibility complex antigens and costimulatory molecules on the host antigen presenting cells (APCs) (8). Host APCs, which survive the conditioning regimen, become activated and capable of processing antigens present in the transplanted cells. Activation of either recipient or donor T cells after interaction with host APCs leads to their proliferation, differentiation and migration.

The identified cytokines and chemokines underline the involvement of an inflamed microenvironment where T lymphocytes, NK cells, immature and mature APCs, among which monocytes and dendritic cells (DC), are recruited from the periphery to the BM (5). Several of these molecules are also able to sustain the inflammation and maintain activation of lymphocytes. In this context, our analysis reveals higher levels of IL7 (**Figure 1A**), which contributes to an inflamed BM microenvironment (9), sustains T-cell proliferation, differentiation and survival, in particular of the *naïve* and memory compartments (10), but also of mature differentiated T lymphocytes, through the Bcl2 pathway (11, 12). IL7 has also been reported to act as co-factor for T-cell activation by stimulating production of Th1 cytokines, including IFNγ, IL2, and TNFα (13). Moreover, in the allo-HSCT setting, high levels of these cytokines have been associated with graft-versus-host disease (GvHD) onset and its exacerbation, by either promoting proliferation and survival of allo-reactive donor mature T cells or by increasing their activation state (14). These data, associated with high levels of IL27, support the assumption of an activated environment, in particular in the BM niche (**Figure 1B**). This latter cytokine, indeed, is able to control both innate and adaptive immune responses by stimulating STAT3 (15, 16) and to block Th17 T-cell activity (17). Furthermore, it has been also associated with the development of GvHD, reducing the number of $CD4^+Tbet^+$ cells, increasing the number of $CD8^+Tc1^+$ cytotoxic T cells and inducing IFNγ response *in vivo* (18).

As reported in our previous study (5), this inflammatory state is mainly driven by IFNγ, which is able to activate macrophages and epithelia to produce CXCL9, CXCL10, but also CXCL11 (19) (**Figure 1C**). These chemokines are able to strongly recruit antigen-primed Th1 T cells directly to the inflamed tissue. Moreover, high levels of these cytokines have been associated with organ rejection in kidney, lung and heart transplantation (20–22). Furthermore, low levels of CCL5 and CCL24, like those found in present analysis, could, instead, be caused by a damage of endothelial and epithelial cells by activated and cytotoxic T lymphocytes, this translating into a further increase of the recruitment of $Th1^+$ T cells expressing CXCR3 (**Figures 1D, E**). It is important to underline, however, that the ligands of CXCR3 (namely, CXCL9, CXCL10, and CXCL11) have been reported to be more potent than CCR5 ligands (i.e., CCL3, CCL4, and CCL5) and the frequency of $CCR5^+$ T lymphocytes is significantly lower in PB circulating T cells (23, 24). The reduced levels of CCL5 can be also explained by the elevated conversion of monocytes into activated macrophages during this inflammation period (25, 26). As shown in **Figure 1F**, the macrophages present in the BM are able to produce high levels of CCL20 (MIP3α), which is actively involved in the recruitment of T lymphocytes and reported to be increased in renal graft rejection and, in general, during inflammation, causing a recruitment of mature DC (27–29). Our data emphasize the role of myeloid cells in boosting and maintaining inflammation: in fact, high levels of CCL2 and CCL7 underline the recruitment of monocytes, immature DCs, and macrophages together with effector T and NK lymphocytes (**Figures 1G, H**) (30–36). Furthermore, CCL2 has been also reported to play a crucial role in the M1 macrophage polarization during inflammation, in the recruitment of $IFN\gamma^+$ γδ T cells and to regulate adhesion and chemotaxis through activation of β1 integrin and p38-MAPK (31, 37). In this altered microenvironment, we also detected high levels of IL8 and IL18 (**Figures 1K, I**). The first is physiologically produced by mononuclear cells and induces migration of lymphocytes to an injured site. High levels of this cytokine have been associated with GF, prolonged neutropenia and impaired differentiation of hematopoietic $CD34^+$ cells (38). Its high expression has also been associated with increased levels of CCL2, CXCL9, CXCL10, and IL2Rα (39). Lastly, elevated levels of IL18 can be explained by an enriched IFNγ environment (40). The production of this cytokine, in fact, is mediated by the inflammasome and, in turn, it is responsible for sustaining IFNγ

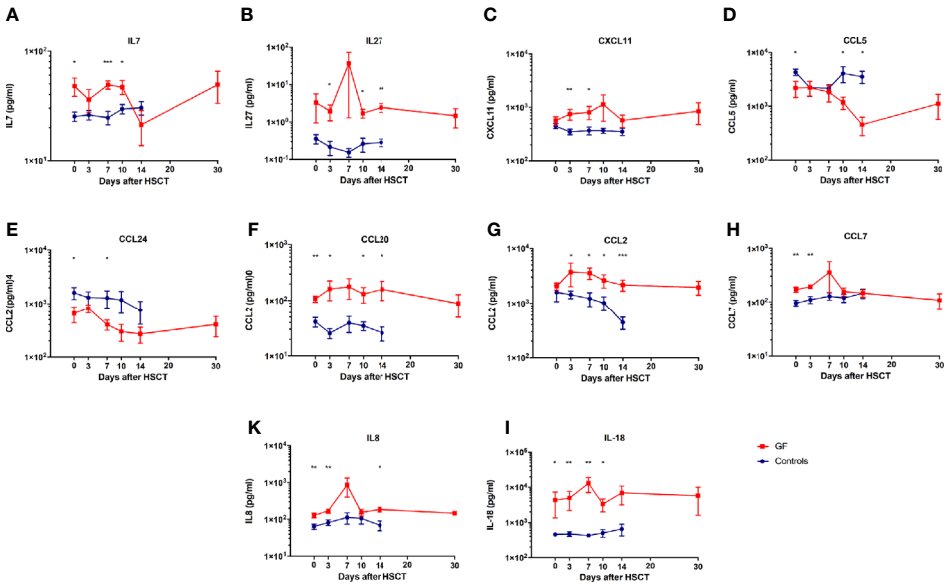

FIGURE 1 | Cytokines and chemokines modulated during GF. Serum levels of IL7 **(A)**, IL27 **(B)**, CXCL11 **(C)**, CCL5 **(D)**, CCL24 **(E)**, CCL20 **(F)**, CCL2 **(G)**, CCL7 **(H)**, IL8 **(K)**, and IL18 **(I)** in patients who either did (red line) or did not (blue line) experience GF are shown. In all graphs mean and SEM for each variable are represented. * p<0.05, ** p<0.01, *** p<0.001.

production in different lymphocyte subsets and is important for the differentiation of various T cell populations (40). Its accumulation has been associated with several immune-mediated diseases, including GvHD, and low overall survival of patients undergoing transplantations (41). IL18 is released by a damaged endothelium and is involved in macrophage activation, increasing expression of other pro-inflammatory cytokines (like CCL2) and in enhancing the activity of Th1 T and NK cells (42, 43). Its function is normally regulated by the presence of the high-affinity molecule IL18BP. For this reason, Liu et al. recently proposed to neutralize IL18 with IL18BP for the treatment of immune-mediated conditions, in which injury-associated cytokines are produced, including IFNγ and CXCL10 (44). In support to the probable role of macrophages and endothelial damage in the development of GF, recently, IL18 has been also described as potential biomarker and therapeutic target of macrophage activation syndrome/HLH, which shares, as mentioned before, several important features with GF (45). Notably, after grouping cytokines analyzed in this and in our previous study (5) as Th1, Th2, or "others," the Th1 profile seems to be predominant (**Figure 2**), although contra-regulatory Th2 cytokines (in particular IL10) are increased (as already reported other hyper-inflammatory conditions, such as in primary HLH (46)).

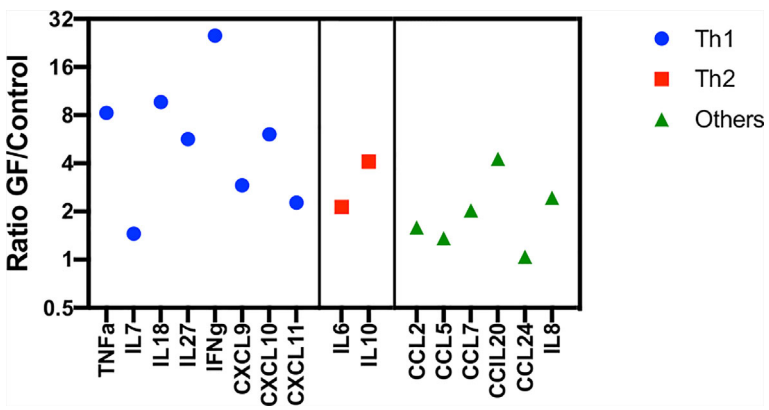

FIGURE 2 | Cytokines/Chemokines found to be preferentially expressed in GF at day +3 after HSCT, grouped as "Th1," Th2," and "other." Cytokine/chemokine levels are reported as ration between values measured in the GF and control group, respectively. This includes also cytokines/Chemokines previously reported in (5).

We acknowledge that, beside the limited sample size, the lack of samples collected before the conditioning regimen represents a limitation of the study, preventing the evaluation of its influence on the cytokine "signature" at time of transplant. Moreover, although not statistically significant, some differences in the conditioning regimens used may have influenced the cytokine profile. Additionally, since one patient in the GF group was affected by HLH, this could impact the cytokine profile of this individual subject (more in general, patients with primary immunodeficiencies may have altered cytokine production). Finally, we acknowledge that infections may influence the pattern of cytokine production. In this regard, the cumulative incidence of bacterial, viral and fungal infection was similar in the two groups investigated (see **Table 2** and **Supplementary Figure 1** for details). For this reason and given that the differences in cytokine levels were already present at very-early time-points, it is unlikely that this factor has influenced the cytokine profile of GF patients and controls.

Our data, together with those previously published by our group, support the hypothesis that during GF, complex mechanisms are activated and involve both soluble molecules and cellular components (**Figures 3** and **4**). By interactome analysis performed using STRING algorithm, several of these molecules were shown to be critical for the triggering and sustaining the pathophysiology of GF (**Figure 3**). Based on these data, strategies to prevent and treat this life-threatening complication can be considered. Notably, the use of emapalumab, a humanized mAb that binds and neutralizes IFNγ, currently approved for the treatment of adult and pediatric patients with primary HLH with refractory, recurrent or progressive disease or intolerance with conventional HLH therapy (47), has been explored as compassionate use (5, 48). Moreover, inhibition of cytokines like IL18 or IL27, as well as strategies aimed at compensation of the microenvironment increasing Th2 cytokines and chemokines (IL1β and CCL24), can be hypothesized.

TABLE 2 | Details on infections recorded in the GF and control cohorts during the study period.

	GF	CTRL
Total	4	3
Viral	**4***	**1**
CMV	3*	1
Adv	1*	
HHV6	1	
Bacterial	**0**	**2**
E. faecium		1
S. capitis		1
Fungal	0	0

One patient developed a coinfection with CMV and Adv.

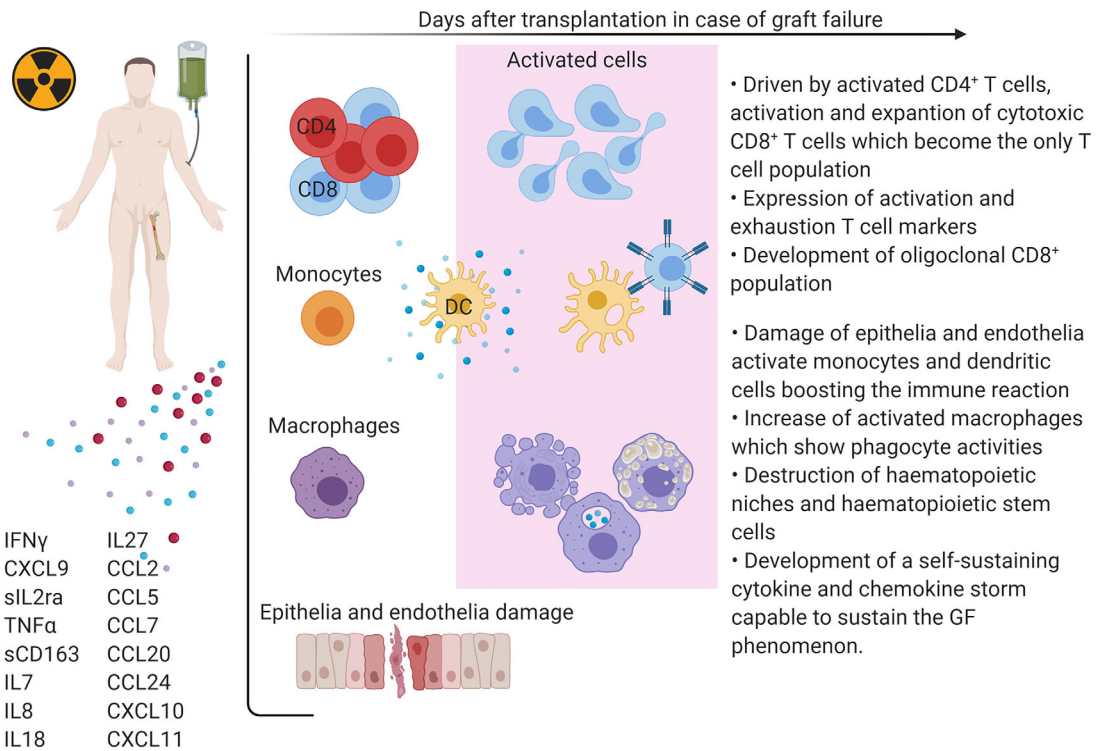

FIGURE 3 | Schematic representation of GF pathophysiology after HSCT.

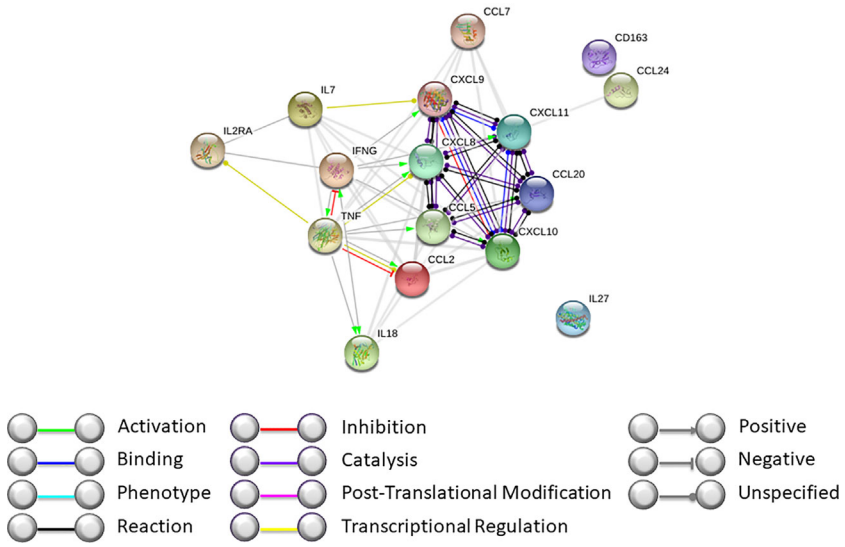

FIGURE 4 | Interactome analysis on identified cytokines and chemokines modulated during GF was performed using STRING software (https://string-db.org). Interactome of cytokines and chemokines modulated during GF after HSCT with high confidence score.

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 studies involving human participants were reviewed and approved by Ethics Committee of Bambino Gesù Childrens' Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

IC, PM, and FL designed the study and analyzed the data. LS, FD, MA, DP, BD, CQ, and PM treated patients, collected samples and data, analyzed data, and edited the paper. GW, CMA, and IC performed immunomagnetic assays, analyzed data, and wrote the paper. 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.2020.613644/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | Cumulative incidence of infections (bacterial, viral and fungal) in GF patients and controls.

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Conflict of Interest: PM and FL have received honoraria from SOBI.

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frontiers in Immunology

REVIEW
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Management of Hepatitis B Virus in Allogeneic Hematopoietic Stem Cell Transplantation

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The high morbidity of HBV reactivation following allogeneic hematopoietic stem cell transplantation (allo-HSCT) is partially due to the intense immunologic potency of complex therapeutic regimens, the use of antithymocyte globulin and calcineurin inhibitors to prevent graft versus-host disease (GVHD), prolonged immune reconstitution, and hematological malignancies infected with hepatitis B virus (HBV). Immunosuppression results in the reactivation of HBV replication from covalently closed circular DNA (cccDNA) residing in hepatocytes. However, the role of viral mutations during HBV reactivation needs to be validated. All individuals scheduled to receive allo-HSCT or wish to donate stem cells should be screened for hepatitis B surface antigen (HBsAg), antibodies to hepatitis B core (anti-HBc), and HBV-DNA. HBsAg-positive recipients of allo-HSCT have a high risk of HBV reactivation; thus, they should receive prophylactic antiviral therapy. The high barrier to resistance nucleos(t)-ide analogs (NAs) seems to be superior to the low barrier agents. Resolved-HBV recipients have a lower risk of HBV reactivation than HBsAg-positive recipients. Although prophylactic antiviral therapy remains controversial, regular monitoring of alanine transaminase (ALT) and HBV-DNA combined with preemptive antiviral treatment may be an optimized strategy. However, optimal antiviral therapy duration and time intervals for monitoring remain to be established. Accepting stem cells from HBsAg-positive donors is associated with a risk of developing HBV-related hepatitis. The overall intervention strategy, including donors and recipients, may decrease the risk of HBV-related hepatitis following HSCT from HBsAg positive stem cells. In this review, we summarize the issues of HBV in allo-HSCT, including HBV reactivation mechanism, HBsAg-positive recipients, HBV-resolved infection recipients, and donor-related factors, and discuss their significance.

Keywords: hepatitis B virus, hematopoietic stem cell transplantation, HBV resolved infection, HBV reactivation, HBV-related hepatitis, stem cell donor

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INTRODUCTION

Globally, an estimated 257 million people live with chronic HBV infection (1). The HBV carrier rate is high (6.2%) in the African and Western Pacific regions (2). In China, the prevalence rate of hepatitis B surface antigen (HBsAg) was estimated at 5–6%, and 4.38% of people 15–29 years of age are carriers (3, 4). Researchers are aware of HBV reactivation (HBVr) complications in patients receiving chemotherapy, monoclonal antibody (especially anti-CD20 antibody) treatment, and other intensive immunosuppressive therapies. Since covalently closed circular DNA (cccDNA) persists in hepatocytes and other tissues, HBsAg-positive patients and historically HBV infected patients are at a risk of HBVr during immunosuppressive therapy (5–7). The strength of HBVr is determined by the degrees of immune control and virus immune activity *in vivo*. In addition, because of the intense immunologic potency of the complex therapeutic regimens, and the use of rituximab and high-dose glucocorticoids, which usually leads to cytopenia, the incidence of HBVr due to immunosuppression is much higher in hematological malignancies than in other diseases (8, 9).

Guidelines have been recommended for patients with HBV infection undergoing immunosuppressive and cytotoxic therapy (8, 10–14). Hematopoietic stem cell transplantation (HSCT) technology has developed rapidly and is expected to become the mainstay treatment for patients with hematologic malignancies. Myeloablative conditioning regimens, antithymocyte globulin and calcineurin inhibitor treatment to prevent graft-versus-host disease (GVHD), high-dose glucocorticoids for GVHD therapy, prolonged immune reconstitution, evolving therapeutic treatments (e.g. ruxolitinib, rituximab, ibrutinib, and monoclonal antibodies) for chronic GVHD therapy, and the risk of donor HBV sources lead to a heighted risk of HBVr complication in hematological patients who accept allo-HSCT. Allo-HSCT is an independent risk factor for HBVr in patients with hematologic malignancies (15). However, knowledge regarding HBVr in allo-HSCT is not comprehensive and there are no standard guidelines for managing HBVr during allo-HSCT. Due to the high probability of HBV infection in hematological patients living in HBV epidemic area and the high frequency of HBVr during HSCT, it is necessary to review the developments made by studies on HBVr in allo-HSCT in recent decades. To comprehensively understand HBVr in allo-HSCT and help physicians deal with HBVr in allo-HSCT, here, we have summarized and reviewed the key issues in this domain.

DEFINITION OF HBV REACTIVATION

Previously there were no standard criteria for HBVr. For HBsAg-positive patients, HBVr was defined as a) 10-fold elevation of circulating HBV DNA compared with baseline levels before HSCT and b) detectable circulating HBV DNA in patients whose serum HBV DNA was undetectable before HSCT. In HBV-resolved patients, HBVr was defined as a) a positive result for the HBsAg test in a patient who previously tested negative (called reverse seroconversion, RS) and b) detectable circulating HBV DNA in patients with undetectable serum HBV DNA before HSCT (6, 13, 16, 17) (**Figure 1**). In 2018, the American Association for the Study of Liver Diseases (AASLD) recommended stricter criteria for HBVr (10). For HBsAg-positive patients, (1) ≥2 log (100-fold) increase in HBV DNA compared to the baseline, (2) HBV DNA ≥ 3 log (1,000) IU/ml in a patient with previously undetectable levels, (given that HBV DNA levels fluctuate) or (3) HBV DNA ≥ 4 log (10,000) IU/ml, if the baseline level is not available. For patients who are anti-HBc-positive and HBsAg-negative, the criteria are: (1) detection of HBV DNA, or (2) reappearance of HBsAg (**Figure 1**). Increase in alanine transaminase (ALT) levels to ≥3 times the baseline level and >100 U/L was deemed a hepatitis flare and the definition of HBV related to hepatitis was hepatitis flare plus HBVr.

HBV REACTIVATION MECHANISM

HBV enters the body and eventually enters hepatocytes through the key liver-specific receptor, sodium-taurocholate co-transporter (18). The nucleocapsid is inserted into the nucleus of hepatocytes and the DNA is converted into cccDNA (19). HBV cccDNA is stable and persistent in hepatocytes, which is the reservoir of HBVr despite serum clearance of HBV (20, 21) (**Figure 2**). The host's immune response to HBV infection undergoes an inactive immune tolerance state, active state, and conversion to the immune control phase (22, 23). HBV-specific T-cell responses suppress viral replication by both cytopathic effects and non-cytopathic cytokine pathways (24, 25). B cells produce antibodies against HBV and inhibit the spread of HBV infection to other hepatocytes (**Figure 2**). The first report of HBVr was made in 1975 by Wands in a patient with lymphoproliferative disease undergoing chemotherapy (26). It has been reported that HBV DNA restarts replication due to treatment-induced loss of immune control and immunosuppression (27–29).

In addition, HBV mutations in the major hydrophilic region of the S domain have been found in HBVr after HSCT in recent years (30–35). However, there is still no evidence that immune-escape mutations occurred prior to reactivation, or that they are responsible for assisting in the viral reactivation process (30). The role of viral mutations and immune escape during HBVr needs to be validated. There was no phenomenon of HBV-specific CD8 T-cell exhaustion during HBVr reported. Hepatitis occurs after immune system reconstitution and destruction of HBV infected hepatocytes (36) (**Figure 2**). HBV immune response activities with amplification of HBV DNA cannot be detected in time since they precede ALT alterations and clinical symptoms. HBV-induced hepatocellular damage is considered to be the result of a complex interplay among HBV, hepatocytes, and immune cells of the host; thus, several HBVr patients may have little or no liver dysfunction, while others may have hepatitis flares, interruption of immunosuppressive drugs, hepatic failure, and even death. The serological alteration of

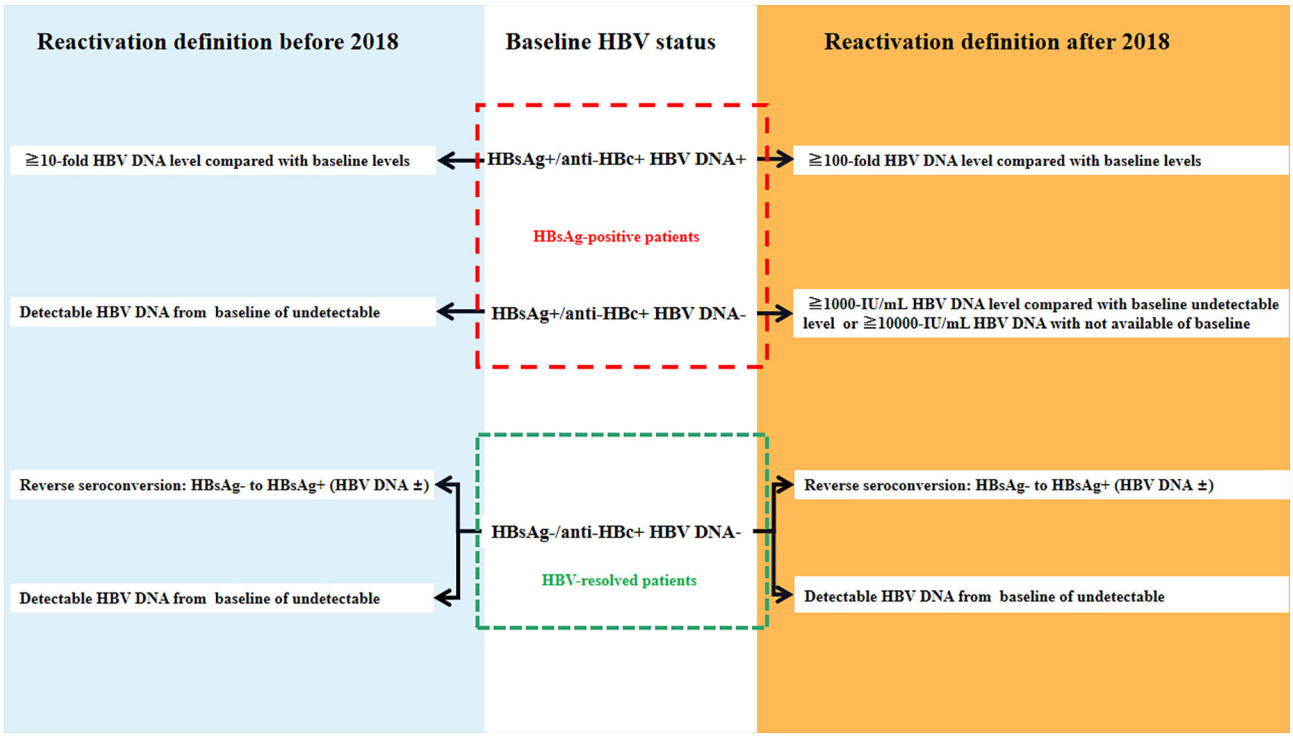

FIGURE 1 | The definition of hepatitis B virus (HBV) reactivation [adapted from (13)].

HBV and the HBV DNA load *in vivo* cannot effectively reflect the clinical influence of HBVr after allo-HSCT (37). Recent studies have found a favorable prognosis for HBVr in allo-HSCT recipients (38–42). The RS of HBV-resolved patients facilitates hepatitis B surface antigen seroclearance following antiviral treatment (38, 39). However, previous studies had different HBVr definitions and heterogeneous patient characteristics. Therefore, it is necessary to reevaluate the clinical influence of HBVr in a homogeneous group following consistent HBVr criteria, such as the incidence of HBV-related hepatitis, liver-related mortality, non-relapse mortality, and interruption or reduction of primary immunosuppressants.

HBsAg-POSITIVE RECIPIENTS

HBsAg-positive recipients have been widely recognized as high-risk (>10%) for HBVr following allo-HSCT. A study from Mary Hospital of the University of Hong Kong in 2002 indicated that the historical control without prophylactic antiviral treatment had an incidence of 45% HBVr after allo-HSCT (43). A multicenter retrospective study from the Italian Group for Blood and Marrow Transplantation showed that two-year incidence of HBVr after allo-HSCT in HBsAg-positive recipients was up to 81% without prophylactic antiviral treatment (44). Because of the high risk of HBVr, there were no HBVr results reported in HBsAg-positive allo-HSCT recipients without prophylactic antiviral treatment, due to thoughtful ethical considerations. Many perspective studies have demonstrated the validity of prophylactic lamivudine (LAM) 100 mg daily in decreasing the risk of HBVr in HBsAg-positive patients receiving chemotherapy (45–47). Similarly, prophylactic LAM 100 mg daily decreased the risk of HBVr to 5% (1/20) in HBsAg-positive recipients following allo-HSCT (43). Furthermore, other studies have shown the effectiveness of prophylactic entecavir (ETV) 0.5 mg daily in minimizing HBVr in HBsAg-positive recipients following allo-HSCT (48–50). In addition, the high barrier to resistance NAs (ETV, tenofovir disoproxil fumarate [TDF], or tenofovir alafenamide [TAF]) seem to be superior to the low barrier to resistance antiviral drugs (LAM, Telbivudine [LdT], and Adefovir [ADV]). A retrospective study from China in 2016 indicated that the ETV 0.5 mg daily group had a much lower incidence of HBVr than the LAM 100 mg daily group (2.1%[2/97] vs. 23.5%[28/119], $p<0.001$) for HBsAg-positive recipients following allo-HSCT (51). However, data comparing the high barrier to resistance of NAs (ETV, TDF, or TAF) with low barrier agents (LAM, LdT, ADV) was limited. Physicians performing HSCT may be concerned whether the antiviral drug would influence the engraftment of neutrophils or platelets during allo-HSCT. A retrospective study in Brazil indicated that LAM/ETV/TAF treatment had no influence on neutrophil or platelet engraftment in allo-HSCT, which needs to be confirmed in large-sample size studies (52). Based on these studies, nearly all guidelines for the prevention of HBVr associated with immunosuppressive therapy had consensus regarding screening for HBsAg and HBcAb before accepting allo-HSCT treatments (11, 13, 53, 54), and prophylactic antiviral treatment

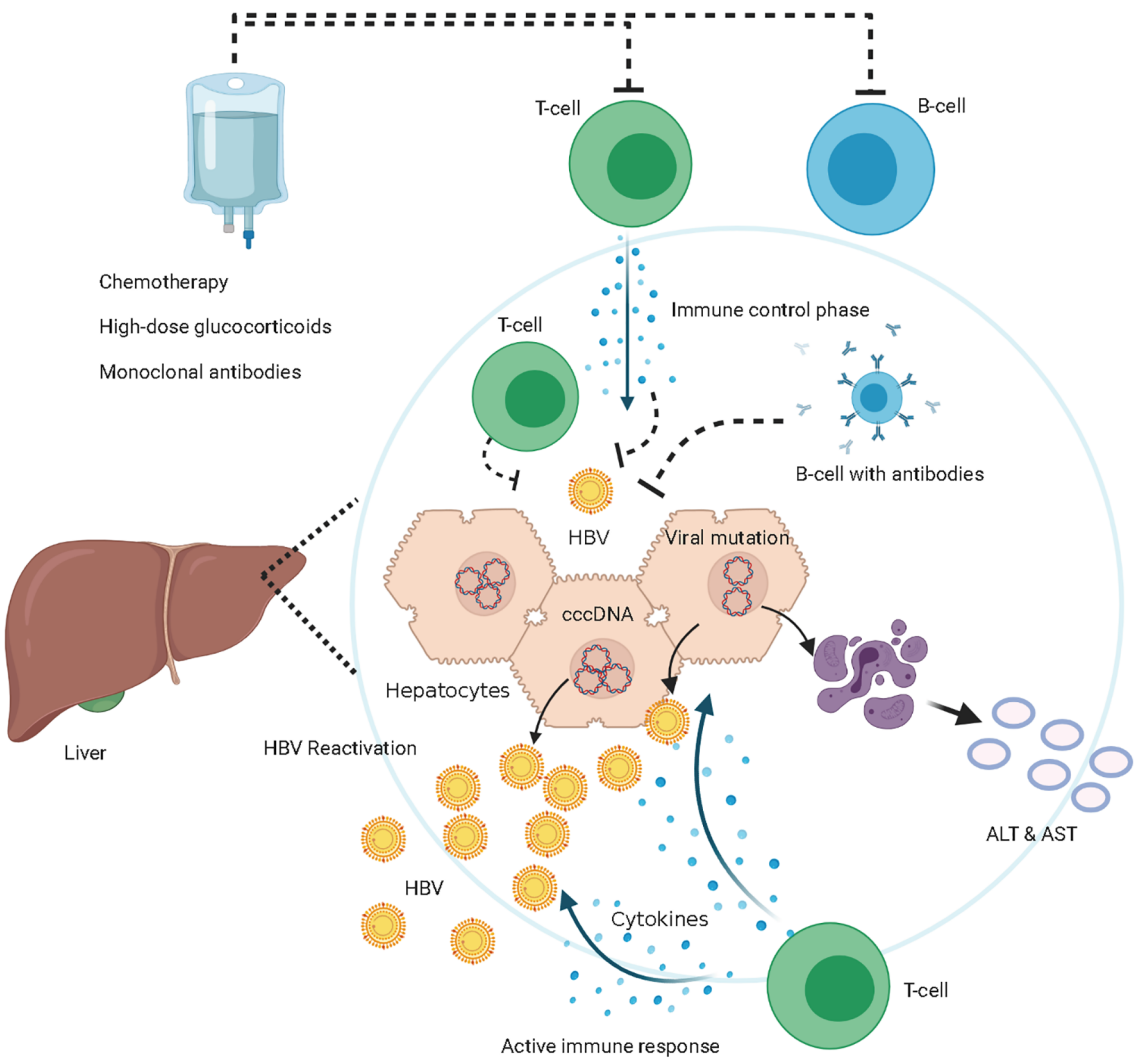

FIGURE 2 | The mechanism of hepatitis B virus (HBV) reactivation. Immune control phase, HBV-specific T-cell suppress viral replication by both cytopathic effects and non-cytopathic cytokine pathways, B cells produce antibodies against HBV and inhibit the spread of HBV infection among hepatocytes, HBV cccDNA persistent in hepatocytes. Immune suppressive phase, T-cells and B-cells are inhibited or eliminated by the immunosuppressive treatments, HBV DNA restarts replication due to treatment-induced loss of immune control. Maybe HBV mutations lead to immune-escape from HBV-specific T-cell, HBV DNA restarts replication. HBV reactivation occurs when HBV DNA amplify actively *in vivo*. Active immune phase, immune system reconstitution and T-cells attack HBV-DNA and HBV infected hepatocytes. The destructed hepatocytes release ALT and aspartate aminotransferase (AST).

was recommended to decrease the risk of HBVr for HBsAg-positive recipients. However, there is no explicit view on screening for HBV DNA and hepatitis B surface antibody (HBsAb) before accepting allo-HSCT therapy. Additionally, the monitoring interval of serological HBV, the duration of prophylactic antiviral treatment, and choice of NAs remain unclear. One suggestion has been that referred monitoring should continue for 6 months after cessation of immunosuppressive therapy, with 2-month intervals potentially being appropriate (55). A recent publication suggested that serological HBV should be obtained at baseline and evaluated every 6 months during antiviral therapy. Additionally, it should also be checked at least monthly for the first 3 months after the cessation of antiviral therapy and every 3 months thereafter (56). Although there is no consensus on duration of antiviral treatment, the majority of recommendations for prophylactic antiviral treatment duration vary from 6 months to 12 months after discontinuation of immunosuppressive therapy (10, 11, 13, 54).

HBV-RESOLVED RECIPIENTS

HBV-resolved patients were defined as HBsAg-negative but positive for antibodies against hepatitis B core antigen (57). While HBV was not actively amplifying *in vivo*, the persistent cccDNA in hepatocytes could be amplified again

when the patient's immune system is suppressed. Previous prospective studies have shown that HBV-resolved hematological patients who accept immunosuppressive treatment/chemotherapy have a risk of HBVr, with the incidence of HBVr varying from 8.3% to 41.5% (7, 58–62). However, the risk of HBVr in HBV-resolved patients who accept allo-HSCT is not well known. A multicenter retrospective study from Italy illustrated that 6/50 (12%) of HBV-resolved patients underwent HBVr after allo-HSCT without prophylactic antiviral treatment at a median of 12 (7–32) months after transplantation; the 5-year cumulative incidence of HBVr was 22% (63). A retrospective study from Chiba University found that 4/35 (11%) HBV-resolved patients developed HBVr (64) without prophylactic antiviral treatment. Another retrospective study from San Martino University Hospital indicated that 14/137 (10%) patients had HBVr within a median of 19 months (range 9–77 months) after HSCT (40) without prophylactic antiviral treatment. We found that 13/300 (4.3%) HBV-resolved patients developed HBVr at a median of 588 days (range 455–1,294 days) after HSCT without prophylactic antiviral treatment (39). A recent retrospective study with a similar sample size from the National Taiwan University Hospital reported that 21/485 (4.72%) HBV-resolved patients presented HBVr at 16 months (range 8–50 months) after HSCT with no antiviral prophylaxis (42). A higher risk of HBVr was reported by a retrospective study at Hamanomachi Hospital, Japan: 18/69 (26.1%) HBV-resolved patients developed HBVr after allo-HSCT at a median of 440 days (75–1,829) without prophylactic antiviral treatment (65). The only prospective research in the domain of HBV-resolved patients undergoing allo-HSCT was conducted by the University of Hong Kong. The two-year cumulative incidence of HBVr was 40.8% (13/62) without prophylactic antiviral treatment, occurring at a median of 44 (8–100) weeks post-transplantation (41). Based on these studies, HBsAg-negative, anti-HBc-positive patients who underwent allo-HSCT also had a risk of HBV reactivation. However, it remains unclear whether prophylactic antiviral therapy can benefit HBV-resolved patients following allo-HSCT treatment as much as it benefits HBsAg-positive patients. A retrospective study from the University of Genoa evaluated 7 years' worth of single-center data on HBV-resolved patients who received allo-HSCT; none of the 50 HBV-resolved patients experienced HBVr while on prophylactic LAM treatment (66). However, another study indicated that although the majority of HBV-resolved recipients accepted antiviral treatment, the rates of HBVr in the HSCT group at one and seven years were 2.5% and 57.9%, respectively (38).

There are many controversial issues in HBV-resolved patients receiving allo-HSCT. The risk stratification of HBsAg-negative/HBcAb-positive HSCT recipients and recommendations on antiviral treatment are inconsistent from different specialized associations (**Table 1**). The Asian Pacific Association for the Study of the Liver recommended in 2015 that HBsAg-negative/HBcAb-positive patients with undetectable serum HBV DNA should be followed carefully by means of ALT and HBV DNA testing, then treating with NAs therapy upon confirmation of HBVr. Most European and American specialized associations provide aggressive views on prophylactic antiviral therapy in HBV-resolved patients. The experts in the domain of immunosuppressive treatment-related HBVr recommend initiating prophylactic treatment or monitoring HBV DNA levels for HBsAg-negative/HBcAb-positive patients undergoing intermediate-risk immunosuppression (6, 58). However, whether HBV-resolved patients receiving allo-HSCT belong to the high-risk or intermediate-risk group of HBVr is controversial. Previous studies reported a much lower incidence of HBVr in the HBsAg-negative/HBcAb-positive group than HBsAg-positive patients who underwent allo-HSCT. Large-sample prospective studies are needed to robustly investigate the incidence of HBVr in HBV-resolved patients undergoing allo-HSCT according to the 2018 AASLD definition of HBVr. Moreover, the effectiveness of prophylactic antiviral therapy in minimizing the risk of HBVr for HBV-resolved HSCT recipients is not well known. There are no studies comparing high barrier NAs (ETV, TDF, or TAF) with low barrier agents (LAM, LdT, and ADV) in HBsAg-negative/HBcAb-positive allo-HSCT recipients. The monitoring interval of serological HBV and duration of prophylactic antiviral treatment is also unclear (**Table 1**).

UNDERLYING REASONS WHY CONTROVERSIES EXIST IN PROPHYLACTIC ANTIVIRAL THERAPY OF HBV-RESOLVED ALLO-HSCT RECIPIENTS

The protective role of HBsAb has been found in hematological patients receiving chemotherapy. A systematic review described the protective role of the HBsAb, with a lower HBV reactivation rate in HBsAb(+) patients compared with HBsAb(-) patients with hematologic disease (7.1% versus 21.8%; $P < 0.001$) (9). In a group of HBsAg(-)/HBcAb(+) patients with lymphoma, patients without HBsAb before rituximab-based chemotherapy had a higher incidence of HBV reactivation than those with HBsAb (68.3% vs. 34.4%; $P = 0.01$) (61). It was reported that exceeding the threshold HBsAb titer of 100 IU/ml was associated with a 0% rate of HBVr (68, 69). However, few studies have investigated the role of HBsAb in HBcAb-positive patients who undergo HSCT. The role of HBsAb during HBVr in HSCT is unclear. We stratified 665 HBsAg-negative patients according to HBcAb/HBsAb presence into four groups; the HBcAb(+)HBsAb(-) group had the highest risk of HBVr among the patient groups (15.7%; $P < 0.001$). The cumulative HBV reactivation rates were 5.3% in the HBcAb(+)HBsAb(+) group, 0% in the HBcAb(-) HBsAb(-) group, and 2.1% in the HBcAb(-)HBsAb(+) group, with no significant difference among these groups. HBsAb in HSCT recipients conferred a protective effect against HBVr (39). A recent retrospective study from Turkey also reported the protective role of HBsAb in HBVr during allo-HSCT (70). Twenty two HBV-resolved patients showed different two-year cumulative incidence of HBVr (20% vs 75%) between groups of

TABLE 1 | Recommendations for hepatitis B surface antigen (HBsAg)-negative/HBcAb-positive patients undergoing allo-hematopoietic stem cell transplantation (HSCT) from different specialized associations.

Association	Risk stratification	Screen recommendation	Recommendation	Duration	Reference
American Society of Clinical Oncology Provisional Clinical Opinion 2015	High risk	Screen for HBsAg and HBcAb, followed by a sensitive HBV DNA test if positive	Prophylactic antiviral therapy or monitored closely and start antiviral therapy if HBVr occurs	Continued up to 12 months after cessation of therapy	(13)
American Gastroenterological Association Institute 2015	Not reported	Screen for HBsAg and HBcAb, followed by a sensitive HBV DNA test if positive	Antiviral prophylaxis	Continue for at least 6 months after discontinuation of immunosuppressive therapy (12 months for B cell–depleting agents).	(11)
Asian Pacific Association for the Study of the Liver 2015	Not reported	Screen for HBsAg and HBcAb prior to treatment, tested for HBV DNA if HBcAb-positive	Patients with detectable HBV DNA should antiviral treatment, patients with undetectable HBV DNA should be followed carefully by ALT and HBV DNA testing, and be treated with NA therapy upon confirmation of HBVr	Not reported	(67)
European Society of Clinical Microbiology and Infectious Diseases 2017	Not reported	Screen for HBsAg, HBcAb and HBsAb, followed by a sensitive HBV DNA test if positive of HBsAg/HBcAb	Prophylaxis with LAM, independent of the presence of HBV DNA	At least 18 months	(53)
European Association for the Study of the Liver 2017	High risk	Screen for HBsAg, HBsAb and HBcAb	Antiviral prophylaxis	Continue for at least 18 months after stopping immunosuppression	(54)
The Indian National Association for Study of the Liver 2018	Not reported	Screen for HBsAg and HBcAb, tested for HBV DNA if HBcAb-positive	Monitored with HBsAg, ALT and HBV DNA every 3 months during therapy and up to 6 months, pre-emptive antiviral therapy on detection of HBsAg or HBV DNA positivity	Continued for at least 18 months after discontinuation of HSCT	(14)
The American Association for the Study of Liver Diseases 2018	Lower risk of HBVr than HBsAg-positive patients, and depending on their clinical situation	Screen for HBsAg and HBcAb	Antiviral prophylaxis	Continued for at least 12 months after completion of immunosuppressive therapy	(10)
American Society of Clinical Oncology 2020	High risk	Screen for HBsAg, anti-HBc, and HBsAb	Antiviral prophylaxis Or careful monitoring and antiviral therapy at the earliest sign of HBVr	Continue for minimum 12 months after anticancer therapy completion	(56)

HBcAb(+)HBsAb(+) and HBcAb(+)HBsAb(-) (70). Certainly, these need to be verified in larger prospective studies. Nearly all studies on HBVr in HBsAg-negative/HBcAb-positive allo-HSCT recipients reported that HBVr was a late phase complication (**Table 2**). Additionally, nearly all studies recommended the administration of 6–12 months of antiviral prophylaxis. It is necessary to consider the value of early antiviral prophylactic treatment in preventing late phase complications of HBVr.

However, long-term antiviral treatment may cause resistance. The cumulative incidence of HBV resistance of anti-HBV drugs with a low resistance barrier (LAM, LdT, and ADV) is prevalent and growing over time in patients with chronic hepatitis B (54). LAM resistance occurs in up to 20% of patients after just one year of use (8). One study reported that an HBsAg-negative patient who underwent allo-HSCT using stem cells from an HBsAg-positive donor eventually acquired HBV infection due to a YYMD mutation as a result of long-term prophylactic treatment with LAM (73). One report suggested that HBV reemerges with T127P, F170FL, and S204R mutations with prophylactic LAM treatment, causing HBVr after HSCT (34). Furthermore, recent studies found favorable prognosis of HBVr in HBsAg-negative/HBcAb-positive HSCT recipients (38–42). HBVr in these groups can be controlled, and most HBVr patients acquire serologic clearance of HBsAg with regularly HBVr monitoring and preemptive antiviral treatment (38, 39). After antiviral initiation, HBV-resolved patients with reactivation showed a one year cumulative HBsAg clearance of 68.3% (38). No case of serologic clearance in an HBV-RS patient who recovered from HBVr converted to active HBsAg carriers has been reported. The liver-related mortality of HBVr was nearly zero. In a large prospective study monitoring HBV DNA monthly in HBV-resolved B cell lymphoma patients, no hepatitis due to HBVr was observed in patients who received antiviral treatment when HBV DNA levels were between 11 and 432 IU/ml (7). Another prospective study enrolled 83 HBsAg-negative/HBcAb-positive hematologic patients receiving anti-CD20 therapy. These patients were monitored every 4 weeks without antiviral therapy and every 2 weeks once HBV DNA was detectable. All patients with HBV DNA had RS or two-fold increase in upper limit of normal ALT received antiviral therapy. After therapy, ALT was normalized and HBV DNA returned to undetectable levels. There were no cases of clinical hepatitis, liver

TABLE 2 | Time of hepatitis B virus (HBV) reactivation in hepatitis B surface antigen (HBsAg)-negative/HBcAb-positive recipients after hematopoietic stem cell transplantation (HSCT).

Publish year	Number of HBVr	Type of study	Type of transplantation	Regimen	Probability of HBVr (cumulative rate)	HBV reactivation time after allo-HSCT	Reference
2011	6/50 (12.0%)	retrospective	45 MSD/2 HRD/3 MUD	35MAC/ 15RIC	13% at 1 year, 22% at 5 years	12 (range 7–32) months	(63)
2014	14/137 (10.2%)	retrospective	76 MSD/20 HRD/24 MUD/17 Others	63MAC/ 74RIC	6.3% at 2 years, 9.6% at 5 years	19 (range 9–77) months	(40)
2014	4/35 (11.4%)	retrospective	Not reported	Not reported	Not reported	19 months	(64)
2015	3/11 (27.3%)	prospective	3 Allo/7 Auto/1 Auto plus Allo	Not reported	Not reported	8, 9, 10 months	(15)
2016	14/52 (26.9%)	retrospective	Not reported	30MAC/ 22RIC	10.8% at 1 year, 43.9% at 5 years	15 (range 3–68) months	(71)
2017	7/107 (6.5%)	retrospective	Auto	Not reported	3.5% at 1 year, 5% at 2 years	16 (range 7–47) months	(72)
2017	13/62 (20.9%)	prospective	Not reported	41MAC/ 21RIC	17.7% at 1 year, 40.8% at 2 years	44 (range 8–100) weeks	(41)
2019	50/385 (12.9%)	retrospective	Not reported	Not reported	2.5% at 1 year, 57.9% at 7 years	19.9 (range 2.4–75.6) months	(38)
2019	18/69 (26.1%)	retrospective	Not reported	Not reported	11.2% at 1 year, 43.0% at 5 years	440 (range 75–1,829) days	(65)
2019	21/445 (4.72%)	retrospective	Not reported	196MAC/ 249RIC	2.2% at 1 year, 10.5% at 5 years	16 (range 8–50) months	(42)
2020	13/300 (4.3%)	retrospective	77 MSD/149 HRD/74 MUD	300MAC/ 0RIC	Not reported	645 (range 455–1,957) days	(39)

MSD, matched sibling donor; HRD, haploidentical related donor; MUD, matched unrelated donor; MAC, myeloablative conditioning; RIC, reduced intensity conditioning; Allo, allogeneic.

failure, or death (74). These data indicate that regular monitoring and preemptive antiviral therapy are effective methods for preventing HBVr-related hepatitis in HBV-resolved patients following immunosuppressive therapy.

DONOR RELATED FACTORS

It was first confirmed in 1995 that HBV can be transmitted *via* stem cells from HBsAg-positive donors to recipients during HSCT (75). A group at Queen Mary Hospital in Hong Kong pioneered the application of stem cells from HBsAg-positive donors in allo-HSCT (16, 76, 77). The incidence of HBV-related hepatitis in recipients who receive HBsAg-positive donor stem cells is in the range of 48% to 55.5% (16, 76). Therefore, serological HBsAg positivity seems to be a contraindication for HSCT donors, due to the fear of HBV-related hepatitis. Selecting a suitable donor for a HSCT recipient from a pool of potential HBsAg-positive donors is an unresolved problem. The donor's predicted favorable factors for HSCT outcomes may conflict with the status of serologic HBsAg positivity. There are no standard guidelines for managing patients who receive stem cells from HBsAg-positive donors. The Fifth European Conference on Infections in Leukemia indicated that both the donor and recipient undergo antiviral treatment and that HBsAg-negative recipients are vaccinated to prevent HBV transmission (78). These measures lowered the incidence of HBV-related hepatitis to 6.9% of recipients who receive stem cells from HBsAg-positive donors, whereas the historical control group was 48% (16). We established a protocol for the management of HBsAg-positive donors comprising of antiviral treatment to lower circulating HBV DNA levels in HBsAg-positive donors, induction of passive immunity in HbsAg-negative recipients using hepatitis B immune globulin, and prophylactic antiviral treatment of HBsAg-positive recipients. The five-year cumulative incidence of HBV-related hepatitis was comparable in patients who received stem cells from HBsAg-positive donors and matched control recipients who received stem cells from HbsAg-negative donors (8.5% [95% CI, −0.9% to 17.9%] vs. 7.9% [95% CI, −0.9% to 16.7%]; P = 0.939) (79). Thus, the overall intervention strategy for accepting HBsAg-positive donors may expand the application of allo-HSCT in HBV-endemic areas by allowing for the inclusion of HBsAg-positive donors. All of the strategies for dealing with accepting HBsAg-positive donors need to be tested in a well-designed prospective study.

ADOPTIVE IMMUNE TRANSFER

It was reported that donor HBsAb decreased the risk of HBVr in HSCT recipients (40). Univariate and multivariate analyses of HBVr risk factors confirmed the protective role of an HBV-immune/exposed donor (HR adjusted = 0.12, 95% CI 0.02–0.96; P = 0.045) (40). A recent large retrospective study indicated that the cumulative incidence of HBV-RS at 5 years was 16.3% and 8.4% for patients with or without donor anti-HBs, respectively. Multivariate analysis revealed that the independent risk factor for HBV-RS was allo-HSCT from donors lacking anti-HBs compared with other donors with anti-HBs antibodies (HR= 0.294; 95% CI, 0.13–0.85; P = 0.0117) (42). However, we stratified 565 HBsAg-negative donors according to HBcAb and HBsAb status into four groups. The cumulative HBVr rates at 5 years in the four groups were 5.3% for HBcAb(-)HBsAb(-), 5.1% in HBcAb(-)HBsAb(+), 3.8% in HBcAb (+)HBsAb(-), and 1.6%

in HBcAb(+)HBsAb(+) (P=0.794). We did not find a protective role for HBV-immune/exposed donors in HSCT recipients (39). In addition, there were few reports of HBsAg clearance in HBsAg-positive patients after allo-HSCT. There were several case reports demonstrating that an HBV-immune/exposed donor with HBsAb can lead to serologic clearance of HBsAg in HBsAg-positive recipients (80, 81). The factors influencing HBsAg clearance in HBsAg-positive patients following allo-HSCT are unclear. Additionally, there was no strong evidence that adoptive immune transfer plays a protective role in HBVr during allo-HSCT.

HBV VACCINE ISSUES

The Francophone Society of Bone Marrow Transplantation and Cellular Therapy recommended HBV vaccine for HBV-resolved recipients to prevent HBVr after allo-HSCT (82). A retrospective study from Hokkaido University Hospital enrolled 21 patients with HBV-resolved infection (83). They received a standard three-dose regimen of hepatitis B vaccine after discontinuation of immunosuppressants. The first vaccine was administered at a median of 15 months (range, 6–79 months) after transplantation. Nine of them tested positive for HBsAb. None of the 21 patients in the vaccine group developed HBVr, which indicated that HBV vaccination of HSCT recipients was a promising method for preventing HBVr. However, the following multicenter prospective clinical research of hepatitis B vaccine to prevent HBVr after allo-HSCT failed to find the protective role of hepatitis B vaccine in minimizing the risk of HBVr in HBV-resolved recipients (31). Only 37% (10/27) of patients had HBsAb with three doses of hepatitis B vaccine 12 months after HSCT, and the 2-year cumulative incidence of HBVr was 27.3%. Encouragingly, a recent preliminary study showed excellent results with an anti-HBs seroconversion rate of 82% in HBsAg-negative pediatric and young adult recipients after HSCT at the median of 10.4 (range 3.0–22.4) months after the third vaccination (84). Another prospective study enrolled 86 adults that accepted a low dose of the HBV vaccine (10 mg/dose) at 6, 7, 8, 12 months after allo-HSCT. The proportion of recipients achieving anti-HBs antibody titers 100 mUI/ml was 64.6% (95% CI, 53% to 75%; n = 51/79) at 6 months after vaccine initiation and 56.8% (95% CI, 39.5% to 72.9%; n = 21/37) at 24 months after vaccine initiation (85). This study suggested a better efficacy of higher HBV vaccine antigen doses. However, the effectiveness of these vaccines in preventing HBVr remains to be evaluated.

OBI (OCCULT HEPATITIS B INFECTION)

OBI was defined as the presence of replication-competent HBV DNA in the liver and/or HBV DNA in the blood of people who test negative for HBsAg. These patients can be classified as seropositive OBI (HBsAb-positive or HBcAb-positive) and seronegative OBI (HBsAb-negative and HBcAb-negative) (86). A study found that 19/124 (15.3%) HBsAg-negative donors were detected to have OBI by using the PCR method, for which 14/19 (73.7%) OBI donors were HBsAb-positive (77). Thus, transmission of stem cells from OBI blood donors is a risk factor for HBV-related hepatitis. Patients with OBI have a risk of HBVr when they receive cancer chemotherapy or other immunosuppressive therapies (86). The risk is high (>10%) in OBI patients receiving anti-CD20 containing regimens and myeloablative regimens for HSCT (41, 61, 62, 87). Considering the probability of OBI, both HSCT recipients and donors should be screened for HBV DNA before HSCT.

NEW THERAPEUTIC TREATMENTS

Several new treatment strategies have emerged for patients after allo-HSCT. These include CAR-T therapy and blinatumomab therapy for relapse, rituximab, ruxolitinib, ibrutinib, and other monoclonal antibodies for chronic GVHD treatment. However, as these strategies target B cells and/or T cells, they may also cause HBVr. Indeed, rituximab is known for resulting in HBVr (59). HBV reactivation has also been reported after CAR-T therapy in patients with current or past HBV infection (88–91). Cases of HBVr have been associated with ibrutinib treatment for hematological malignancies but not for patients after allo-HSCT (92–94). Therefore, physicians should carefully monitor the ALT and HBV DNA in chronic HBV infection or resolved HBV infection patients when applying new therapeutic treatments after allo-HSCT.

FUTURE DIRECTIONS

Several unresolved issues remain regarding HBVr during allo-HSCT, which require more work in future studies. The new DNA sequence of HBVr in the process of HBVr needs to be found to redefine the role of virus mutation itself in the mechanism of HBVr. The frequency of monitoring and the duration of NAs prophylactic administration in preventing HBVr during allo-HSCT remains unclear and needs to be established urgently. Many consensuses still recommend LAM as the first-line option. The data regarding the comparison of high barrier (ETV, TDF, or TAF) NAs with low barrier agents (LAM, LdT, ADV) in the prophylaxis of HBVr in HSCT is limited. The frequency of developing drug resistance during long-term antiviral treatment, especially for low barrier agents, in HSCT is unclear. These data may change the first-line recommendation of prophylactic antiviral treatment for HBVr in the future. Prophylactic antiviral therapy for HBV-resolved patients is controversial and large-sample prospective studies are needed to investigate the incidence of HBVr in these patients following allo-HSCT. The protective role of HBsAb in HBVr during allo-HSCT and the adoptive immune effect of donors for decreasing HBVr are awaiting further exploration. Regularly monitoring of ALT and HBV DNA and preemptive antiviral treatment in HBV-resolved patients need to be

TABLE 3 | Agenda for further research.

Group	Further research	Purpose
HBsAg-positive recipients	The frequency of monitoring and the duration of NAs prophylactic administration	Establish antiviral therapy duration and monitored time intervals
	Comparison effectiveness and resistance of high barrier drugs and low barrier agents in the prophylaxis of HBVr	Promote to first-line recommendation of prophylactic antiviral treatment with high barrier agents
	HBsAg clearance in HBsAg-positive patients after allo-HSCT accepting stem cells from HBsAb-positive donors	To verify the adoptive immune role and the influence factors of HBsAg Seroclearance
	New therapeutic treatments (e.g. ruxolitinib, ibrutinib, monoclonal antibodies)	To investigate the latent risk of HBVr during treatments
HBsAg-negative/HBcAb-positive recipients	Prospective studies to reflect the real cumulative rate of HBVr without prophylactic treatment	To investigate the incidence of HBVr in HBV-resolved patients undergoing allo-HSCT
	Prospective studies to investigate the protective role of HBsAb (both recipients and donors)	To explore the protective role of HBsAb in HBVr
	The frequency of monitoring and the duration of NAs prophylactic administration	Establish antiviral therapy duration and monitored time intervals
	HBV vaccine dosage and schedule for minimizing the risk of HBVr	Explore and establish valid method of preventing HBVr with vaccine method
	New therapeutic treatments (e.g. ruxolitinib, rituximab, ibrutinib, monoclonal antibody)	To investigate the latent risk of HBVr during treatments
HBsAg-positive donors	Prospective clinical trials to verify the strategy for preventing HBV related hepatitis from accepting HBsAg positive donors	To establish the effective strategy for accepting stem cells from HBsAg positive donors
All HBVr recipients	Investigate the viral mutation with gene sequencing method	To verify the role of virus itself in process of HBVr during allo-HSCT
	Investigate the incidence of HBV-related hepatitis, liver-related mortality, non-relapse mortality, and interruption of immunosuppressants	To reevaluate the clinical influence of HBVr after allo-HSCT

verified. We believe that HBsAb-positive patients may be the most likely stratified group of resolved HBV infection patients who need not accept prophylactic antiviral therapy for preventing HBVr. The dosages and schedule of vaccines in preventing HBVr in HBV-resolved recipients remains to be evaluated in prospective trials. The clinical influence of HBVr during allo-HSCT requires further investigation, such as the interruption of immunosuppressants, severe hepatitis, liver-related mortality, and non-relapse mortality. We portray an agenda for future further research in HBVr during allo-HSCT (**Table 3**).

CONCLUSIONS

Considering the risk of HBVr, all individuals who plan to receive allo-HSCT or donate stem cells should be screened for HBsAg, HBcAb, and HBV DNA. HBsAg-positive recipients of allo-HSCT have a high risk of HBVr. They should accept prophylactic antiviral therapy to decrease the risk of HBVr. The high barrier NAs (ETV, TDF, or TAF) seem to be superior to the low barrier agents (LAM, LdT, ADV). Resolved HBV infection recipients also have a risk of HBVr, but the risk is lower than that of HBsAg-positive recipients. There are controversies in prophylactic antiviral therapy for resolved HBV infection recipients to prevent HBVr. The optimal antiviral therapy duration and monitored time intervals in both HBsAg-positive and HBsAg-negative/HBcAb-positive recipients remain to be established. There is little evidence to suggest that adoptive donor immunity plays an important role in the prevention of HBVr after allo-HSCT. Accepting stem cells from HBsAg-positive donors is associated with a risk of viral infection, and thus may develop HBV-related hepatitis. The overall intervention strategy, including donors and recipients, can decrease the risk of HBV-related hepatitis following HSCT from HBsAg-positive stem cells. It will increase the treatment options for patients in need of allo-HSCT in HBV-endemic areas by allowing the inclusion of HBsAg-positive individuals as donors.

AUTHOR CONTRIBUTIONS

YW, writing of the original draft. HH, funding acquisition, project administration, and validation. YL, funding acquisition, project administration, review, and validation. All authors contributed to the article and approved the submitted version.

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Decreased iKIR-HLA C Pair Confers Worse Clinical Outcomes for Patients With Myeloid Disease Receiving Antithymocyte Globulin-Based Haploidentical Hematopoietic Stem Cell Transplantation

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Hematopoietic stem cell transplantation (HSCT) is a curative therapy for patients with malignant hematologic diseases. Killer immunoglobin-like receptor (KIR) expressed by NK cells is closely associated with the transplant outcomes, and it has been widely explored and debated for a few decades. Recently published studies have revealed that inhibitory KIRs (iKIRs) are educated by their cognate human lymphocyte antigen (HLA) ligands, and that decreased iKIR-HLA pairs post-transplantation may indicate a reduced NK cell function and impaired control of the primary disease. However, this theory still needs to be validated by additional clinical studies. Here we conducted a retrospective analysis of 246 patients who received haploidentical (haplo)-HSCT at our treatment center between January 2015 and June 2018. Our data suggests that decreased iKIR-HLA C pair post-HSCT correlated with a significantly higher risk of relapse [hazard risk (HR) = 2.95, p = 0.019] and reduced overall survival (OS) (HR = 3.74, p = 0.001) and disease-free survival (DFS) (HR = 4.05, p = 0.0004) in patients with myeloid disease. In conclusion, decreased iKIR-HLA C pair should be avoided during anti-thymocyte globulin (ATG)-based haplo-HSCT, especially for patients with myeloid disease.

Keywords: KIR, hematopoietic stem cell transplantation, iKIR-HLA model, relapse, survival

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INTRODUCTION

Natural killer (NK) cells act as the first line of defense in the immune system. They can rapidly recognize autologous cells and eliminate non-self-components without prior antigen presentation (1, 2). Multiple receptors expressed on NK cells have been implicated in the regulation of their function, with a particular focus on the activities of killer immunoglobin-like receptors (KIRs).

It is well accepted that KIR genes and receptors can be divided into inhibitory and activating functions based on their diverse activities (3). Inhibitory KIRs (iKIRs) bind human lymphocyte antigen (HLA) class I molecules in a specific manner, KIR2DL1 recognizes HLA-C2 group allies, KIR2DL2 and KIR2DL3 recognize HLA-C1 group allies, KIR3DL1 recognizes HLA-Bw4 group allies, and KIR3DL2 recognizes HLA-A3/A11 allies. Activating KIRs (aKIRs) such as KIR2DS1, KIR2DS2, and KIR2DS4 recognize HLA C2, HLA C1, and HLA A11, respectively, but the ligands of the remaining KIRs remain largely unknown. Based on their chromosomal locations, KIR genes can be further identified as centromeric (cen) or telomeric (tel) genes. In addition, KIR genotype AA is made up of only one aKIR gene: KIR2DS4, while KIR genotype B/x is made up of a number of more variable aKIR genes (4).

Normally, autoimmune activation is inhibited because autologous cells express at least one inhibitory HLA ligand; however, tumor transformed cells downregulate HLA expression and/or upregulate activating signals that may trigger NK cell activation (5, 6). Following allogeneic hematopoietic stem cell transplantation (allo-HSCT), donor-derived NK cells may be activated as the recipients may not express the same inhibitory HLA ligands as the donor, preventing their association with the donor iKIRs. This has led to widespread speculation that NK cell alloreactivity in graft *versus* host (GVH) direction may provide additional benefits to tumor-killing strategies.

The Perugia group first established the KIR ligand-ligand model (also known as the KIR ligand model) based on HLA phenotype differences between donors and recipients. In this model, they assumed that donor-derived NK cells might kill recipient cells because the HLA ligands presented by the donor might be absent in the recipient. When they evaluated T cell-depleted (TCD) transplants without post-transplant immunosuppression, they were able to show that KIR ligand mismatch between donor-recipient pairs provided some protective effect against relapse, especially in patients with acute myeloid leukemia (AML) (7). Further development of KIR-typing technology allowed researchers to develop the receptor-ligand model (also known as the missing ligand model), which was used to evaluate the compatibilities between donor iKIRs and recipient HLA ligands. Results using this model suggested that the receptor-ligand model was a more accurate predictor for relapse risk than the KIR ligand model in leukemia patients (8). Additionally, Cooley et al. reported that KIR B/x donors significantly improved the relapse-free survival (RFS) rates for recipients with AML when compared to donors with a KIR AA genotype, suggesting that aKIRs may play a critical role in reducing relapse (9). Following these observations, numerous clinical studies have explored the impact of KIR on transplant outcomes. However, a large variability was found in these results and several factors may be responsible for these discrepancies, including disease type, transplant regimen, donor-recipient relationship, graft source and graft composition, etc (10–12).

In the last few decades, our understanding of NK cell reconstitution and KIR education has evolved a great deal. Pioneer studies in this field have found that reconstituted NK cells are highly immature and exhibit compromised cytotoxicity against leukemia cells in the early phases following transplantation. Afterward, these NK cells gradually acquire receptors and KIR reconstitution can take between several months and even years (13, 14). Importantly, a variety of data has suggested that NK cells acquire specific functionality only after engagement between the iKIRs and their cognate ligands. However, NK cells expressing iKIRs without cognate ligands (non-self KIR) are hyporesponsive and referred to "uneducated cells" (15, 16). Further, the education process mediated by cognate ligands is not restricted to autologous NK cells, but has also been demonstrated in donor-derived reconstituted NK cells after HSCT (17–19).

Recently, the Nowak team proposed the iKIR-HLA model to explore the optimal donor. Since the HLA environment may be altered after transplantation (from donor to recipient), the variations in iKIR-HLA pairs could be divided into three groups (decreased group: cognate iKIR-HLA pairs present in donor but absent in recipient; unchanged group: cognate iKIR-HLA pairs present both in donor and recipient; increased group: cognate iKIR-HLA pairs present in recipient but absent in donor). Consistent results from their studies showed that decreased iKIR-HLA pairs post transplantation correlated with a higher risk of relapse and inferior overall survival (OS), indicating that poor NK cell education resulted in weaker graft *versus* leukemia (GVL) effects (20–22). To further investigate the effects of these KIR interactions on transplant outcomes, we designed a retrospective study to evaluate a cohort of 246 patients, and evaluated our clinical outcomes using the iKIR-HLA model, the receptor-ligand model and KIR gene content.

METHODS

Patients

This retrospective study was comprised of 246 patients with hematological malignancies. All transplants were performed between January 2015 and June 2018 and all methodologies applied during this study were consistent with the Declaration of Helsinki. The protocol was approved by the Ethics Review Committee of the First Affiliated Hospital of Zhejiang University and informed consent was obtained from each patient before transplantation. The authors had full access to the data and assume responsibility for its authenticity.

KIR and HLA Typing

Peripheral blood mononuclear cells were collected from recipients and their donors prior to transplantation and used for HLA and KIR testing. Alleles in the HLA-A, -B, and -C loci were determined using high-resolution HLA typing and KIR gene analysis was performed using the PCR-SSO method (KIR SSO Genotyping Test; OneLamda, Canoga Park, CA, USA).

Transplant Protocol

Most patients were subjected to a myeloablative conditioning (MAC) regimen that included administration of cytarabine

(4 g/m^2/d IV on days −10 to −9), busulfan (Bu) (3.2 mg/kg/d IV on days −8 to −6), cyclophosphamide (Cy) (1.8 g/m^2/d IV on days −5 to −4), methyl-N-(2-chloroethyl)-N-cyclohexyl-N-nitrosourea (Me-CCNU) (250 mg/m^2 orally on day −3), and antithymocyte globulin Fresenius [ATG-F; 2.5 mg/(kg d) IV on days −5 to −2] or ATG Genzyme [ATG-G; 1.5 mg/(kg d) IV on days −5 to −2]. The other patients were subjected to reduced-intensity conditioning (RIC) that consisted of exposure to fludarabine 30 mg/m^2/d IV between days −10 and −5, Bu 3.2 mg/kg/d IV between days −6 and −5, and ATG-F 5 mg/(kg d) IV between days −4 and −1 or ATG-G 2.5 mg/(kg d) IV between days −4 and −1. All patients received G-CSF mobilized peripheral blood stem cells and no graft was subjected to *ex vivo* T-cell depletion. Graft *versus* host disease (GVHD) prophylaxis consisted of cyclosporin A (CsA) or Tacrolimus (Tac), with methotrexate (MTX) and low-dose mycophenolate mofetil (MMF) (23, 24).

Definitions

Relapse was defined as disease reoccurrence in bone marrow and/or extramedullary sites. Non-relapse mortality (NRM) was defined as death from any cause apart from relapse. Overall survival (OS) was defined as the time from transplant until death or last follow up, and disease-free survival (DFS) was defined as survival without relapse. Patients were classified as low/intermediate risk or high/very high risk based on the refinement of the disease risk index (DRI) (25). Diagnosis of acute and chronic GVHD (aGVHD and cGVHD) was made using established criteria (26, 27). The viral loads for Epstein-Barr virus (EBV) and cytomegalovirus (CMV) were monitored weekly for the first 3 months after transplantation, biweekly from the fourth to the sixth month post-transplant, and monthly from the seventh to the twelfth month post-transplant. Viremia was defined as a viral load in excess of 5×10^2 copies/ml.

Statistical Analysis

All clinical data were analyzed using SPSS 22.0 (IBM, Armonk, NY, USA) and R project 3.6.1 software (http://www.r-project.org). The clinical features for the samples were presented as median or percentage values. OS and DFS were calculated using the Kaplan–Meier method and compared using the log-rank test. The cumulative incidences of EBV viremia, CMV viremia, aGVHD, cGVHD, relapse, and NRM were estimated *via* the competing-risks model and compared using the Gray test. All variables with a p-value of <0.10 in the univariate analysis were then included in the multivariate analysis. Results were considered statistically significant when $p < 0.05$.

RESULTS

Characteristics of Patients and Donors

The clinical features of these 246 donor-patient pairs are summarized in **Table 1**. In this retrospective study, 142 (57.7%) patients with myeloid disease and 104 (42.3%) patients with lymphoid disease received haplo-HSCT at our center. Disease types included acute myeloid leukemia (AML, n = 115), myelodysplastic syndrome (MDS, n = 22), myeloproliferative neoplasm (MPN, n = 5), acute lymphoblastic leukemia (ALL, n = 93), and lymphoma (n = 11). The median age of the patients and donors in these groups were 30 years (range, 9–50 years) and 35 years (range, 11–59 years), respectively. The median mononuclear (MNC) cell and CD34$^+$ cell counts in the grafts were 15.34×10^8/kg (range, 2.97–59.80×10^8/kg) and 6.30×10^6/kg (range, 0.27–34.37×10^6/kg), respectively. A total of 233 (94.7%) patients received the MAC regimen and 13 (5.3%) received the RIC regimen. ATG-F was used in 205 (83.3%) patients while the other 41 (16.7%) received ATG-G as part of their conditioning regimen. One hundred eighty-one (73.5%) patients received haplo-HSCT during their first remission (CR1); 73 (29.7%) patients were defined as high or very high risk based on the refinement of DRI (49 in the myeloid cohort and 24 in the lymphoid cohort, 34.5 *vs* 23.1%, p = 0.053). Most patients expressed HLA C1C1 or HLA C1C2 and only 4.5% presented with a HLA C2C2 ligand.

Of the 246 donors, 143 (58.1%) were KIR genotype AA, 76 (30.9%) were KIR BA, and 27 (11.0%) were KIR BB. Receptor-ligand (R-L) mismatches at the HLA-A3/A11 locus, HLA-Bw4 locus, and HLA-C locus were identified in 53.3, 39.0, and 71.5% of the donor-recipient pairs, respectively. After transplantation, 40 (16.2%) patients experienced a decrease in their iKIR-HLA A3/A11 pair, 26 (10.6%) exhibited decreased iKIR-HLA Bw4 pair, and 43 (17.5%) had decreased iKIR-HLA C (C1 or C2) pair.

EBV and CMV Viremia

During the first 180 days after HSCT, 90 (36.6%) patients developed EBV viremia. Disease category (myeloid or lymphoid) (p = 0.001), ATG source (p = 0.0003), and patient sex (p = 0.029) were identified as potent factors influencing EBV viremia (**Table 2**). Multivariate analysis suggested that myeloid disease [hazard risk (HR) = 0.48, p = 0.0005] was a protective factor for EBV viremia, while ATG-G (HR = 2.58, p < 0.0001) and sex (male patients (HR = 1.57, p = 0.042)) were independent risk factors for EBV viremia (**Table 3**). In lymphoid disease, KIR2DS2$^+$ donors were found to exhibit a higher incidence of EBV viremia when compared with KIR2DS2$^-$ donors (63.2 *vs* 43.5%, p = 0.078), but this was not identified to be an independent effect in the multivariate analysis.

The CI for CMV viremia within 180 days of transplant was 65.0% (78.1% in patients treated with ATG-G and 62.4% in patients treated with ATG-F, p = 0.003). Donor-patient pairs with R-L mismatch at HLA-C locus tended to experience a lower CI for CMV viremia than did donor-patient R-L C matched pairs (62.5 *vs* 71.4, p = 0.079). The multivariate analysis revealed that only ATG-G was an independent risk factor for CMV viremia (HR = 1.70, p = 0.008).

aGVHD and cGVHD

Following transplantation, 83 (33.7%) developed grade II–IV aGVHD (aGVHD$^{2-4}$). As expected, a significant reduction in aGVHD$^{2-4}$ occurrence was found in patients receiving RIC conditioning compared with patients receiving MAC conditioning (7.7 *vs* 35.2%, p = 0.041). Patients with low and intermediate risk also experienced a lower CI of aGVHD$^{2-4}$ (30.6

TABLE 1 | Clinical features of patients, donors, and transplants.

Variables	All patients (246)	Myeloid cohort (142)	Lymphoid cohort (104)
Median patient age (years)	30 (9–60)	33 (9–60)	24 (13–56)
Median donor age (years)	35 (11–59)	32 (11–55)	38 (13–59)
Median MNC cells (×10 E^8/kg)	15.34 (2.97–59.80)	14.36 (2.97–59.80)	15.61 (5.80–46.14)
Median $CD34^+$ cells (×10 E^6/kg)	6.30 (0.27–34.37)	6.06 (0.27–34.37)	7.03 (1.77–22.87)
Median follow up time (years)	3.0 (0.1–5.5)	3.0 (0.2–5.5)	2.9 (0.1–5.5)
Patient sex			
Male	136 (55.3)	77 (54.2)	59 (56.7)
Female	110 (44.7)	65 (45.8)	45 (43.3)
Donor/Patient sex combination			
Female/Male	44 (17.9)	27 (19.0)	17 (16.3)
Other combinations	202 (82.1)	115 (81.0)	87 (83.7)
ABO blood mismatch			
Identical	131 (53.3)	73 (51.4)	58 (55.8)
Mismatch	115 (46.7)	69 (48.6)	46 (44.2)
Diagnosis		/	/
AML	115 (46.7)		
MDS	22 (8.9)		
MPN	5 (2.0)		
ALL	93 (37.8)		
Lymphoma	11 (4.5)		
Disease status at HSCT			
CR1	181 (73.5)	99 (69.7)	82 (78.9)
>CR1	65 (26.4)	43 (30.3)	22 (21.2)
Disease risk index			
Low/Int risk	173 (70.3)	93 (65.5)	80 (76.9)
High/Very high risk	73 (29.7)	49 (34.5)	24 (23.1)
Conditioning regimen			
MAC	233 (94.7)	133 (93.7)	100 (96.2)
RIC	13 (5.3)	9 (6.3)	4 (3.8)
ATG			
ATG-F	205 (83.3)	117 (82.4)	88 (84.6)
ATG-G	41 (16.7)	25 (17.6)	16 (15.4)
HLA ligands of patients			
$A3/A11^+$	115 (46.7)	66 (46.5)	49 (47.1)
$Bw4^+$	148 (60.2)	90 (63.4)	58 (55.8)
C1/C1	167 (67.9)	95 (66.9)	72 (69.2)
C1/C2	68 (27.6)	42 (29.6)	24 (23.1)
C2/C2	11 (4.5)	5 (3.5)	6 (5.8)
Receptor-ligand (R-L) model			
R-L A3/A11 mismatch	131 (53.3)	76 (53.5)	55 (52.9)
R-L Bw4 mismatch	96 (39.0)	49 (34.5)	47 (45.2)
R-L C mismatch	176 (71.5)	98 (69.0)	78 (75.0)
Donor KIR genotype			
AA	143 (58.1)	81 (57.0)	62 (59.6)
B/x	103 (41.9)	61 (43.0)	42 (40.4)
BA	76 (30.9)	45 (31.7)	31 (29.8)
BB	27 (11.0)	16 (11.3)	11 (10.6)
Donor activating KIR gene			
$KIR2DS1^+$	83 (33.7)	49 (34.5)	34 (32.7)
$KIR2DS2^+$	46 (18.7)	27 (19.0)	19 (18.3)
$KIR2DS3^+$	41 (16.7)	24 (16.9)	17 (16.3)
$KIR2DS4^+$	238 (96.7)	135 (95.1)	103 (99.0)
$KIR2DS5^+$	57 (23.2)	36 (25.4)	21 (20.2)
$KIR3DS1^+$	85 (34.6)	51 (35.9)	34 (32.7)
iKIR-HLA pairs variation			
A3/A11			
Decreased (D)	40 (16.2)	22 (15.5)	18 (17.3)
Unchanged (U)	166 (67.5)	98 (69.0)	68 (65.4)
Increased (I)	40 (16.2)	23 (16.2)	17 (16.3)
Bw4			
Decreased (D)	26 (10.6)	14 (9.9)	12 (11.5)
Unchanged (U)	189 (76.8)	109 (76.8)	80 (76.9)
Increased (I)	31 (12.6)	19 (13.4)	12 (11.5)
C			
Decreased (D)	43 (17.5)	20 (14.1)	23 (22.1)
Unchanged (U)	163 (66.3)	98 (69.0)	65 (58.7)
Increased (I)	40 (16.3)	24 (16.9)	16 (15.4)
EBV viremia	90 (36.6)	41 (28.9)	49 (47.1)
CMV viremia	160 (65.0)	93 (65.5)	67 (64.4)
aGVHD			
Grade 0	92 (37.4)	55 (38.7)	37 (35.6)
Grade I	71 (28.9)	44 (31.0)	27 (26.0)
Grade II	55 (22.4)	30 (21.1)	25 (24.0)
Grade III	12 (4.9)	7 (4.9)	5 (4.8)
Grade IV	16 (6.5)	6 (4.2)	10 (9.6)
cGVHD			
Not included	7 (2.8)	2 (1.4)	5 (4.8)
No	139 (56.5)	76 (53.5)	63 (60.6)
Mild	58 (23.6)	39 (27.5)	19 (18.3)
Moderate	26 (10.6)	14 (9.9)	12 (11.5)
Severe	16 (6.5)	11 (7.7)	5 (4.8)
relapse	55 (22.4)	28 (16.9)	27 (26.0)
NRM	14 (5.7)	4 (2.8)	10 (9.6)
OS	185(75.2)	115 (82.4)	70 (67.3)
DFS	177 (72.0)	110 (80.3)	67 (64.4)

AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; MPN, myeloproliferative neoplasm; ALL, acute lymphoblastic leukemia; MNC, mononuclear; CR1, first complete remission; MAC, myeloablative conditioning; RIC, reduced-intensity conditioning; ATG, Antithymocyte Globulin; EBV, Epstein-Barr virus; CMV, cytomegalovirus; aGVHD, acute graft versus host disease; cGVHD, chronic graft versus host disease; NRM, non-relapse mortality; OS, overall survival; DFS, disease-free survival.

vs 41.1%, p = 0.080). However, none of these factors remained significant in the multivariate analysis. In lymphoid cohort, there was a trend that donor-patient pairs with R-L mismatch on HLA-C locus experienced a lower aGVHD[2–4] (33.3 *vs* 53.9%, P = 0.095).

Among patients who survived more than 100 days after transplantation, 100 (41.8%) patients developed cGVHD and 42 (17.6%) of them had moderate to severe cGVHD. Univariate analysis identified KIR2DS2 (p = 0.048) and KIR2DS3 (p = 0.083) as two potent protective factors for moderate to severe cGVHD. Nevertheless, no such correlations were found in the multivariate analysis.

Relapse and NRM

After a median follow up time of 3.0 years (yr) (range, 0.1–5.5 yr), 55 (22.4%) patients experienced relapse. Patients with lymphoid disease experienced a higher 3-yr relapse rate than patients with myeloid disease (26.2 *vs* 17.3%, p = 0.087). The CI for 3-yr relapse was also higher in patients with high/very high-risk disease (32.9 *vs* 16.1%, p = 0.002). Patients who received HSCT at CR1 experienced a lower 3-yr relapse rate than the other group (16.5

TABLE 2 | Univariate analysis of factors that influence transplant outcomes.

Outcome and potent factors, %	All patients	P	Myeloid cohort	P	Lymphoid cohort	P
1. EBV viremia*						
Myeloid *vs* Lymphoid	28.9 *vs* 47.1	**0.001**				
ATG-G *vs* ATG-F	58.6 *vs* 32.2	**0.0003**	48.0 *vs* 24.8	**0.009**	75.0 *vs* 42.1	**0.003**
Male *vs* Female	42.7 *vs* 29.1	**0.029**	35.1 *vs* 21.5	**0.087**	52.5 *vs* 40.0	0.177
KIR2DS2$^+$ *vs* KIR2DS2$^-$	43.5 *vs* 35.0	**0.199**	25.0 *vs* 29.7	0.643	63.2 *vs* 43.5	**0.078**
2. CMV viremia*						
ATG-G *vs* ATG-F	78.1 *vs* 62.4	**0.003**	72.0 *vs* 64.1	0.199	87.5 *vs* 60.4	**0.001**
KIR2DS1$^+$ *vs* KIR2DS1$^-$	67.5 *vs* 63.8	0.935	77.6 *vs* 59.1	**0.029**	52.9 *vs* 70.0	**0.030**
KIR2DS3$^+$ *vs* KIR2DS3$^-$	63.4 *vs* 65.4	0.695	79.2 *vs* 62.7	0.191	41.2 *vs* 69.0	**0.057**
KIR3DS1$^+$ *vs* KIR3DS1$^-$	67.9 *vs* 63.6	0.997	78.0 *vs* 58.7	**0.041**	52.9 *vs* 70.0	**0.030**
R-L C (mismatch *vs* match)	62.5 *vs* 71.4	**0.079**	63.3 *vs* 70.5	0.267	61.5 *vs* 73.1	0.151
3. Grade II-IV aGVHD*						
High/Very high risk *vs* Low/Int risk	41.1 *vs* 30.6	**0.080**	38.8 *vs* 25.8	**0.068**	45.8 *vs* 36.3	0.454
MAC *vs* RIC	35.2 *vs* 7.7	**0.041**	31.6 *vs* 11.1	0.178	40.0 *vs* 0.0	0.125
R-L C (mismatch *vs* match)	31.25 *vs* 40.0	0.256	29.6 *vs* 31.8	0.853	33.3 *vs* 53.9	**0.095**
4. Moderate to severe cGVHD*						
KIR2DS2$^+$ *vs* KIR2DS2$^-$	6.5 *vs* 19.2	**0.048**	7.4 *vs* 20.3	0.133	5.3 *vs* 17.7	0.197
KIR2DS3$^+$ *vs* KIR2DS3$^-$	7.3 *vs* 18.7	**0.083**	12.5 *vs* 19.0	0.487	0.0 *vs* 18.4	**0.057**
5. 3-yr CIR						
Myeloid *vs* Lymphoid	17.3 *vs* 26.2	**0.087**				
High/Very high risk *vs* Low/Int risk	32.9 *vs* 16.1	**0.002**	26.5 *vs* 12.4	**0.031**	45.8 *vs* 20.4	**0.009**
CR1 *vs* >CR1	16.5 *vs* 33.9	**0.002**	13.6 *vs* 25.7	**0.070**	23.5 *vs* 36.4	0.202
iKIR-HLA C variation (D *vs* U+I)	38.1 *vs* 17.5	**0.005**	40.0 *vs* 13.5	**0.004**	35.8 *vs* 23.5	0.317
6. 3-yr NRM						
Myeloid *vs* Lymphoid	2.8 *vs* 9.9	**0.024**				
High/Very high risk *vs* Low/Int risk	1.4 *vs* 7.7	**0.057**	2.0 *vs* 3.2	0.683	0.0 *vs* 12.8	**0.070**
iKIR-HLA C variation (D *vs* U+I)	4.7 *vs* 6.0	0.745	10.0 *vs* 1.6	**0.037**	0.0 *vs* 12.7	**0.078**
KIR2DS3$^+$ *vs* KIR2DS3$^-$	0.0 *vs* 7.0	**0.086**	0.0 *vs* 3.4	0.362	0.0 *vs* 11.8	0.145
KIR B/x *vs* KIR AA	2.9 *vs* 7.9	0.112	0 *vs* 4.9	**0.079**	7.1 *vs* 11.7	0.497
7. 3-yr OS						
Myeloid *vs* Lymphoid	81.6 *vs* 67.7	**0.016**				
ATG-G *vs* ATG-F	75.5 *vs* 75.8	0.861	92.0 *vs* 79.3	0.147	49.2 *vs* 71.0	**0.041**
High/Very high risk *vs* Low/Int risk	67.0 *vs* 79.3	**0.036**	71.4 *vs* 87.1	**0.029**	58.3 *vs* 70.5	0.202
CR1 *vs* >CR1	79.7 *vs* 64.5	**0.010**	85.8 *vs* 72.0	**0.053**	72.4 *vs* 50.0	**0.025**
iKIR-HLA C variation (D *vs* U+I)	65.1 *vs* 77.9	**0.093**	55.0 *vs* 86.0	**0.0006**	73.9 *vs* 65.9	0.418
8. 3-yr DFS						
Myeloid *vs* Lymphoid	79.9 *vs* 63.9	**0.006**				
ATG-G *vs* ATG-F	73.2 *vs* 73.3	0.835	78.3 *vs* 88.0	0.293	50.0 *vs* 66.5	**0.085**
High/Very high risk *vs* Low/Int risk	65.7 *vs* 76.2	**0.080**	71.4 *vs* 84.4	**0.066**	54.2 *vs* 66.8	0.218
CR1 *vs* >CR1	76.7 *vs* 63.0	**0.024**	83.3 *vs* 72.0	**0.107**	67.7 *vs* 50.0	**0.085**
iKIR-HLA C variation (D *vs* U+I)	57.3 *vs* 76.5	**0.016**	50.0 *vs* 84.9	**0.0001**	64.2 *vs* 63.9	0.813

**Estimations of cumulative incidence are given at 100 days post-HSCT for aGVHD; 180 days post-HSCT for EBV and CMV viremia; 3 years post-HSCT for cGVHD. Potent factors with p < 0.10 were in bold type.*

vs 33.9%, p = 0.002). No significant differences in relapse rate were found using the receptor ligand model and activating KIRs. However, decreased iKIR-HLA C pair were associated with a higher risk for 3-yr relapse (38.1 *vs* 17.5%, p = 0.005). When analyzed separately, the discrepancy in relapse rates was more evident in the myeloid cohort (40.0 *vs* 13.5%, p = 0.004) than in the lymphoid cohort (35.8 *vs* 23.5%, p = 0.317) (**Figure 1**). Multivariate analysis revealed that CR1 (HR = 0.53, P = 0.029) and decreased iKIR-HLA C pair (HR = 1.95, P = 0.033) were independent factors for relapse for the entire cohort, and the adverse effects of decreased iKIR-HLA C pair on the 3-yr relapse rate was more evident in myeloid disease (HR = 2.95, p = 0.019).

A total of 14 (5.7%) patients experienced NRM at a median follow-up time of 0.3 yr (range, 0.1–2.8 yr), 6 (2.4%) patients died of severe GVHD (5 aGVHD and 1 cGVHD), 7 (2.8%) patients died from severe infection (6 pulmonary infections and 1 sepsis), and 1 (0.4%) patient with primary poor graft function (28) died from an intracranial hemorrhage. No variables were found to be significant predictors of NRM.

OS and DFS

The CI for 3-yr OS was 75.6% for all patients. Disease category (p = 0.016), disease status (p = 0.010), and disease risk index (p = 0.036) were all found to influence 3-yr OS in the univariate analysis. In addition, the 3-yr OS rate in transplants with decreased iKIR-HLA C pair was shown to be 65.1% [95% confidence interval (CI): 52.3–81.0%], which was lower than those with unchanged or increased iKIR-HLA C pair (77.9%, 95% CI: 72.3–83.9%, p = 0.093), and the negative impact of decreased iKIR-HLA C pair was more apparent in the myeloid cohort [55.0% (95% CI: 37.0–81.8%) *vs* 86.0 (95% CI: 80.0–92.4%), p = 0.0006] than in the lymphoid cohort [73.9% (95% CI: 58.0–94.2%) *vs* 65.9% (95% CI: 56.1–77.3%), p = 0.418] (**Figures 2A–C**). In the lymphoid cohort, patients who received

TABLE 3 | Multivariate analysis of factors that influence transplant outcomes.

Outcomes and significant factors	All patients		Myeloid cohort		Lymphoid cohort	
	P	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)
1. EBV viremia*						
ATG-G *vs* ATG-F	**<0.0001**	**2.58 (1.61–4.13)**	**0.007**	**2.51 (1.28–4.93)**	**0.004**	**2.66 (1.37–5.14)**
Male *vs* Female	**0.042**	**1.57 (1.02–2.41)**				
Myeloid *vs* Lymphoid	**0.0005**	**0.48 (0.31–0.72)**				
2. CMV viremia*						
ATG-G *vs* ATG-F	**0.008**	**1.70 (1.15–2.51)**			**0.005**	**1.76 (1.19–2.59)**
3. 3-yr CIR						
CR1 *vs* >CR1	**0.029**	**0.53 (0.30–0.94)**				
iKIR-HLA C variation (D *vs* U+I)	**0.033**	**1.95 (1.06–3.61)**	**0.019**	**2.95 (1.19–7.27)**		
4. 3-yr OS						
Myeloid *vs* Lymphoid	**0.006**	**0.49 (0.29–0.82)**				
CR1 *vs* >CR1	**0.004**	**0.46 (0.27–0.78)**			**0.029**	**0.45 (0.22–0.92)**
iKIR-HLA C variation (D *vs* U+I)			**0.001**	**3.74 (1.66–8.39)**		
5. 3-yr DFS						
Myeloid *vs* lymphoid	**0.003**	**0.47 (0.28–0.77)**				
CR1 *vs* >CR1	**0.009**	**0.51 (0.31–0.84)**			**0.034**	**0.47 (0.24–0.94)**
iKIR-HLA C variation (D *vs* U+I)			**0.0004**	**4.05 (1.87–8.80)**		

**Estimations of cumulative incidence are given at 100 days post-HSCT for aGVHD; 180 days post-HSCT for EBV and CMV viremia.*
Significant factors with P < 0.05 were in bold type.

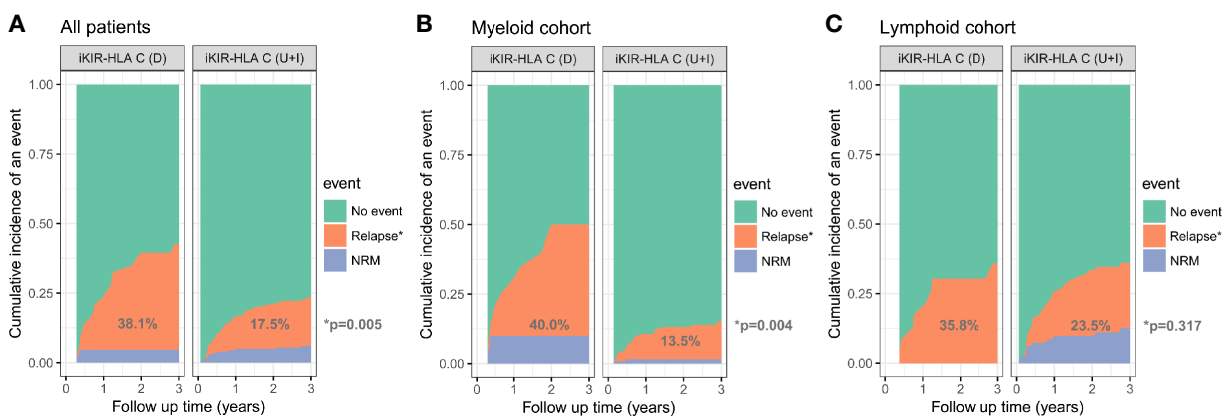

FIGURE 1 | Cumulative incidence of relapse among all patients **(A)**, the myeloid cohort **(B)**, and the lymphoid cohort **(C)**, based on the variation (D, decreased; U, unchanged; I, increased) in iKIR-HLA C pair post-transplantation.

ATG-G prior to transplantation experienced a lower 3-yr OS (49.2 *vs* 71.0%, p = 0.041). Multivariate analysis identified myeloid disease (HR = 0.49, p = 0.006) and CR1 (HR = 0.46, p = 0.004) as protective factors for 3-yr OS. CR1 in the lymphoid cohort (HR = 0.45, p = 0.029) remained significant in multivariate analysis, and decreased iKIR-HLA C pair conferred a poorer 3-yr OS in the myeloid cohort (HR = 3.74, p = 0.001).

In addition, dramatically reduced 3-yr DFS was observed when iKIR-HLA C pair was decreased both in the entire cohort [57.3% (95% CI: 44.0–74.6%) *vs* 76.5% (95% CI: 70.8–82.6%), p = 0.016] and the myeloid cohort [50.0% (95% CI: 32.3–77.5%) *vs* 84.9% (95% CI: 78.6–91.6%), p = 0.0001]. For patients with lymphoid disease, variation in iKIR-HLA C pair was not associated with DFS [64.2% (95% CI: 47.0–87.8%) *vs* 63.9% (95% CI: 54.2–75.4%), p = 0.813] (**Figures 2D–F**). In the multivariate analysis, myeloid disease (HR = 0.47, p = 0.003) and CR1 (HR = 0.51, p = 0.009) were shown to be independent factors influencing DFS. A significantly reduced DFS was also observed in myeloid patients with decreased iKIR-HLA C pair (HR = 4.05, p = 0.0004).

DISCUSSION

There has been a longstanding debate about the impact of KIR alloreactivity on clinical outcomes. It was only recently revealed that reconstituted KIR are educated by HLA ligands and that the loss of the cognate ligands dampens NK cell functions (17–19). This means that searching for donors who exhibit the greatest NK cell function in recipients rather than "match or mismatch" would be a more reliable measure for predicting transplant success.

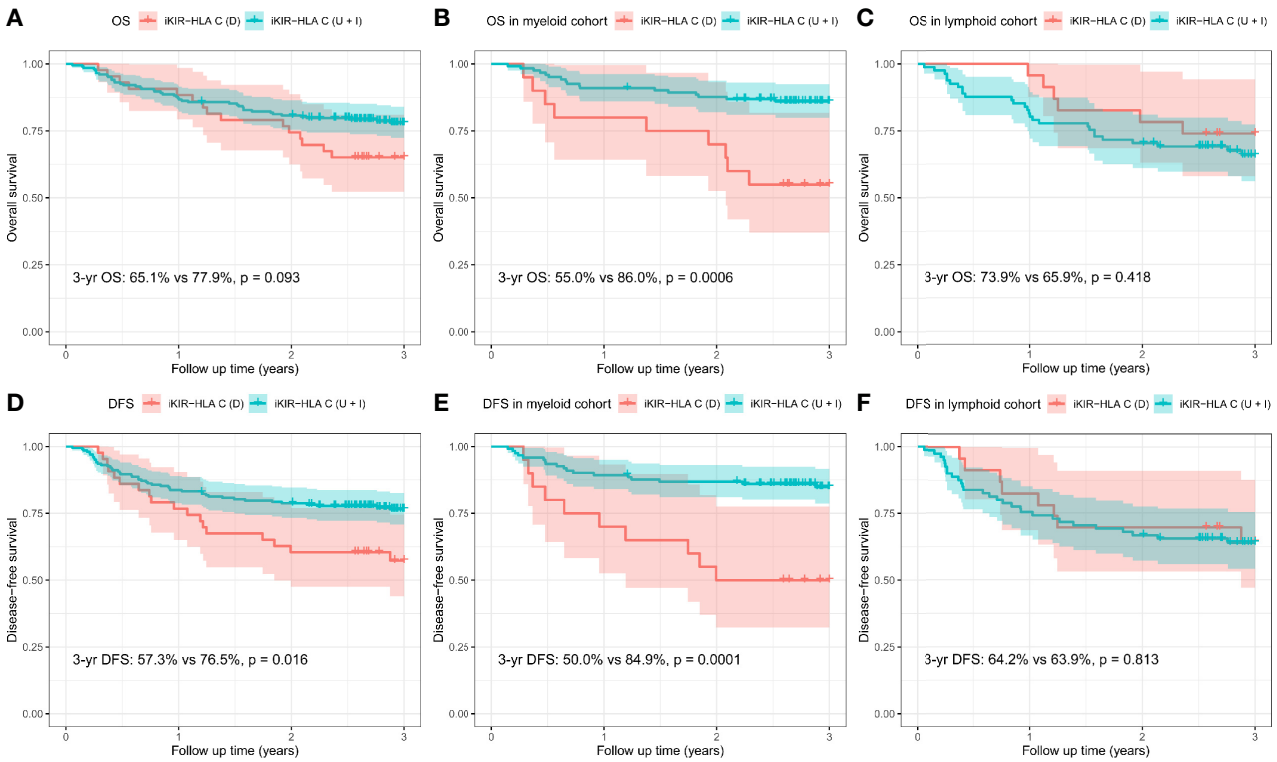

FIGURE 2 | Overall survival (OS) and disease-free survival (DFS) rates for all patients **(A, D)**, the myeloid cohort **(B, E)**, and the lymphoid cohort **(C, F)**, based on the variation (D, decreased; U, unchanged; I, increased) in their iKIR-HLA C pair post-transplantation.

Previously, Nowak et al. proposed the iKIR-HLA model that could be used to predict transplant outcomes (20–22). Among the multiple interactions between the iKIRs and HLA ligands, we identified that only decreased iKIR-HLA C pair post transplantation was a negative indicator for relapse and survival, especially in patients with myeloid disease. Nevertheless, variations in iKIR-HLA A3/A11 pair and iKIR-HLA Bw4 pair did not influence the treatment outcomes.

It is widely accepted that almost all HLA C molecules are recognized by iKIRs. However, only a minority of HLA B and HLA A epitopes act as KIR ligands (29–31). Similarly, all patients in our cohort expressed at least one HLA C ligand, while the HLA Bw4 and A3/A11 ligands were expressed at a frequency of 60.2 and 46.7%, respectively. This suggests that the HLA C ligands play a dominant role in KIR education (32). Given this, reconstituted NK cells with decreased iKIR-HLA C pair may exhibit impaired anti-tumor effects (18, 19). In addition, the expression levels of HLA A and B ligands on normal cells are more than tenfold higher than that of the HLA C (33), this means that when cancerous cells downregulate HLA class I antigens to escape immune surveillance, the stability of the self-tolerance mediated by iKIR-HLA C interactions is more vulnerable to be broken. In other words, HLA-C may play a major role in missing-self recognition and modulate NK cell activation. Moreover, Pende et al. found that lymphoblastic leukemias express a higher surface density of HLA class I molecules than myeloid leukemias (34). Verheyden et al. went on to test the expression of HLA ligands in normal T cells, AML cells, B-ALL cells, and B-chronic lymphoid leukemic (B-CLL) cells. Interestingly, only HLA C were dramatically downregulated on all types of leukemic cells as compared with their healthy control, with this downregulation being the most apparent in AML cells (35). Makanga et al. demonstrated that $CD57^+$ and KIR^+ NK cells from healthy individuals exhibited the highest degree of cytotoxicity against AML blasts, while ALL targets were less susceptible to KIR^+ NK subsets compared with $NKG2A^+$ NK subsets (36). On the basis of previous studies, we hypothesize that KIR may have a minor impact on the elimination of lymphoblastic leukemias, and patients with myeloid disease are more likely to benefit from well KIR-educated NK cells.

In many European studies, aKIRs, especially KIR2DS1 (37–39) and KIR2DS2 (40, 41), have been shown to be associated with improved survival or reduced relapse. Yet, as reported in several studies from East Asia (42–45), aKIRs were not found to grant any survival advantage or relapse protection to the patients in our cohort. One reason for this may be the genetic differences between these different ethnic groups. Single et al. revealed that almost 46.7% Europeans express the KIR2DS2 gene, and 66.5% present the HLA C2 ligand for KIR2DS1 (46). However, both the KIR2DS2 gene (18.7%) and the HLA C2 ligand (32.1%) were expressed at much lower frequencies in this study. The KIR2DS1 gene frequency in our cohort was also a bit lower than those of the European populations (33.7 *vs* 37.8%). Thus, we speculate that KIR2DS1 may have a reduced chance of activation resulting from the absence of its cognate ligand,

and that the beneficial impact of KIR2DS2 on transplant outcomes may be more apparent in a larger cohort of Chinese patients.

Additionally, we could not find evidence of any significant association between receptor ligand mismatch and clinical outcomes. Since mature donor lymphocytes are mostly eliminated following ATG treatment, the transient expression of alloreactive NK cells in the recipients may not be sufficient to influence GVHD (47–49). After which the reconstituted NK cells expressing non-self KIRs may not exhibit enough cytotoxicity to eliminate the remaining leukemic cells (17–19).

In summary, we conclude that when using ATG-based haplo-HSCT, deceased iKIR-HLA C pair should be avoided during donor selection, especially for patients with myeloid disease. The exact role of the aKIRs in the Chinese population still needs to be explored in future studies.

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 the Ethics Review Committee of the First Affiliated Hospital of Zhejiang University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

HH designed the study and supervised the analyses and manuscript preparation. YZ, FG, and YW collected and analyzed the data, YZ and FG wrote the manuscript. All authors discussed and interpreted the results. All authors contributed to the article and approved the submitted version. YZ and FG contributed equally to this work and should be considered as co-first authors.

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Co-Reactivation of Cytomegalovirus and Epstein-Barr Virus Was Associated With Poor Prognosis After Allogeneic Stem Cell Transplantation

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Reactivation of cytomegalovirus (CMV) or Epstein-Barr virus (EBV) is common after hematopoietic stem cell transplantation (HSCT). Previous researches have demonstrated that either CMV or EBV reactivation is associated with poor outcomes of HSCT. However, few studies investigate the impact of CMV and EBV co-reactivation after HSCT. In this study, we described the clinical characteristics of HSCT recipients with CMV and EBV co-reactivation (defined as CMV and EBV viremia occur at the same period of time). We conducted a longitudinal study of 247 patients who underwent HSCT in our center. A total of 24 (9.7%) patients had CMV and EBV co-reactivation. These patients showed higher incidence of viral pneumonitis (P=0.005). Patients with CMV and EBV co-reactivation had significant lower 1-year overall survival (OS) (P=0.004) and lower 1-year leukemia free survival (LFS) (P=0.016). Our further analysis suggested that duration of CMV (P=0.014), EBV (P<0.001), and CD4+CD25+ T cell counts at day 30 post-transplantation (P=0.05) are independent risk factors of virus co-reactivation. In conclusion, patients who developed co-reactivation of CMV and EBV had poor prognosis in terms of lower 1-year OS and LFS, and the CMV and EBV co-reactivation was associated with prolonged CMV or EBV duration and poor CD4+CD25+ T cell reconstitution at day 30 post-transplantation.

Keywords: cytomegalovirus, Epstein-Barr virus, co-reactivation, immune reconstitution, stem cell transplantation

INTRODUCTION

The burden of clinically relevant viral infections, especially double-stranded DNA herpesviruses, continues to rise. Reactivation of multiple different herpes viruses is commonly acquired following allogeneic hematopoietic stem cell transplantation (HSCT). Cytomegalovirus (CMV) is the most frequently reactivated virus (1) after allo-HSCT and increases non-relapse mortality despite

the widely adopted protocol of pre-emptive therapy (2–4). Epstein-Barr virus (EBV) (1), especially EBV-related post-transplantation lymphoproliferative disorder (PTLD), is associated with a high mortality rate of 50%–90% (5, 6).

CMV and EBV are the most clinically relevant viruses in the present era with well-defined treatment approaches. A bidirectional relationship seems to exist between these two viruses; higher incidence/poor clearance of CMV infection and a higher incidence of EBV-PTLD and delayed immune reconstitution as a cause or effect is key to all these findings (7, 8). It is therefore reasonable to assume that co-reactivation of CMV and EBV may indicate an even more severe clinical condition compared to that for the reactivation of each virus alone. However, few studies have investigated co-reactivation of CMV and EBV among HSCT recipients. In our study, we aimed to explore the clinical characteristics of patients with co-reactivation of CMV and EBV, study the effect of such co-reactivation on prognosis, and identify associated risk factors. We also discuss the role of immune reconstitution in the co-reactivation of the two viruses.

MATERIALS AND METHODS

Study Cohort

A total of 253 patients underwent their first allo-HSCT between July 2015 and June 2016 at Peking University People's Hospital (Haidian district, Beijing) at the Institute of Hematology. These patients were retrospectively reviewed in the current study. The Ethics Committee of Peking University People's Hospital approved this study. All patients provided written informed consent prior to transplantation.

Transplantation Procedure

For patients with acute leukemia (AL) or myelodysplastic syndrome (MDS) who underwent haplo-HSCT and matched unrelated donor HSCT, the conditioning regimen consisted of cytarabine (4 g/m^2/day) intravenously on days -10 to -9, busulfan (3.2 mg/kg/day) intravenously on days -8 to -6, cyclophosphamide (1.8 g/m^2/day) intravenously on days -5 to -4, semustine (250 mg/m^2) orally once on day -3, and rabbit anti-thymocyte globulin (ATG) (2.5 mg/kg/day; Sang Stat, Lyon, France) intravenously on days -5 to -2. Patients with AL or MDS who underwent HLA-identical HSCT received a conditioning regimen that did not include ATG but consisted of hydroxyurea (80 mg/kg) orally divided twice on day -10, cytarabine (2 g/m^2/day) intravenously on day -9, busulfan (3.2 mg/kg/day) intravenously on days -8 to -6, cyclophosphamide (1.8 g/m^2/day) intravenously on days -5 to -4, and semustine (250 mg/m^2) orally once on day -3. For patients with aplastic anemia who underwent haplo-HSCT, conditioning therapy consisted of busulfan (3.2 mg/kg/day) intravenously for 2 days on days -7 and -6, cyclophosphamide (50 mg/kg/day) intravenously for four consecutive days on days -5 to -2, and rabbit ATG (2.5 mg/kg/day; Sang Stat, Lyon, France) intravenously for four consecutive days on days -5 to -2 (9). For patients with aplastic anemia who underwent identical HSCT or matched unrelated donor HSCT, the conditioning regimen excluded busulfan, and only consisted of cyclophosphamide (50 mg/kg/day) intravenously for four consecutive days on days -5 to -2, and rabbit ATG (2.5 mg/kg/day; Sang Stat, Lyon, France) intravenously for four consecutive days on days -5 to -2. The conditioning regime of the only one MM patient in this study consisted of cytarabine (4 g/m^2/day) on days -10 to -9, busulfan (3.2 mg/kg/day) on days -10 to -8, cyclophosphamide (1g/m^2/day) on days -7 to -6, fludarabine 50 mg/day on days -6 to -2, and simustine (250 mg/m^2) orally once on day -3 along with rabbit ATG (2.5 mg/kg/day) on days -5 to -2.

Virus Monitoring and Therapy

CMV and EBV reactivation was monitored twice per week using real-time quantitative polymerase chain reaction (PCR) of plasma samples. All patients received ganciclovir between days -9 and -2 (10). Pre-emptive therapy with either intravenous ganciclovir (5 mg/kg, twice daily) or intravenous foscarnet (90 mg/kg/d) was initiated when CMV viremia was confirmed and the treatment lasted until CMV DNA was not detected twice on consecutive tests. Adoptive transfer of CMV-specific cytotoxic T lymphocytes (CTLs) was performed if available in those with refractory CMV infection or CMV disease (2). Antiviral drugs, such as foscarnet, were infused in patients with EBV viremia. In addition, rituximab was infused if EBV viremia was persistent or developed into EBV disease (6). EBV-specific CTL therapy was adopted as salvage option.

Graft-Versus-Host Disease (GVHD) Prophylaxis

Cyclosporin A (CsA), methotrexate (MTX), and mycophenolate (MMF) were administered to patients for GVHD prophylaxis. CsA was administered at 2.5 mg/kg/day intravenously in two doses from day -9 until the patients could take CsA orally. The trough concentration of CsA was monitored, requiring a target trough blood concentration of 150–250 ng/ml. MTX was administered intravenously at a dose of 15 mg/m^2 on day +1 and 10 mg/m^2 on days +3, +6, and +11 (day +11 was omitted in patients with matched sibling donor transplantation). Mycophenolate (MMF) was administered orally from day -9 to day +30 at a dose of 0.5 g (0.25 g for children) every 12 h.

Immunophenotyping

Peripheral blood samples were collected from recipients on days 30, 60, and 90 after HSCT. The samples were stained without further separation to minimize selective loss shortly after collection. The combinations of the directly conjugated monoclonal antibodies CD3-FITC, CD4-PE, CD8-APC, CD19-Per-CP, CD25-PE (BD Biosciences, Mountain View, CA, USA), and their isotype-matched antibodies were used to analyze the immunophenotype of T lymphocyte subsets. Flow cytometry was performed using a BD FACSSort machine (Becton Dickinson Biosciences, San Jose, CA, USA). The data were analyzed using CellQuest software (BD Biosciences).

Definitions

Myeloid engraftment was defined as the first of three consecutive days with an absolute neutrophil count (ANC) ≥0.5×10^9/L, and

platelet engraftment was defined as the first of seven consecutive days with a platelet count ≥20×10^9/L without transfusion. CMV and EBV viremia was defined as the first of two consecutive detections in which virus DNA reached or exceeded 1,000 copies/ml and 500 copies/ml, respectively. Co-reactivation of CMV and EBV was defined as the detection of EBV or CMV viremia during CMV or EBV viremia, respectively. The time of co-reactivation was defined as the day when viremia of the first virus was identified. The duration of viremia was defined as the number of days between the first day of viremia and the first day when the virus was no longer found. The longest duration was included in the analysis of patients with more than one episode of viremia. CMV disease was diagnosed according to the published definition. Both acute and chronic GVHD were diagnosed and graded using traditional criteria (11, 12). Time to relapse was defined as days between date of transplantation and date of disease recurrence. Non-relapse mortality (NRM) was defined as death from all causes other than those directly related to a hematologic malignant disease itself, occurring at any time after transplantation. Overall survival (OS) was defined as the number of days from transplantation to death from any cause. Leukemia-free survival (LFS) was defined as the number of days from transplantation to disease progression after transplantation.

Statistical Analyses

Categorical variables were compared between the two groups using the χ^2 test or Fisher's exact test. Continuous variables were compared using a nonparametric test (Mann-Whitney U test). Multivariate Cox proportional hazards models were adopted with proportional hazards assumption and for testing interactions. Statistical analyses were performed using IBM SPSS 22.0 statistical software (IBM SPSS Statistics, USA).

RESULTS

Patients Characteristics

Six patients infected with EBV were excluded from the study. Finally, 247 patients were enrolled in this study. Patient characteristics are listed in **Table 1**. There were 144 (58.3%) men. The median age was 29 (1–63) years. Acute leukemia, both

TABLE 1 | Characteristics of patients.

Characteristic	Co-reactivation group	Other reactivation group[#]	No reactivation group	*P* value
Gender, no.(%)				0.91
Male	14 (58.3)	83 (57.2)	47 (60.3)	
Female	10 (41.7)	62 (42.8)	31 (39.7)	
Age, median (range)	29 (6–51)	27 (1–61)	35 (3–63)	0.246
Underlying disease, no.(%)				0.22
AML	9 (37.5)	57 (39.3)	34 (43.6)	
ALL	14 (58.3)	66 (45.5)	27 (34.6)	
SAA	0	8 (5.5)	8 (10.3)	
MDS	1 (4.2)	8 (5.5)	8 (10.3)	
Other*	0	6 (4.1)	1 (1.3)	
Disease status				0.496
≤CR2	23 (95.8)	133 (91.7)	69 (88.5)	
CR3 or NR	1 (4.2)	12 (8.3)	9 (11.5)	
Donor-recipient relationship, no.(%)				0.001
Father	13 (54.2)	66 (45.5)	20 (25.6)	
Mother	1 (4.2)	8 (5.5)	4 (5.1)	
Sibling	3 (12.5)	45 (31)	45 (57.7)	
Son/Daughter	5 (20.8)	23 (15.9)	6 (7.7)	
Unrelated donor	2 (8.3)	3 (2.1)	3 (3.8)	
HLA match, no.(%)				<0.001
Haploidentical	22 (91.7)	133 (91.7)	39 (50)	
Identical	0	9 (6.2)	36 (46.2)	
Unrelated donor	2 (8.3)	3 (2.1)	3 (3.8)	
Blood type, no.(%)				0.889
Matched	12 (50)	80 (55.5)	43 (55.1)	
Minor mismatched	4 (16.7)	29 (20)	13 (16.7)	
Major mismatched	5 (20.8)	28 (19.3)	15 (19.2)	
Major and minor mismatched	3 (12.5)	8 (5.5)	7 (9)	
ATG used in conditioning therapy, no.(%)	24 (100)	138 (95.2)	43 (55.1)	<0.001
MNC, median (range), 10^8/kg	8.21 (5.91–13.35)	8.63 (4.3–15.67)	8.45 (2.89–12.74)	0.547
CD34+ cell absolute count, median (range), 10^6/kg	3.14 (1.06–7.48)	2.41 (0.28–8.07)	2.49 (0.97–6.06)	0.409
Donor gender, no.(%)				0.148
Male	19 (79.2)	117 (80.7)	54 (69.2)	
Female	5 (20.8)	28 (19.3)	24 (30.8)	

[#]*Other reactivation group includes reactivation of CMV only, and reactivation of both CMV and EBV but does not fulfill definition of CMV and EBV co-reactivation*

**Other underlying diseases include multiple myeloma (one patient), chronic myelomonocytic leukemia (two patients), chronic myeloid leukemia (two patients), acute heterozygosis leukemia (two patients).*

acute myeloid leukemia (n=100, 40.5%) and acute lymphoblastic leukemia (n=107, 43.3%), accounted for most patients. More than half (n=194, 78.5%) of patients underwent HSCT from haploidentical donors. Forty-five (18.2%) patients received HSCT from HLA-matched siblings, and eight (3.2%) underwent HSCT from unrelated donors. Myeloid engraftment and platelet engraftment were achieved in 245 (99.2%) patients at a median of 13 (10–31) days and in 227 (91.9%) patients at a median of 14 (6–267) days after HSCT, respectively. The incidence of grade 3–4 acute GVHD and grade 1–4 acute GVHD was 6.12% (n=15) and 50.6% (n=125), respectively. The median follow-up time for survivors was 12 months. The 1-year OS, LFS, NRM, and relapse rates were 67.6%, 66.0%, 19.4%, and 6.5%, respectively.

Virus Reactivation

At least one episode of CMV viremia was found in 68.4% of the patients (n=169), among which 15 patients were infected twice or more during the year after transplantation. The median onset time of CMV viremia was 34 (7–175) days, and the median duration was 20 days (range, 6–77 days). CMV DNA copy numbers varied in patients with a median of 5.48×10^3 (0–5.01 $\times10^5$) copies. Thirty-six (14.6%) patients had EBV reactivation. EBV viremia occurred at a median of 48.5 (25–102) days after transplantation and lasted a median of 14 (3–60) days. For patients with reactivated EBV, EBV DNA copies reached 6×10^3 (6×10^2–1.76×10^6). According to the definition above, 24 (9.7%) patients were categorized as having co-reactivation of CMV and EBV. Twelve (4.9%) patients had both CMV and EBV reactivation but did not fulfill the definition of co-reactivation. A total of 133 (53.8%) patients were infected with CMV only, and 78 (31.6%) patients had no episodes of reactivation of either virus.

Effect of CMV and EBV Co-Reactivation on Clinical Outcomes

Patients were divided into three groups based on CMV and EBV reactivation according to our definition above: (1) co-reactivation group, defined as the detection of EBV or CMV viremia during CMV or EBV viremia, respectively; (2) other reactivation group was defined as reactivation with CMV and/or EBV but did not meet the criteria for co-reactivation; and (3) no reactivation group was defined as neither CMV nor EBV reactivation detected. The characteristics of the three groups are listed in **Table 1**.

Myeloid engraftment was comparable between the three groups (100% vs. 100% vs. 97.4% for co-reactivation, other reactivation, and no reactivation groups, respectively, *P*=0.113). However, myeloid engraftment seemed to be delayed in patients with no virus reactivation (13 vs. 13 vs. 14 days for co-reactivation, other reactivation, and no reactivation groups, respectively, *P*=0.008). Regarding platelet engraftment, the proportion of patients (87.5% vs. 91.7% vs. 93.6% for co-reactivation, other reactivation, and no reactivation groups, respectively, *P*=0.628) and days of engraftment (13 vs. 13 vs. 14 for co-reactivation, other reactivation, and no reactivation groups, respectively, *P*=0.389) were comparable between the three groups. The incidence of acute GVHD was significantly higher in the reactivation group than in the no reactivation group (50% vs. 66.9% vs. 20.5%, respectively, *P*<0.001), while the incidence of chronic GVHD was similar in the three groups (4.2% vs. 9.7% vs. 9% for co-reactivation, other reactivation, and no reactivation groups, respectively, *P*=0.682). Patients in the reactivation group were more likely to develop viral pneumonia than those in the other two groups (20.8% vs 9% vs 1.3% for co-reactivation, other reactivation, and no reactivation groups, respectively, *P*=0.005), but we did not observe a similar trend for viral enteritis (0% vs 2.1% vs. 0% for co-reactivation, other reactivation, and no reactivation groups, respectively, *P*=0.344). CMV or EBV disease was diagnosed in 22 patients, among whom there were 19 cases of pneumonia and three cases of gastroenteritis. EBV-PTLD was diagnosed in 5 patients, and all 5 patients received rituximab treatment. Hemorrhagic cystitis was also more prevalent in the reactivation group (37.5% vs. 35.2% vs. 14.1% for co-reactivation, other reactivation, and no reactivation groups, respectively, *P*=0.002) (**Table 2**).

The 1-year OS was significantly lower in the reactivation group (50% vs. 66.2% vs.75.6% for co-reactivation, other reactivation, and no reactivation groups, respectively, *P*=0.021). The 1-year LFS was also lower in the co-reactivation group (50% vs. 65.5% vs. 71.8% for co-reactivation, other reactivation, and no reactivation groups, respectively), although the difference was not statistically significant (*P*=0.057). Viral reactivation was an independent risk factor for 1-year OS (**Figure 1**) (HR 4.94 for co-reactivation vs. no reactivation, and HR 1.94 for other reactivation vs. no reactivation, P=0.004) and LFS (**Figure 2**) (HR 3.66 for co-reactivation vs. no reactivation, and HR 1.51 for other reactivation vs. no reactivation, P=0.016). The causes of death are summarized in **Supplementary Table S1**. Risk factors for 1-year OS and 1-year LFS are summarized in **Table 3**.

Predictive Factors Associated With CMV and EBV Co-Reactivation

Patients with CMV and EBV co-reactivation were compared with all other patients to identify factors associated with co-reactivation. The donor-recipient relationship (father, mother, sibling, and son/daughter, respectively, vs. unrelated donor); HLA matched status, use of ATG; period of CMV and EBV viremia, respectively; and peak CMV and EBV DNA copies, respectively, were associated with CMV and EBV co-reactivation. CD3+ (*P*=0.052) and CD4+CD25+ (*P*=0.052) cell counts on day 30 after transplantation also seemed to play a role in virus co-reactivation in univariate analysis. Cox multivariate analysis of the above factors showed that the donor-recipient relationship (father, mother, sibling, and son/daughter, respectively, vs. unrelated donor, *P*=0.001), duration of CMV (*P*=0.014) and EBV (*P*<0.001), and CD4+CD25+ cell counts at day 30 post-transplantation (*P*=0.05) were independent risk factors for CMV and EBV co-reactivation (**Table 4**). However, of all 247 patients enrolled in the study, 45 (18.2%) patients received HSCT from HLA-matched family donors and all of these donors were siblings, which might introduce a potential bias. To account for this, we reanalyzed patients who received

TABLE 2 | The impact of co-reactivation on clinical outcomes.

Clinical Outcomes	Co-reactivation group	Other reactivation group[#]	No reactivation group	*P* value
neutrophil engraftment, no.(%)	24 (100)	145 (100)	76 (97.4)	0.113
Time of neutrophil engraftment, +d, median (range)	13 (10–20)	13 (10–31)	14 (10–24)	0.008
Platelet engraftment, no.(%)	21 (87.5)	133 (91.7)	73 (93.6)	0.628
Time of PLT engraftment, +d, median (range)	13 (9–56)	14 (6–267)	14 (7–80)	0.389
aGVHD, no. (%)	12 (50)	97 (66.9)	16 (20.5)	<0.001
Time of aGVHD, +d, median (range)	23 (9–57)	19 (6–87)	14.5 (9–40)	<0.001
aGVHD grade, no (%)				0.015
0–II	23 (95.8)	131 (90.3)	78 (100)	
III–IV	1 (4.2)	14 (9.7)	0 (0)	
CMV viremia, no. (%)	24 (100)	145 (100)	0 (0)	——
Time of first CMV viremia, +d, median (range)	33.5 (21–62)	34 (7–175)	——	——
Duration of CMV viremia, d, median (range)	23.5 (14–56)	18 (6–77)	——	——
Receiving CMV-CTL	13(54.2%)	12 (8.2%)	0	<0.001
Highest CMV viral load, ×10^3 copies/ml, median (range)	28.25 (4.16–206)	9.08 (1.12–501)	——	——
EBV viremia, no. (%)	24 (100)	12 (8.3)	0 (0)	——
Time of first EBV viremia, +d, median (range)	45.5 (25–76)	58.5 (35–102)	——	——
Duration of EBV viremia, d, median (range)	15.5 (3–39)	14 (4–60)	——	——
Highest EBV viral load, ×10^3 copies/ml, median (range)	6.75 (1.2–1760)	5.04 (0.6–536)	——	——
Viral pneumonitis, no. (%)	5 (20.8)	13 (9)	1 (1.3)#	0.005
Viral enteritis, no. (%)	0 (0)	3 (2.1)	0 (0)	0.344
Hemorrhagic cystitis, no. (%)	9 (37.5)	51 (35.2)	11 (14.1)	0.002
cGVHD, no. (%)	1 (4.2)	14 (9.7)	7 (9)	0.682
Immune reconstitution at day 30 after HSCT, median (range)				
WBC, 10^9/L	5.24 (2.55–24.33)	5.49 (1.49–30.9)	4.73 (1.44–19.08)	0.311
CD19, 10^9/L	0.0052 (0.039)	0.0034 (0.24)	0.0041 (0.042)	0.505
CD3, 10^9/L	0.018 (1.66)	0.088 (7.69)	0.18 (3.02)	0.009
CD4, 10^9/L	0.0031 (0.21)	0.012 (0.56)	0.072 (0.68)	<0.001
CD8, 10^9/L	0.011 (1.47)	0.056 (7.28)	0.074 (2.46)	0.086
CD4CD25, 10^9/L	0.00045 (0.029)	0.0017 (0.19)	0.0083 (0.27)	<0.001
WBC count at day 60 post-transplantation, median (range)	3.09 (0.6–7.76)	3.41 (0.65–15.77)	4.2 (0.53–9.96)	0.203
Overall survival in 1 year after HSCT no. (%)	12 (50)	96 (66.2)	59 (75.6)	0.021
Leukemia free survival in 1 year after HSCT no. (%)	12 (50)	95 (65.5)	56 (71.8)	0.057
Mortality cause, no. (%)				
NRM	9 (37.5)	27 (18.6)	12 (15.4)	0.053
Relapse	0 (0)	11 (7.59)	1 (1.28)	0.057
Relapse time, d, median (range)	——	118 (60–359)	224 (55–364)	0.262

[#]One patient who did not have CMV and EBV viremia was highly suspicious of EBV pneumonitis because of a positive EBV-DNA result in bronchoalveolar lavage fluid.

haplo-HSCT. The donor-recipient relationship was excluded as a risk factor for CMV and EBV co-reactivation in univariate analysis (*P*=0.561).

DISCUSSION

In this study, we demonstrated that patients with CMV and EBV co-reactivation were associated with poor prognosis in terms of acute GVHD, viral disease, OS, and LFS. This suggests that our study has important implications for clinical physicians.

Although CMV reactivation was strongly associated with EBV reactivation (13), co-reactivation of CMV and EBV was relatively less common than that of other double-stranded DNA viruses. Twenty-four (9.7%) patients were identified as having CMV and EBV co-reactivation in our study. This is consistent with a previous study in which 32/330 (9.7%) patients had co-reactivation of CMV and EBV (14), although the definition of virus co-reactivation was slightly different, as our study emphasized that the two viruses must be present at the same time. Hill et al. showed that 62% of patients could be detected with ≥2 double-stranded viruses after allogeneic HSCT. However, only 2.4% of patients were found to have CMV and EBV, with or without other double-stranded viruses (1).

Our study found that CMV and EBV co-reactivation was associated with decreased 1-year OS, which was mainly due to increased NRM. In the co-reactivation group, the 1-year NRM was higher than in the other two groups, although the difference was not statistically significant (*P*=0.053), and no death occurred because of relapse. This was partly in accordance with a previous study in which patients with CMV and EBV co-reactivation had a significant higher 6-month non-relapse mortality than those with CMV or EBV reactivation alone (14). Although CMV reactivation alone after HSCT was not associated with 1-year OS because of the decreased relapse and increased 1-year NRM (7), co-reactivation with EBV was different.

Prolonged viremia with higher CMV-load was observed in the co-reactivation group than in the other-reactivation group, reflecting the influence of parallel EBV-reactivation on CMV-replication and kinetics, which is commonly seen amongst the β-herpesviruses as they can regulate immunity. Immunoreactivation of one virus by another virus has been documented previously by

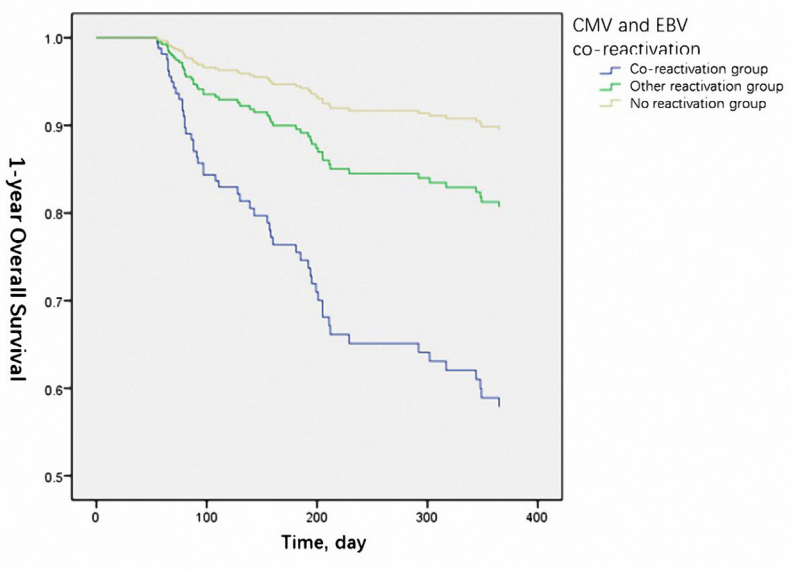

FIGURE 1 | Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) co-reactivation was identified as one of the independent risk factors for 1-year overall survival. (P=0.004).

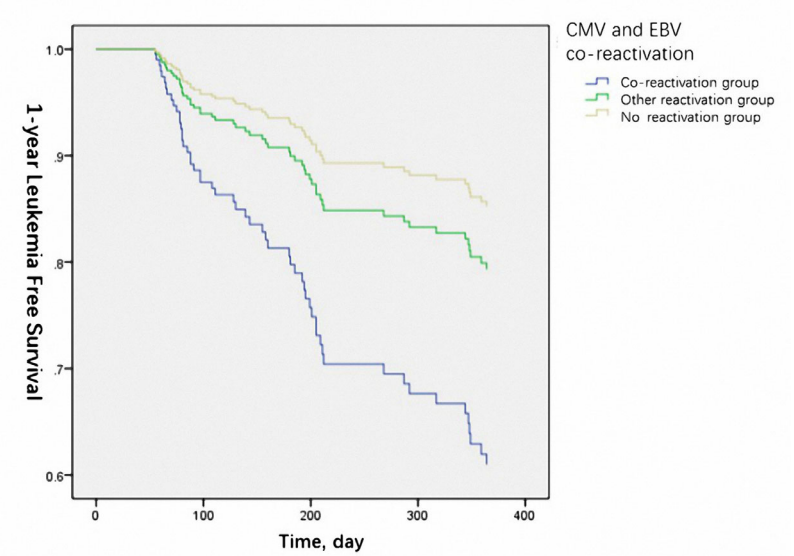

FIGURE 2 | Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) co-reactivation was identified as one of the independent risk factors for 1-year leukemia free survival. (P=0.016).

us and others in both HSCT and SOT. This could also reflect the poor immune reconstitution as reflected in poor CD3+ and CD4 +25+ cell counts (on day 30), which were lower than those in the other reactivation and no reactivation groups.

The incidence of acute GVHD was significantly higher in the co-reactivation and other reactivation groups than in the no reactivation group in our study. Patients in both groups had reactivated CMV, indicating an association between CMV and acute GVHD. In fact, multiple studies have shown that acute GVHD and its treatment put patients at risk of CMV reactivation (15, 16). A retrospective study also identified CMV reactivation as a risk factor for acute GVHD, proving the bidirectional

TABLE 3 | Risk factors for 1-year OS and 1-year LFS.

Factors	Univariate analysis		Multivariate analysis			
	OS	LFS	OS		LFS	
	P value	P value	P value	HR [95%CI]	P value	HR [95%CI]
Underlying disease	0.037(ALL vs. MDS)	0.04 (ALL vs. SAA) 0.028(ALL vs. MDS) 0.087(AML vs.MDS)	N	——	N	——
Disease status (CR3 or NR vs. CR1-2)	<0.001	<0.001	<0.001	6.045 (3.088–11.832)	<0.001	5.685 (2.984–10.832)
HLA match	0.05 (matched sibling vs. haploidentical donor)	N	N	——	N	——
Platelet engraftment (<=median versus >median)	<0.001	<0.001	<0.001	0.103 (0.052–0.205)	<0.001	0.107 (0.054s–0.210)
aGVHD grade (0–II vs. III–IV)	<0.001	<0.001	N	——	N	——
Virus reactivation (no reactivation vs. Co-reactivation)	0.021	0.057	0.001	0.202 (0.078–0.527)	0.005	0.274 (0.112–0.671)
Viral pneumonitis	<0.001	<0.001	N	——	N	——
Hemorrhagic cystitis	0.002	0.002	N	——	N	——
Highest viral load of CMV((>median versus <= median))	0.004	0.006	N	——	N	——
WBC count at day 60 (>median versus <= median)	0.001	0.001	0.005	0.851 (0.734–0.988)	0.034	0.857 (0.743–0.988)

N, not statistically significant.

TABLE 4 | Risk factors for cytomegalovirus (CMV) and Epstein-Barr virus (EBV) co-reactivation.

Factors	Univaraite analysis	Multivariate analysis	
	P value	P value	HR [95%CI]
Donor-recipient relationship	0.013 (sibling vs. father) 0.019 (sibling vs. son/daughter) 0.009 (sibling vs. unrelated matched donor)	0.001[#] <0.001(unrelated matched donor vs. father) 0.005 (unrelated matched donor vs. sibling)	131.479(13.236-1306.056) 35.809(2.966-432.346)
HLA match	0.02 (identical sibling vs. unrelated matched donor)	——	——
ATG used in conditioning therapy	0.022	N	——
Duration of CMV viremia (<=median versus >median)	<0.001	0.014	1.040 (1.008-1.073)
Duration of EBV viremia (<=median versus >median)	<0.001	<0.001	1.155 (1.108-1.205)
Highest viral load of CMV (<=median versus >median)	<0.001	N	——
Highest viral load of EBV (<=median versus >median)	<0.001	N	——
CD3+ cell counts at day 30 post-transplantation (<=median versus >median)	0.052	N	——
CD4+CD25+ cell counts at day 30 post-transplantation (<=median versus >median)	0.052	0.05	0 (0-0.8)

[#]*Donor-recipient relationship as an independent risk factor for virus co-reactivation was believed to be affected by HLA match as siblings contained all cases of HLA-identical HSCT. Reanalysis of haplo-identical HSCT patients further confirmed this hypothesis.*

relationship between CMV reactivation and acute GVHD (17). A previous study also identified grade III–IV acute GVHD as a risk factor for EBV reactivation (18). However, CMV and EBV co-reactivation in our study was not associated with a higher incidence of overall acute GVHD or severe acute GVHD (grade III-IV) than that in the other reactivation group. It might be that the other reactivation group also included patients with both reactivated CMV and EBV. However, they were not reactivated at the same period of time.

The independent risk factors for co-reactivation of CMV and EBV virus identified in this study include duration of CMV and EBV, CD4+CD25+ T cell counts on day 30 post-transplantation,

and donor-recipient relationship. Reanalysis of haplo-HSCT patients was performed to account for the role of donor-recipient relationship on virus co-reactivation. As a result, the donor-recipient relationship was excluded in the univariate analysis (P=0.561). Previous studies have shown that risk factors for CMV reactivation after HSCT include a donor or recipient seropositive for CMV, mismatched or unrelated donors, pre-allo-HSCT viremia, and use of alemtuzumab (19, 20). However, almost all patients in our study were either donor seropositive or recipient seropositive, making it less meaningful to analyze the effect of serum status on virus reactivation.

Our study identified CD4+CD25+ cell counts on day 30 post-HSCT as an independent risk factor for CMV and EBV co-reactivation. CD4+CD25+ T cells are a subset of CD4+ T cells and represent regulatory T cells (Tregs). Normally, Tregs play an important role in controlling the cellular immune response to infectious agents, providing a balance to activating stimuli that allow elimination of the pathogen without immunopathological damage to the host. As a result, patients with a viral infection usually have an elevated number of Tregs to control the cellular immune response. However, one study showed that no significant difference could be detected by comparing both absolute and relative Treg cell numbers among allogeneic HSCT patients with and without CMV infection, indicating that Tregs did not inhibit CMV clearance in HSCT patients (21). Moreover, Ngoma et al. showed that a lower proportion of Treg on day 30 after allogeneic HSCT was associated with an increased risk of CMV infection, implying an association between impaired Treg reconstitution and CMV infection (22). The paradox might be due to the positive correlation between Treg and CMV-specific CD8+ T cell recovery after HSCT (23). Although Tregs were activated at an early stage in EBV infection (24), our study demonstrated that the effect of decreased Treg numbers on CMV reactivation was greater than that of elevated Treg numbers on EBV reactivation, as the co-reactivation group had significantly lower CD4+CD25+ cell counts.

The present study has several limitations. First, the retrospective nature of this study has inherent risks of bias; however, the patient profile and the transplant complications do not appear different from those reported in prospective studies. Second, we did not monitor other herpesviruses, which could have a bearing on all these findings. However, we concentrated only on the two most clinically important viruses with defined treatment options. We did not monitor lymphocyte reconstitution, especially virus-specific immune reconstitution, or the replication kinetics of the viruses, which could be important in managing and understanding these situations better.

Despite several limitations, we have demonstrated in this study that co-reactivation of CMV and EBV according to our definition is associated with lower 1-year OS and LFS. CD4+CD25+ T cell counts on day 30 post-transplantation are identified as one of the independent risk factors for CMV and EBV co-reactivation, which may provide an alternative way to prevent CMV and EBV reactivation in HSCT patients.

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

AUTHOR CONTRIBUTIONS

X-JH and Y-QS designed the study. J-RZ, D-YS, RW and Y-QS wrote the manuscript. All authors contributed to the data preparation and interpretation. All authors approved the final version. 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.2020.620891/full#supplementary-material

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Characteristics and Risk Factors of Cytokine Release Syndrome in Chimeric Antigen Receptor T Cell Treatment

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Clinical trials have confirmed that chimeric antigen receptor (CAR) T cell therapies are revolutionizing approaches for treating several relapsed or refractory hematological tumors. Cytokine release syndrome (CRS) is an adverse event with high incidence during CAR-T treatment. A further understanding of the characteristics and related risk factors of CRS is important for effective management. A total of 142 patients with relapsed or refractory acute lymphocyte leukemia (ALL), lymphoma, or multiple myeloma (MM) received lymphodepletion chemotherapy followed by infusion of CAR-T cells. The characteristics of CRS at different time points after treatment were monitored and risk factors were analyzed. The incidence of CRS for ALL, lymphoma, and multiple myeloma were 82%, 90%, and 90% respectively. Fever was observed on a median of day 3 for ALL, day 1 for lymphoma, and day 8.5 for MM after CAR-T cell infusion, and the duration was different between grade 1–2 CRS and grade 3–5 CRS. Disease types, peak concentration of IL-6, and CRP were associated with CRS. For patients with ALL, numbers of lymphoblast in bone marrow before lymphodepletion, peak concentration of IL-6, and CRP were independent risk factors of CRS. Clinical stage of lymphoma patients and high tumor burden in marrow of MM patients were independent risk factors of CRS. In conclusion, the characteristics and risk factors of CRS in different B-cell hematological tumors are different and should be managed individually during CAR-T cell therapy.

Keywords: cytokine release syndrome, chimeric antigen receptor T cell, acute lymphocyte leukemia, lymphoma, multiple myeloma

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INTRODUCTION

Clinical trials have confirmed that CAR-T has become an important approach for treating relapse or refractory hematological tumors (1–3). However, adverse events in CAR-T treatment are a major obstacle that can even cause death. CRS is one of the adverse events with high incidence in CAR-T cell treatment (4). According to the published data, more than 54–91% of patients may develop different grades of CRS during treatment (5). Therefore, it is important to improve the prognosis through evaluation of severity and timely intervention of CRS. However, currently available diagnostic criteria and severity grading systems of CRS are based on clinical manifestations that may delay the diagnosis and treatment of CRS (6). Therefore, a deep understanding of the characteristics of CRS and related risk factors has great clinical significance for effective management.

Several groups have tried to explore and identify risk factors of CRS (1, 7, 8), especially using laboratory biomarkers to predict severe CRS. The data showed that a 250-fold increase of single cytokine or a 75-fold increase of two cytokines suggests severe CRS (1). IL-1 increases earlier than IL-6 and blocking IL-1 also abolishes both CRS and neurotoxicity, resulting in substantially extended leukemia-free survival (9). Several studies (8, 10–12) also found that tumor burden, intensity of lymphodepletion chemotherapy, CAR-T cell dose, and thrombocytopenia were risk factors of CRS. In addition, patients with severe CRS subsequently have elevated endothelial cell activation markers such as Angiopoietin-2 and von Willebrand Factor before lymphodepletion chemotherapy (8). These studies have important implications for predicting the occurrence and development of CRS. However, these characteristics and risk assessment of CRS are based on CD19 CAR-T cell therapy. Our clinical experience (13, 14) and more and more recently published data show that the onset time, clinical characteristics, and severity of CRS are different among MM, ALL, and lymphoma (1–3). It is suggested that, in addition to CRS grading, the characteristics and risk factors of CRS should also be taken into consideration in the treatment of different B-cell hematological tumors.

Therefore, we analyzed the characteristics and risk factors of CRS in four centers in China. We observed and analyzed the available factors in patients with different B-cell hematological tumors to provide direct and reliable indicators for clinicians to manage CRS.

PATIENTS AND METHODS

Study Design and Patient Information

A total of 142 patients with B cell hematologic malignancies received CAR-T cell treatment in four clinical centers of China. All clinical studies have been approved by the ethics committee and registered with the Chinese Clinical Trial Registration Center, respectively (ChiCTR-OIC-16008291, ChiCTR-OOC-16008447 and NCT03258047). All eligible patients were enrolled according to inclusion and exclusion criteria of the clinical studies. Patients were eligible if they were 18–69 years of age and had confirmed relapsed or refractory MM, ALL, or lymphoma, a Karnofsky Performance Score of 50 points or more, and a life expectancy of more than 12 weeks without active infections and serious liver, kidney, heart, and other diseases. Female patients who had negative serum HCG without pregnancy planned within 6 months after treatment were included. Patients with a history of mental illness, a high degree of allergies, or severe allergies (especially those who are allergic to IL-2) were excluded.

Pretreatment and CAR-T Cell Infusion

The lymphodepletion chemotherapy included FC [fludarabine (three daily doses of 30mg/m^2) and cyclophosphamide (one daily dose of 750 mg/m^2)] cyclophosphamide alone or no pretreatment for 2 patients who were prior transplant. Infused CAR-T cells included anti-CD19 CAR-T cell, anti-BCMA CAR-T cell, and anti-CD20 CAR-T cell. Glucocorticoids were not used to prevent allergic reactions prior to infusion. Due to the risk of arrhythmia, cardiac monitoring was performed from the time of CAR-T cell infusion until no sign of CRS.

Evaluation of Adverse Events and Serum Biomarkers

The adverse events were evaluated using the cytokine release syndrome evaluation criteria proposed by Lee and colleagues (6) and Common Terminology Criteria for Adverse Events version 4.0 (14). Clinical manifestations and vital signs associated with CRS were recorded at any time during treatment. Peripheral blood was collected to detect IL-6, ferritin, C-reactive protein (CRP), blood cells, creatinine, liver transaminase, bilirubin, and coagulation profiles before pretreatment and every 2 days after CAR-T cell infusion. If the patient had heart palpitations, myocardial enzymes, electrocardiogram, and troponin were measured. Complete blood cell count and chemistry panel were performed more than one time per day for patients at high risk of severe CRS and/or CRES, or those with a high tumor burden.

Statistical Methods

Descriptive statistics (median/IQR/range, count, and percent) are reported for key variables. Fisher's exact test, Kruskal-Wallis test, and Nemenyi test was used to compare categorical (gender, transplant, disease type, CAR-T cell dose, costimulatory molecules, species of scFv, risk stratification, clinical stage, type of light chain, and ISS stage) and continuous variables (age, blast cell, peak concentration of IL-6 and CRP, CD4/CD8, and β2-MG) among Non-CRS, grade 1–2 CRS, or 3–5 CRS. Ordinal logistic regression was used to estimate the risk factors of the occurrence of CRS. Tests were generally performed at a significance level of 0.05. All p-values reported were two-sided without adjustments for multiple comparisons. The time points of measuring biomarkers were chosen based on the clinical trial protocol and the need of clinical management. Statistical analyses were performed using SPASS (version 22.0).

RESULTS

Patient Treatment Characteristics and Response

A total of 142 patients with relapsed or refractory hematology malignancies were included in the analyses. Eighty-seven (61.3%) patients were males and 55 (38.7%) females. The median age was 45 (IRQ=24–59). Fifty-five (55.7%) patients with ALL (5 Ph-positive ALL and 14 received allogeneic hematopoietic stem cell transplantation previously) received anti-CD19 CAR-T cell, and 25 patients with MM, including 11 (7.7%) type IgG, 5 (3.5%) IgA, 5 (3.5%) light chain, and 4 (2.8%) other types. Seven (28.0%) MM patients were in stage II and 18 (72.0%) in stage III. All MM patients received a combination of humanized anti-CD19 and anti-BCMA CAR T cells treatment. There were 62 patients with lymphoma, including 47 (33.1%) patients with diffuse large B-cell lymphoma, 5 (3.5%) follicular cell lymphoma, 2 (1.4%) mantle cell lymphoma, and 8 (5.6%) other types of B-cell lymphoma. Forty (28.2%) patients with lymphoma received CD19+CD20 CAR-T and 22(15.5%) received CD19 CAR-T (**Table 1**).

The overall response rates (ORR) of ALL, lymphoma, and myeloma were 85%, 70%, and 95.2%, respectively. The complete response (CR) was 85% in ALL patients, and the CR and partial response (PR) was 30% and 40% in patients with lymphoma, respectively. In patients with MM, CR, very good partial response (VGPR), and PR were 45%, 23%, and 20% respectively at one month after CAR-T cell infusion (**Figure S1**).

TABLE 1 | Characteristics of Patients (n=142).

Variables	All patients (%)
Gender	
M	87 (61.3%)
F	55 (38.7%)
Age, median (IRQ)	45 (24–59)
Disease	
ALL	55 (38.7%)
Ph positive	
Yes	5 (3.5%)
No	50 (35.2%)
Prior Transplant	
Yes	14 (9.9%)
No	41 (28.9%)
MM	25 (17.6%)
MG	
IgG	11 (7.7%)
IgA	5 (3.5%)
Light chain	5 (3.5%)
Other malignant plasmacyte disease	4 (2.8%)
Disease stage at diagnosis (ISS staging)	
II	6 (4.2%)
III	18 (12.7%)
Lymphoma	62 (43.7%)
NHL	
DLBCL	47 (33.1%)
FL	5 (3.5%)
MCL	2 (1.4%)
Other BL	8 (5.6%)
Target of CAR-T cell	
ALL	
CD19	55 (38.7%)
MM	
CD19+BCMA	25 (17.6%)
Lymphoma	
CD19	22 (15.5%)
CD19+CD20	40 (28.2%)

The Incidence of CRS and Characteristics

The CRS incidence of ALL, lymphoma, and MM were 82%, 90%, and 90%, respectively. However, the severity of CRS was different among MM, ALL, and lymphoma. Grade 1–2 and grade 3–5 CRS were found in 33 (60%) and 11 (20%) patients with ALL respectively. In patients with lymphoma, grade 1–2 CRS were observed in 45 of 62 (72.6%) and grade 3–5 in 10 of 62 (16.1%). But only one patient with MM encountered grade 3 CRS and most patients had grade 1 or 2 CRS (**Figure 1**). One patient with ALL died of heart failure resulting from the CRS-related myocarditis. Three patients with lymphoma and one patient with ALL had developed gastrointestinal bleeding.

Fever was the most common sign of CRS. In all patients, there was no difference in the onset time of fever between grade 1–2 and grade 3–5 CRS. But there was a difference among patients with different diseases. The median onset time of fever was day 3 (IRQ, day 0–7) in ALL patients, day 1 (IRQ, day 0–5) in patients with lymphoma, and day 8.5 (IRQ, day 1.75–12.75) in MM patients. The onset time of fever was different between MM and ALL (p=0.0044), or MM and lymphoma (p=0.0002), but no difference between ALL and lymphoma (p=0.5549). Further analysis according to the disease type and CRS level showed that the median onset time of fever in ALL patients with grade 1–2 and grade 3–5 CRS were day 4 (range day 0–10) and day 1 (range day 0–7) respectively and there was no difference. Fever occurred on a median of 1.5 days (range, 0–16 days) after CAR-T cell infusion in lymphoma patients with grade 1–2 CRS and without difference compared to patients with grade 3–5 CRS. Only one patient with MM developed grade 3 CRS, and the onset

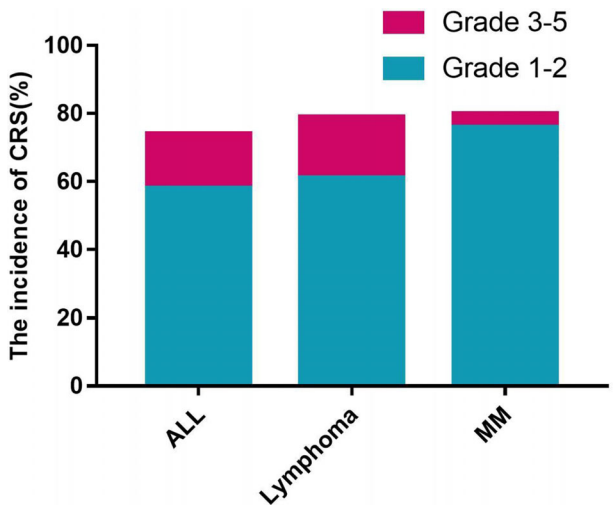

FIGURE 1 | The incidence of CRS.

time of fever was earlier than those with grade 1–2 CRS (**Table 2**).

The duration of fever in all patients was significantly different between grade 1–2 and grade 3–5 CRS (p=0.007). However, there was no statistical difference in the median duration of fever among ALL [3 days (0–7 days)], lymphoma [5 days (3–8 days)], and MM [4 days (3–8 days)]. The peak temperature of fever was different between grade 1–2 and grade 3–5 CRS (p= 0.02) and no difference among different diseases [ALL: 40°C (39.15–40.5°C) vs lymphoma: 39.4°C (38.8–39.8°C) vs MM: 39.1°C (38.8–39.7°C)] (**Table 2**).

Changes of peak concentration of IL-6 and CRP in serum were consistent with severity of CRS. There was difference of IL-6 concentration in serum between non-CRS and grade 3–5 CRS patients with ALL on days 7 and 10 and without differences among them at other time points. There were no differences in CRP among different B cell tumors patients with non-CRS, grade 1–2 CRS, and grade 3–5 CRS at specific time points (day 0, 3, 7… after CAR-T cells infusion). However, the peak concentration of IL-6 and CRP during CRS were significantly higher than the baseline (**Table S1**, **Figure S2**).

Clinical Factors Related to CRS

We analyzed age, gender, prior transplantation, disease type, CAR-T cell dose, and costimulatory molecules of all patients, separately. However, there were no differences between patients with grade 1–2 CRS and grade 3–5 CRS, except for disease type (ALL versus MM, p=0.049). The peak concentration of IL-6 (p=0.000) and CRP (p=0.001) was different among the patients with non-CRS, grade 1–2, or grade 3–5 CRS. Further analysis showed that there was statistical difference in the peak concentration of IL-6 between the patients with Non-CRS and grade 1–2 CRS (p=0.00), Non-CRS and grade 3–5 CRS (p=0.00), or grade 1–2 and 3–5 CRS (p= 0.03). The peak concentration of CRP was different between the patients with Non-CRS and grade 1–2 CRS (p=0.01), Non-CRS and grade 3–5 CRS (p=0.00), but there was no difference between grade 1–2 and grade 3–5 CRS (p=0.18) (**Table 3**). In the regression model, peak concentration of IL-6 (OR: 1.001, 95% CI: 1.0–1.001) and CRP (OR: 1.011, 95% CI: 1.005–1.017) could predict CRS (**Table S2**). The concentration of IL-6 >54.95pg/ml has 81.8% specificity and 61.0% sensitivity and the concentration of CRP >88.45mg/ml has 91.3% specificity and 52.1% sensitivity for CRS (**Figure S3**, **S4**).

TABLE 2 | Characteristics of fever in patients with CRS.

Patients	Grade 1–2 CRS	Grade 3–5 CRS	*P* value*
All patients, Median (range)			
Initial time of fever, day	3 (0–16)	1 (0–7)	0.062
Duration of fever, day	4 (1–36)	8 (2–32)	0.007
Peak of fever, °C	39.4 (37.7–41.9)	40 (37.9–40.5)	0.02
ALL, Median (range)			
Initial time of fever, day	4 (0–10)	1 (0–7)	0.33
Duration of fever, day	5 (1–36)	8 (2–20)	0.094
Peak of fever, °C	39.8 (38–41.9)	40.1 (39.6–40.5)	0.269
Lymphoma, Median (range)			
Initial time of fever, day	1.5 (0–16)	1 (0–7)	0.273
Duration of fever, day	4 (1–18)	5 (3–32)	0.194
Peak of fever, °C	39.1 (37.7–40.5)	39.3 (37.9–40.3)	0.654
MM, Median (range)			
Initial time of fever, day	9 (0–15)	4	–
Duration of fever, day	4 (1–11)	11	–
Peak of fever, °C	39.3 (38.1–41)	40.4	–

**Two-sided P-values calculated based on Kruskal-Wallis test.*

TABLE 3 | Clinical general factors related to cytokine release syndrome, by grade **(n=119)**.

Variable	CRS Grade		Univariate
	Grade 1–2	Grade 3–5	Analysis **P* value
Gender, n (%)			0.860
Male	61 (51.3%)	14 (11.8%)	
Female	38 (31.9%)	8 (6.7%)	
Age, Median [IQR]	49 (28–59)	37 (23–51.75)	0.317
Prior Transplant			0.510
Yes	13 (10.9%)	4 (3.4%)	
No	86 (72.3%)	18 (15.1%)	
Disease Type, n (%)			
ALL	33 (27.7%)	11 (9.2%)	0.049※
Lymphoma	45 (37.8%)	10 (8.4%)	0.163
MM	21 (17.6%)	1 (0.8%)	
CAR-T Cell Dose			
10^8 or >10^8	56 (47.1%)	15 (12.6%)	0.343
10^7	25 (21.0%)	5 (4.2%)	0.687
10^6 or <10^6	18 (15.1%)	2 (1.7%)	
Costimulatory molecules			1.0
CD28	9 (7.6%)	2 (1.7%)	
4-1BB	90 (75.6%)	20 (16.8%)	

**Two-sided P-values calculated based on Kruskal-Wallis test for continuous variables, and Fisher's Exact test for categorical variables.*
※*ALL versus MM.*

Risk Factors of CRS in Different B Cell Tumors

Each B-cell tumor has its own staging or prognosis evaluation systems. We analyze the relationship between these available factors and the occurrence of CRS.

ALL

We analyzed a variety of clinical factors that may be associated with CRS (gender, age, transplantation, CAR-T cell dose, bone marrow tumor burden, species of CAR, costimulatory molecules, serum maximum values of IL-6 and CRP, and minimum level of CD4/CD8) separately. Univariate analysis showed that the number of blasts cells in bone marrow (p=0.003), serum peak concentration of IL-6 (p=0.001) and CRP (p=0.008), and minimum value of CD4/CD8 (p=0.028) are the influencing factors for the occurrence of CRS. These factors were further entered into an ordinal logistic regression model, and the results showed that the number of blasts in bone marrow (OR:1.034, 95% CI 1.011-1.058) was the independent risk factors for CRS (**Table 4**, **Table S3**). The number of blast cells in bone marrow >22.0% (before pretreatment) has 45.0% specificity and 90.9% sensitivity for severe CRS (**Figure S5**).

Lymphoma

We analyzed a variety of clinical factors (gender, age, risk stratification, clinical stage, CAR-T cell dose, serum peak

TABLE 4 | Clinical factors related to cytokine release syndrome (patients with ALL, n=55).

Variable	CRS Grade			Univariate Analysis	Multivariable Analysis		
	non-CRS	Grade 1–2	Grade 3–5	*P* value	OR	95% CI	*P* value
Gender, n (%)				0.46			
Male	8 (14.5)	17 (30.9)	5 (9.1)				
Female	3 (5.5)	16 (29.1)	6 (10.9)				
Age							
Median [IQR]	17 (5–41)	15 (3–69)	25 (15–66)	0.176			
Prior Transplant				0.69			
Yes	2 (3.6)	8 (14.5)	4 (7.3)				
No	9 (16.4)	25 (45.5)	7 (12.7)				
CAR-T Cell Dose				0.29			0.794[a]
10^6>10^7	8 (14.5)	24 (43.6)	5 (9.1)				
10^8 or >10^8	3 (5.5)	9 (16.4)	6 (10.9)				
Bone Marrow Blast Cell, Median [range]							
Before FC treatment	0 (0–93)	62 (0–88)	80 (15–95)	0.003	1.034	1.011–1.058	0.004[a]
Before CAR-T infusion	0 (0–92.5)	6 (0–92)	18 (0–95)	0.053	–	–	0.758[a]
Species of scFv, n(%)				0.17			0.173[a]
humanization	4 (7.3)	22 (40.0)	8 (14.5)				
mouse	7 (12.7)	11 (20.0)	3 (5.5)				
Costimulatory molecules				0.385			0.184[a]
CD28	5 (9.1)	9 (16.4)	2 (3.6)				
4-1BB	6 (10.9)	24 (43.6)	9 (16.4)		–	–	

*****Two-sided P-values calculated based on Kruskal-Wallis test for continuous variables, and Fisher's Exact test for categorical variables.*

※*Ordinal Regression were performed to assess impact of baseline factors on the occurrence of CRS.*

[a]*The variables that enter the regression model include: Univariate Analysis (P≤0.1) or the variables that may affect the results.*

concentration of IL-6 and CRP, CD4/CD8, etc.) that may be associated with CRS in patients with lymphoma, separately. Univariate analysis showed that gender (p=0.016), serum peak concentration of IL-6 (p=0.016), were related to the occurrence or severity of CRS. Ordinal logistic regression model showed that sex (OR:0.113, 95% CI:0.019–0.666) and clinical stage (stage IV vs stage II, OR:0.05, 95% CI: 0.03–0.926) are independent risk factors for the CRS (**Table 5**, **Table S4**).

MM

We analyzed a variety of clinical factors (gender, age, β2-MG, type of light chain, ISS stage, plasma cell number in bone marrow, peak concentration of IL-6 and CRP, and minimum value of CD4/CD8) that may be associated with CRS in patients with multiple myeloma separately. But univariate analysis showed that these factors were not related to the occurrence or severity of CRS. However, based on clinical experience, several

TABLE 5 | Clinical factors related to cytokine release syndrome (patients with Lymphoma, n=62).

Variable	CRS Grade			Univariate Analysis	Multivariable Analysis		
	non-CRS	Grade 1–2	Grade 3–5	*P* value	OR	95% CI	P value
Gender, n (%)				0.016			
Male	2 (3.2%)	35 (56.5)	9 (14.5)		0.113	0.019–0.666	0.016[a]
Female	5 (3.2%)	10 (16.1)	1 (1.6)				
Age							
Median [IQR]	51 (23–62)	52 (24–72)	43.5 (17–70)	0.571			
Risk stratification (IPI)				0.554			
High	0	12 (19.4%)	2 (3.2%)				0.969[a]
Moderate	4 (6.5%)	20 (32.3%)	6 (9.7%)				0.687[a]
Low	3 (4.8%)	13 (21.0%)	2 (3.2%)				
Clinical stage				0.058			
IV	3 (4.8%)	19 (30.6%)	9 (14.5%)		0.05	0.03–0.926	0.044[a]
III	3 (4.8%)	23 (37.1%)	1 (1.6%)				0.133[a]
II	1 (1.6%)	3 (4.8%)	0				
CAR-T Cell Dose				0.579			
10^5>10^7	2 (3.2%)	18 (29.0%)	2 (3.2%)				
10^8>10^9	5 (3.2%)	27 (43.5%)	8 (112.9%)				

*****Two-sided P-values calculated based on Kruskal-Wallis test for continuous variables, and Fisher's Exact test for categorical variables.*

※*Ordinal Regression were performed to assess impact of baseline factors on the occurrence of CRS.*

[a]*The variables that enter the regression model include: Univariate Analysis (P≤0.1) or the variables that may affect the results.*

TABLE 6 | Clinical factors related to cytokine release syndrome (patients with MM, n=25).

Variable	CRS Grade			Univariate Analysis	Multivariable Analysis		
	non-CRS	Grade 1	Grade 2–3	*P* value	OR	95% CI	P value
Gender, n (%)				0.869			
Male	3 (12.0)	4 (16.0)	4 (16.0)				
Female	2 (8.0)	7 (28.0)	5 (20.0)				
Age							
Median [IQR]	62 (51–65)	59 (53–63)	52 (46–59)	0.140			
β2-MG, n (%)	6713.5 (2413–18600)	3159 (1843–6511)	2888 (1763–12522)	0.455			0.056[a]
Type of Light chain				1.0			
Kappa	2 (8.0)	7 (28.0)	6 (24.0)				
Lambda	1 (4.0)	4 (16.0)	3 (12.0)				
Myeloma Cells in Bone Marrow, Median (range)	16 (8–21)	12 (2–69)	24 (3–67)	0.343	1.072	1.008-1.140	0.028[a]
ISS stage				1.0			
II	1 (4.0)	3 (12.0)	3 (12.0)				
III	4 (16.0)	8 (32.0)	6 (24.0)				

**Two-sided P-values calculated based on Kruskal-Wallis test for continuous variables, and Fisher's Exact test for categorical variables.*

[※]Ordinal Regression were performed to assess impact of baseline factors on the occurrence of CRS.

[a]The variables that enter the regression model include: Univariate Analysis (P≤0.1) or the variables that may affect the results.

factors (β2-MG and number of plasma cells) were entered in an ordinal logistic regression model that may be related to CRS. The results showed that the number of plasma cells in the bone marrow (OR:1.072, 95% CI:1.008–1.140) is an independent risk factor for CRS (**Table 6**, **Table S5**).

DISCUSSION

CRS is one of the major complications during CAR-T cell treatment. However, current guidelines or options of management of CRS are based on data of CD19 CAR and risk assessment of CRS occurrence of different diseases (ALL, lymphoma, or MM) use the same standard or method (5, 15–18). This is not reasonable to management of CRS of patients with B-cell hematological tumors. In this *post hoc* analysis, we found that although the clinical manifestations of CRS in different diseases are similar, the characteristics and risk factors of CRS are not the same, suggesting that we need to pay more attention to the management of CRS according to disease type, instead of treating them in the same way.

In our study, there is no difference in the total incidence of CRS among patients with MM, ALL, or lymphoma. However, the incidence of severe CRS in patients with MM is significantly lower than those with ALL or lymphoma. We are not sure if this phenomenon is caused by the antigen itself, kinetics of CAR-T cell proliferation, the immune microenvironment, or others. Two patients with lymphoma and one patient with ALL were complicated with gastrointestinal bleeding during CRS. Because the general condition of the patient was very poor, we were unable to perform colonoscopy and pathology to determine the true cause of gastrointestinal bleeding, but we should pay attention to this fatal complication. One patient with ALL died of acute myocarditis. This patient first showed elevated glutamic oxaloacetic transaminase (ALT) and lactic dehydrogenase (LDH) in serum, without any other special clinical manifestations. Although glucocorticoid and IL-6 were used, the patient suddenly developed heart failure heavily and died. Therefore, for patients with myocardial damage, we should be vigilant for fatal heart failure. Therefore, to balance the possible advantages and disadvantages of intensive treatment (tochizumab, glucocorticoid, etc.), MM patients with CRS have more sufficient observation time, while patients with acute lymphoblastic leukemia and lymphoma need to be more cautious and timely, especially for patients with organ damage.

Fever is the primary manifestation of initiation of CRS in most patients. Although the fever types of different diseases were similar, the median onset time of fever in patients with lymphoma was the earliest, followed by ALL and MM. The mechanism of CRS is still unclear. According to our and other published data, the proliferation of CAR-T cell in MM patients is relatively slower than that in those with ALL (1, 3, 13, 14), which may be the cause of delayed occurrence of CRS. Another interesting phenomenon is that we, as well as other research groups, have found that the incidence of CRS is high during BCMA CAR-T cells in patients with relapsed or refractory MM, but the incidence of severe CRS is very low. Therefore, when fever occurs early after CAR-T cell infusion, CRS should be considered first for patients with acute lymphoblastic leukemia and lymphoma, and the changes of peripheral oxygen concentration, blood pressure, organ function, and blood biological markers (IL-6, CRP, and ferritin, etc.) should be monitored more frequently. However, in MM patients, the onset time of CRS related fever is relatively late, which may coexist with infection and bring more challenges to diagnosis and treatment of CRS.

Cytokines are the critical factors in the CRS (9, 19, 20), including ferritin, IL-6, CRP, TNF, interferon, IL-10, IL-1, MCP, etc. Among them, the most commonly used in the clinic are ferritin, IL-6, and CRP. Several clinical studies (8) have confirmed that the serum levels of these factors are associated with the occurrence and severity of CRS, and dynamic changes reflect the outcome of CRS. In our study, we found that the trends of these factors at the different time points (day 3, day 7, day 10…) were

consistent with the occurrence or progression of CRS. However, only IL-6 levels at specific time point (day 7 and 10) were different in ALL patients between grade 3–5 CRS and non CRS. But a significant difference in the peak concentration of IL-6, ferritin, and CRP occurred among patients with different levels of CRS, indicating that the cytokines level at a specific time point does not truly reflect their trends. We also found that cytokine levels can change sharply in a few days or even hours. Therefore, we should monitor the changes of cytokines level more frequently according to the severity of CRS, rather than at specific time points.

The occurrence and severity of CRS are related to several factors, including CAR-T cells dose, proliferation of CAR-T cells, and the number of blasts in the bone marrow (8, 21). Early CRS risk assessment helps to monitor and intervene in a timely manner for patients with high-risk factors. The patient's baseline characteristics are the most available predictor (7, 8). Our data showed that the type of disease was an important factor of the severity of CRS, and the different B-cell hematological tumors have their own predictive risk factors. Moreover, IL-6 and CRP were the independent risk factor not only for the occurrence of CRS but also for the severity of CRS. For patients with acute lymphoblastic leukemia, tumor burden was the high-risk factor for CRS. Therefore, reducing the tumor burden before CAR-T cell therapy may reduce the occurrence of CRS. Clinical stage is associated with CRS in patients with lymphoma (especially stage IV). For patients with lymphoma involving organs (small intestine, liver, lungs, etc.), while monitoring or intervening CRS, more attention should be paid to the damage (gastrointestinal bleeding, pulmonary edema, liver failure) of the involved organs. The number of abnormal plasma cells in the bone marrow is a high risk factor of CRS in patients with multiple myeloma, and reducing the tumor load as much as possible before CAR-T cell therapy may be one of the strategies to reduce CRS. This is a retrospective study. Different manufacturers and multiple combinations of CAR-T cells, sample size available for each disease, different CAR product or construct also may lead to differences in the incidence and severity of CRS. In addition, limited observational factors may also miss some factors that may affect CRS. Therefore, in the future, more rigorous clinical studies need to be designed to verify the factors that might predict CRS.

CONCLUSIONS

The occurrence of CRS in different B-cell tumors has its own characteristics. Compared with ALL and lymphoma, severe CRS incidence in MM patients is lower and occurs later. The risk factors of CRS in different B-cell tumors are different, suggesting that individualized treatment is required in clinical practice.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committees of the affiliated Hospital of Xuzhou Medical University, Xinqiao Hospital, the First Affiliated Hospital of Zhejiang University, and the First Affiliated Hospital of Nantong University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KX, XZ, WQ, HoL, ZL, JZ, JC, and ZY designed the research. All investigators and their respective research teams recruited and followed up the patient. ZY, CZ, HuL, HZ, HH, YQ, and YiW collected and analyzed research data. ZY and HZ wrote and edited the manuscript. All authors were involved at each stage of manuscript preparation and approved the final version. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.611366/full#supplementary-material

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REVIEW
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Functional Contributions of Antigen Presenting Cells in Chronic Graft-Versus-Host Disease

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Chronic graft-versus-host disease (cGVHD) is one of the most common reasons of late non-relapse morbidity and mortality of patients with allogeneic hematopoietic stem cell transplantation (allo-HSCT). While acute GVHD is considered driven by a pathogenic T cell dominant mechanism, the pathogenesis of cGVHD is much complicated and involves participation of a variety of immune cells other than pathogenic T cells. Existing studies have revealed that antigen presenting cells (APCs) play crucial roles in the pathophysiology of cGVHD. APCs could not only present auto- and alloantigens to prime and activate pathogenic T cells, but also directly mediate the pathogenesis of cGVHD *via* multiple mechanisms including infiltration into tissues/organs, production of inflammatory cytokines as well as auto- and alloantibodies. The studies of this field have led to several therapies targeting different APCs with promising results. This review will focus on the important roles of APCs and their contributions in the pathophysiology of cGVHD after allo-HSCT.

Keywords: chronic graft versus host disease, allogeneic hematopoietic stem cell transplantation, antigen presenting cells, immune tolerance, immune regulation

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INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a widely used life-saving procedure for patients with hematopoietic malignancies including leukemia, lymphoma as well as other non-malignant diseases related with bone marrow failure. However, its success is markedly compromised by the development of graft-versus-host disease (GVHD) after transplantation due to the histoincompatibility between donors and recipients. Donor alloreactive T cells are first primed through recognition of host alloantigens presented by host antigen presenting cells (APCs), and less often, by donor APCs. Upon preparative conditioning (including high dose chemotherapy and/or total body irradiation) caused gastrointestinal tract or tissue damage, the released pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) stimulate the upregulation of costimulatory molecules and production of inflammatory cytokines expressed in APCs. Such APCs subsequently drive the activation and differentiation of donor alloreactive T cells into effector T cells which contribute to GVHD in target organs (1–3). According to the time of onset and pathological mechanisms, GVHD can be divided into acute GVHD (aGVHD) and chronic GVHD (cGVHD). aGVHD usually starts within the first 100 days after allo-HSCT and is mediated mainly by infused donor alloreactive T cells in the grafts. Accompanied with

the process of aGVHD, donor hematopoietic stem cells (HSCs) engraft in host bone marrow and develop into various immune cell lineages. Unfortunately, such donor-derived immune cells could be dysfunctional and autoreactive due to the altered microenvironment unable to support their normal development. Many aGVHD survivors could further develop into subsequent cGVHD which usually begins at a later stage (100 days to 2 years after allo-HSCT), though earlier onset (termed overlap cGVHD when concurrent with aGVHD) is also possible (4, 5).

cGVHD is a life-threatening complication which affects 30%–70% patients who have received allo-HSCT (6–8), with prior episode of aGVHD as the most potent risk factor. It remains a leading cause of late non-relapse morbidity and mortality of patients following allo-HSCT (9). The incidence of cGVHD has been increasing in the past two decades attributed to increased use of old age donors and unrelated/mismatched donors, reduced intensity conditioning regimen and G-CSF mobilized peripheral blood stem cells (G-PBSCs) instead of unmanipulated bone marrow grafts (8, 10, 11). Several curative therapies against aGVHD, such as corticosteroids and calcineurin inhibitors and other immune inhibition drugs, have been successfully developed (12). However, therapies for cGVHD are still challenging due to our poor understanding on its much complex and obscure pathogenesis (13). Conventional treatments for cGVHD are glucocorticoids and immunosuppressive drugs which only achieve disease remission in part of the patients (14, 15). Moreover, systemic glucocorticoids often bring long-term complications which increase morbidity and mortality in patients with cGVHD (12, 16). In recent years, ruxolitinib (a selective JAK1/2 inhibitor) has been used in patients with steroid-refractory cGVHD which showed promising clinical results (17). Other cell based therapy such as extracorporeal photopheresis has also been found to benefit the treatment of cGVHD although the immunological mechanism remains elusive (18).

OVERVIEW OF CGVHD IN PATIENTS AND MOUSE MODELS

GVHD is a complex immunological process involving both innate and adaptive immune responses. cGVHD and aGVHD have distinct pathogenesis albeit they share some common clinical manifestations (19). Unlike aGVHD in which T cells play dominant pathogenic roles (20), the pathogenesis of cGVHD is comprehensive and involves the infiltration of various inflammatory cells as well as the production of auto- and alloantibodies. The complexity of cGVHD immunopathology also indicates a dysfunction of immune tolerance in the hosts after allo-HSCT, which may be part of the reasons for the unresponsiveness of cGVHD patients to the commonly used immunosuppressive agents (21). Tissue and organ damage caused by donor T cell-mediated aGVHD is crucial for initiating cGVHD. Depletion or inhibition of donor T cells in the grafts by anti-lymphocyte antibodies and high-dose cyclophosphamide in the early post-transplantation period could not only prevent aGVHD but also delay the onset of cGVHD (22–25). cGVHD affects not only epithelial tissues (gastrointestinal tract, lung, liver and skin), mostly targeted in aGVHD, but also many other tissues/organs including oral, esophageal, musculoskeletal, fascial, ocular, joint, and even genital tissues (4, 26–29). Attributed to the introduction of National Institute of Health (NIH) consensus criteria, the diagnosis and scoring for cGVHD have been greatly improved in the last two decades. Fibrosis is the most frequently observed characteristic of cGVHD with cutaneous and pulmonary fibrosis (tissue fibrosis manifesting as scleroderma and bronchiolitis obliterans) as the definitive clinical manifestations (4, 30).

Since human cGVHD is very difficult to study mechanistically, various mouse models of cGVHD have been developed in the last decades (31–36). To recapitulate the natural evolution of clinical cGVHD in human allo-HSCT patients, mouse models have been designed with a more precise imitation of clinic procedures including preparative conditioning (total body irradiation), donor and recipient strain combinations (use semiallogeneic F1 mice or minor histoincompatible mice as recipients), and in some models, use of G-CSF-mobilized splenocytes or peripheral blood grafts instead of conventional bone marrow transplantation (BMT) plus purified splenic T cells to induce cGVHD (37, 38). These aspects permit recipients to survive aGVHD and give time for auto- and alloreactive T cells and B cells to develop and cause cGVHD. Inappropriate BMT conditions such as high dose total body irradiation, or high T cell number in grafts, or use of fully MHC-mismatched donors often correlate with an early mortality (within a couple of weeks) after BMT as a result of severe gastrointestinal aGVHD (20, 39). By adjusting to an optimal BMT condition, an autoimmune-mediated pathology could be induced 4-8 weeks after BMT attributable to chronic autoreactive T cell activation and subsequent autoantibody production (40, 41). Considering of the different kinetics with clinical symptoms observed in patients, the disease occurrence in mouse cGVHD models is often absence or only happens at late stage after BMT. In a mouse model of mixed hematopoietic chimerism, the persistence of host B cells and high levels of circulating IgG autoantibodies were found to be associated with the appearance of sclerodermatous cGVHD-like lesions which were observed 7-9 months after BMT (42). In recent years, $CD34^+$-stem-cell-humanized NSG mice were found to develop cGVHD late after transplantation (more than 24 weeks). These mice reproduce the full spectrum of pleiotropism of human cGVHD in the absence of prior aGVHD which may serve as a great model for cGVHD related research (43).

In cGVHD, donor T cells developed from engrafted HSCs could be both auto- and alloreactive capable of inducing similar disease when adoptively transferred into secondary allogeneic or syngeneic recipients (44, 45). In these mouse models, pathogenic Th17 cells have been implicated to be causative to cGVHD as well as their roles in aGVHD (46–48). Specific antibody-mediated suppression of IL-17 producing cells reduces histopathological damage of skin, salivary gland and liver in cGVHD (47). In addition, T follicular helper (Tfh) cells play a part in cGVHD as well through interaction with auto- and alloreactive germinal

center (GC) B cells *via* expression of both cell surface molecules and IL-21 (41). The pathogenesis of cGVHD is also found to be closely related with deficient development of regulatory cell subsets such as regulatory T cells (Tregs) and regulatory B cells (Bregs) (49, 50). In addition to the contributions of dysfunctional lymphocytes, pathogenic macrophages play important roles in the development of cGVHD, indicating a mutlifactorial pathogenesis of the disease (51, 52). Based on the studies of mouse models, the pathophysiological and immunological evolution of cGVHD should include at least 4 major mechanisms: distorted T cell negative selection in injured host thymus, lack of regulatory cell populations, macrophage-mediated multi-organ fibrosis and loss of B cell tolerance (50–53). cGVHD is a result of immune imbalance between inflammatory immune responses and inhibitory immune mechanisms that maintain immune tolerance. Given that APCs play critical roles in initiation of auto- and alloreactive T cell responses, development/maintenance of central/peripheral immune tolerance, production of profibrotic cytokines as well as auto- and alloantibodies, they are likely important contributors to the development of cGVHD. Below, we review the existing literatures of the functions and contributions of APCs in the pathogenesis of cGVHD (**Table 1**).

DYSREGULATION OF CENTRAL AND PERIPHERAL T CELL TOLERANCE BY DENDRITIC CELLS IN CGVHD

Dendritic cells (DCs) at steady state play dual roles in the induction of T cell-mediated adaptive immune response and maintenance of immune tolerance (72, 73). In cGVHD settings after allo-HSCT, DCs are crucial for initiating pathogenic T cell activation in periphery. Their dysfunction also causes failure of autoreactive T cell education in host thymus and loss of T cell peripheral tolerance which contribute to the pathogenesis of cGVHD.

Preclinical Data

During normal thymopoietic development, autoreactive T cells are depleted in the thymus as a result of negative selection which

TABLE 1 | Distinct origins and functions of antigen presenting cells (APCs) in chronic graft-versus-host disease.

Cell type	Origin	Function	Mouse model/ Patient
DCs	Donor	Regulate T cell central tolerance (44)	(*H2-Ab1*$^{-/-}$)
		May influence T cell peripheral tolerance (54, 55)	B6→C3H (44)
		Impaired cDC expression of MHCII leads to a failure of Treg development (50)	Patients (54, 55)
		GM-CSF induced CD4$^+$CD8$^-$ DCs promote Treg expansion (56)	B6→B6D2F1 (50)
			BALB/c→B6 (50)
			B10.D2→BALB/c (56)
	Host	NA	NA
B cells	Donor	Production of autoantibodies (57, 58)	DBA/2→BALB/c (57)
		Production of autoanitbodies (59, 60)	B6→B10.BR (58)
		Promote the expansion of donor autoreactive T cells (61)	Patients (59, 60)
		Interaction with Tfh cells (41, 62, 63)	DBA/2→BALB/c (61)
		Altered B-cell homeostasis, over-activation of IgG producing B cells, increased numbers of circulating pre-GC B cells and post-GC plasmablast-like cells (64)	B6→B10.BR (41)
			B6→B6D2F1 (62)
			Bm12→B6 (62)
			DBA/2→BALB/c (63)
			Patients (64)
	Host	Produce autoantibodies in a mixed chimerism mouse model (42)	FVB→BALB/c (42)
Macrophages	Donor	Mediate fibrosis *via* producing of profibrotic TGF-β, induce the differentiation of fibroblasts into collagen-producing myofibroblasts, promote collagen synthesis and deposition (65, 66)	B6→B10.BR (65)
			B10.D2→BALB/c (65)
		Activate and interact with Th17 cells (67)	B10.D2→BALB/c (66)
		Induce a strong T cell infiltration in the buccal mucosa and labial salivary glands (68)	HSPCs→*hIL-6* Tg NSG* (67)
		CSF-1 dependent BM derived M2 macrophages induce pathogenesis of cGVHD *via* expression of CD206 and production of TGF-β (51)	Patients (68)
		M2 macrophage over-activation and increased oxidative stress (69)	B6→B6D2F1 (51)
			Patients (69)
	Host	NA	NA
mTECs	Donor	Restore T cell central tolerance and ameliorate cGVHD by adoptive transfer of donor derived TEC progenitors (70)	B6→BALB/c (70)
	Host	Defective T cell negative selection in thymus due to damage of mTECs (71)	B6→BALB.B (71)

**In this study, cord blood-derived human CD34$^+$CD38$^-$CD45RA$^-$ haematopoietic stem/progenitor cells (HSPCs) were transferred into sublethally irradiated hIL-6 transgenic NSG mice. NA, data not available.*

is mediated by the medullary thymic epithelia cells (mTECs) and the presence of intrathymic autoantigen presenting DCs (73–76). However, in allogeneic BMT scenario, preparative conditioning regimen and donor T cell-mediated aGVHD could damage host thymus and impair thymopoiesis, resulting in dysfunction of negative selection and subsequent release of auto- and alloreactive T cells into periphery (77–79). Allogeneic BMT recipient animals of MHC class II deficient bone marrow grafts developed cGVHD which can be prevented by prior thymectomy (44), indicating a regulatory role of donor DCs in T cell central tolerance during cGVHD. Donor T cells escaped from the thymus of recipient of MHC class II deficient bone marrow grafts are autoreactive and pathogenic owing to the dysfunction of DCs and can cause cGVHD when transferred into secondary recipient mice (44). Interestingly, even host T cells become pathogenic in the absence of DC-mediated central tolerance. Unlike radioresistant tissue-resident macrophages, host DCs are radiosensitive and replaced by donor cells shortly after transplantation. A study reported that host T cells derived from radioresistant intrathymic T cell precursors escaped negative selection in mice lack of host intrathymic DCs and caused dermal fibrosis in mouse cGVHD model (80). After escaping from dysfunctional thymus, auto- and alloreactive T cells further differentiate into effector T cells in periphery. DCs are well known as the most potent professional APCs in eliciting peripheral naïve T cell activation. While host DCs are rapidly eliminated early after allo-HSCT, donor DCs predominate in peripheral tissues and contribute to the development of cGVHD by presenting both host and donor antigens to activate donor T cells *via* indirectly antigen presentation (81, 82).

Clinical Data

Although the appearance of donor DCs occurs early after allo-HSCT, their reconstitution is impaired and requires a long period of time to complete. Conventional DCs (cDCs) and plasmacytoid DCs (pDCs) are two major DC subsets both of which contribute to the induction of donor T cell tolerance against host organs after allo-HSCT (73, 74, 83). A study of pediatric allo-HSCT revealed that cDC numbers returned to normal level within 300-400 days after transplantation while pDC numbers recovered very slowly in these pediatric patients and were always lower than their age-matched healthy controls up to 7 years after transplantation (54). Another study reported that allo-HSCT patients with sooner or higher pDC recovery profile correlated with improved overall survival, indicating pDC count in peripheral blood of allo-HSCT patients is a significant predictor of long-term outcome after allo-HSCT (55).

Pathophysiologic Interpretation and Therapeutic Implications

DCs maintain T cell immune tolerance in both thymus and periphery. Peripheral T cell tolerance can be induced *via* direct interaction of inhibitory signaling molecules PD-L1/PD-1 and (CD80/86)/CTLA4 expressed on the surface of DCs and T cells, respectively (84–86). Besides, DCs could also promote donor T cell tolerance *via* expansion of Tregs. In addition to IL-2 dependency, Tregs require costimulatory signals from DCs for their optimal activation and proliferation. Tregs play important roles in the control of pathogenic T cell response and dysfunctional Treg development could cause various autoimmune diseases (87, 88). Decreased numbers of circulating Tregs were found to be correlated with cGVHD in both preclinical and clinical studies (40, 89–91), and adoptive transfer of Tregs could effectively ameliorate cGVHD (92, 93). DCs are important for their role in the induction and maintenance of Tregs and this function is mediated through a MHC class II-dependent interaction (94). It was found that an inflammatory cytokine milieu dominated by TNF during GVHD impairs the MHC class II antigen presentation pathway of cDCs, while MHC class I presentation remains largely intact, and leads to a failure in Treg development which results in a loss of immune tolerance in cGVHD (50, 95). Promoting Treg expansion is a promising approach to prevent cGVHD. Low-dose subcutaneous injection of IL-2 has shown to effectively expand Tregs *in vivo* and ameliorate cGVHD (96–99). A recent study reported that GM-CSF treatment increased $CD4^+CD8^-$ DC number and promoted DC-dependent Treg expansion, thus protected mice against the development of skin cGVHD (56), validating an indirect strategy to prevent cGVHD *via* strengthening DC and Treg interaction.

ACTIVATION AND INFILTRATION OF DONOR MACROPHAGES CONTRIBUTE TO CGVHD

Macrophages are remarkably plastic innate immune cells which can be found in all tissues and exhibit a vast functional diversity in development, maintenance of microenvironment homeostasis, tissue damage repair as well as innate immunity and adaptive immunity (100–102). Tissue-resident macrophages differ from monocyte-derived macrophages in terms of origin, which has been widely investigated in the last decade as immune sentinels in immune defense and resolution of inflammation (103). They are of embryonic origin and found to reside in majority peripheral tissues and organs, replenished by self-renewal independent of bone marrow monocyte replacement at steady state. However, after allo-HSCT, tissue-resident macrophages can be replaced by donor monocyte-derived macrophages which contribute to the pathogenesis of cGVHD.

Preclinical Data

In mouse models, accumulating studies support the concept that donor-derived macrophages could facilitate and intensify the pathophysiology of cGVHD (37, 51, 67, 104). It has been revealed that inhibition of donor macrophage infiltration in tissues and organs could ameliorate mouse cGVHD (65). CSF-1 axis controls macrophage development, differentiation and survival and is critical for monocyte-derived macrophage reconstitution after allo-HSCT. In IL-17-dependent cGVHD models of scleroderma and bronchiolitis obliterans, donor bone marrow-derived macrophages were found infiltrating the skin and lung in a CSF-1/CSF-1R-, but not CCL2/CCR2- or GM-CSF/GM-CSFR-, dependent manner and contribute to the

pathogenesis of cGVHD. These macrophages express CD206 and TGF-β but not iNOS, identifying them as M2 macrophages (51). Administration of CSF-1R blocking antibodies significantly reduced HSP47$^+$ myofibroblasts in the skin, indicating a macrophage-dependent accumulation of myofibroblasts in cGVHD (66). The origin of macrophages is important for their profibrotic gene expression as evidenced by a finding that monocyte-derived alveolar macrophages differ significantly from tissue-resident alveolar macrophages and drive lung fibrosis after BMT (105).

Clinical Data

In allo-HSCT scenario, host derived tissue-resident macrophages are eliminated and replaced by donor monocyte differentiated tissue resident macrophages with M2 phenotype which are found associated with the development of cGVHD. CD163, a scavenger receptor with immunoregulatory properties, is expressed mainly on M2 macrophages. Examination of biopsy specimens from patients with skin GVHD showed that increased infiltration of CD163$^+$ M2 macrophages was a significant predictor for refractory GVHD and poor prognosis (106). Soluble CD163 (sCD163) accumulates in the blood of hosts under oxidative stress or severe inflammatory conditions, as a result of direct secretion by activated macrophages or cleavage of membrane-bound CD163 from cell surface by matrix metalloproteinases (107–110). Intriguingly, plasma sCD163 in allo-HSCT patients is a high risk predictor of cGVHD, indicating a role of M2 macrophage activation and oxidative stress in the pathogenesis of cGVHD (69). Macrophage-derived chemokine and CC chemokine receptor 4 were also found to be closely associated with strong T cell infiltration in the buccal mucosa and labial salivary glands in cGVHD patients (68).

Pathophysiologic Interpretation and Therapeutic Implications

Activated donor-derived macrophages could mediate tissue fibrosis *via* production of profibrotic cytokine TGF-β, which induces the differentiation of fibroblasts into collagen-producing myofibroblasts capable of promoting collagen synthesis and deposition in cGVHD (65, 66, 111, 112). Pirfenidone, approved by U.S. Food and Drug Administration (FDA) for idiopathic pulmonary fibrosis, can also ameliorate cGVHD by inhibiting macrophage infiltration and TGF-β production (65). A recent study found that type 2 cannabinoid receptor expressed on macrophages played a critical role in the regulation of cGVHD and therapeutic targeting of this receptor by agonist showed beneficial effect in a sclerodermatous cGVHD model (113). Additionally, macrophages could contribute to the pathogenesis of cGVHD *via* interaction with T cells. In cGVHD, alloreactive T cells activate and differentiate into Th1/Tc1, Th17/Tc17, and Tfh cell paradigms in the presence of inflammatory cytokines such as IL-6 and IL-12, while Th17/Tc17 cells play a central role in cGVHD pathophysiology (46–48). IL-17 is a key mediator of pathology in cGVHD and it controls the infiltration of F4/80$^+$ macrophages into skin which facilitate the development of scleroderma (51). It should be noted that both pathogenic macrophages and T cells share some common cytokine requirement. IL-6 is a multifunctional inflammatory cytokine which can activate macrophages and also drive the differentiation of pathogenic Th17 cells. By using a humanized cGVHD mouse model through engraftment of human hematopoietic stem/progenitor cells into *hIL-6* transgenic recipient mice, Rintaro et al. reported that co-activation of macrophages and T cells were found in lung and liver and contribute to the pathogenesis of cGVHD (67). *IL-6* gene polymorphism is closely associated with the pathogenesis of cGVHD and anti-IL-6R monoclonal antibody (tocilizumab) has been reported to ameliorate cGVHD in some allo-HSCT patients (114, 115).

LOSS OF B CELL TOLERANCE IN CGVHD

At steady state, B cells develop in bone marrow and undergo negative selection which leads to a state of B cell central tolerance to avoid production and release of autoreactive B cells into periphery. Loss of B cell tolerance and aberrant activation of peripheral B cells contribute to the development of cGVHD (116–118).

Preclinical Data

An intact bone marrow microenvironment is critical for normal B cell lymphopoiesis. Osteoblasts, which could form bone marrow stromal niche for HSCs and B cell progenitors, are targeted by donor pathogenic T cells in GVHD (119, 120). Interestingly, protection of osteoblasts from T cell-mediated damage, by a Treg-expanded graft infusion, could maintain the bone marrow niche for early B cell progenitors and increase the number of pro-B, pre-B and immature B cells in bone marrow and ameliorate cGVHD (121). Aberrant B cell negative selection in host bone marrow causes release of auto- and alloreactive B cells into periphery. These B cells migrate into secondary lymphoid organs and encounter auto- and alloantigens, become activated and then differentiate into plasmablasts or memory B cells *via* interaction with Tfh cells. Through their expression of cell surface molecules and IL-21, Tfh cells promote mature B cell proliferation, differentiation and secretion of auto- and alloantibodies in cGVHD (41, 62, 122). Both Tfh cells and GC B cells are involved in cGVHD and their functions are mutually dependent. Depletion of B cells could suppress Tfh cells in addition to GC formation in cGVHD (63). These data indicate that T-B cell interaction is an important contributor to the pathogenesis of cGVHD. Interestingly, it was reported that donor B cells in transplants, activated by donor T cells, are also efficient APCs to augment the initial clonal expansion and survival of donor autoreactive T cells which are capable of mediating autoimmune-like cGVHD (61). Recently, a study by Deng et al. has reported that extrafollicular CD4$^+$ T and B cell interactions are more important and sufficient for inducing cGVHD, while GC formation is dispensable (123). They identified PSGL-1^{low}CD4$^+$ pre-Tfh-like extrafollicular T cells that were critical for the pathogenesis of cGVHD owing to their interaction with B cells,

indicating a much complex mechanism of T-B cell interaction in the pathogenesis of cGVHD.

Clinical Data

It was originally found in a case report that a cGVHD patient who developed refractory immune-mediated thrombocytopenia after allo-HSCT responded to B cell depletion therapy (124). This finding provided evidence of B cell dysfunction in the immunopathology of cGVHD and suggested a potential way of cGVHD prevention by B cell depletion. B cell development deficiency is often observed in cGVHD patients, indicating an aberrant bone marrow microenvironment failed to support normal B cell lymphopoiesis and selection during cGVHD (125, 126). Insufficient B lymphopoiesis causes post-transplantational B cell deficiency with decreased bone marrow B cell precursors which has been reported in both aGVHD and cGVHD patients after allo-HSCT (127, 128). In addition, there is increasing evidence showing that aberrant peripheral B cell expansion is a feature of cGVHD owing to their dysfunctional regulation of activation and proliferation. For instance, B cells from patients with active cGVHD are in a heightened metabolic state and resistant to apoptosis due to deficient expression of proapoptotic molecule Bim (129). B cell activating factor of the tumor necrosis family (BAFF), which is produced by macrophages, monocytes, DCs, T cells and stromal cells, plays important roles in B cell metabolism, survival and maintaining autoreactive B cell clones (130–132). In cGVHD patients, increased BAFF concentrations and higher BAFF/B-cell ratios correlate with increased numbers of circulating pre-GC B cells and post-GC plasmablast-like cells (64). These circulating pathogenic B cells are capable of autoantibody production without requiring additional antigen stimulation. Besides, other molecules regulating B cell activation and proliferation could also contribute to B cell-mediated pathogenesis in cGVHD. Increased NOTCH2 activation was found to be closely related with robust BCR responsiveness to alloantigens in B cells from cGVHD patients and suppression of BCR-NOTCH hyperactivation by all-*trans* retinoic acid could reduce NOTCH2 signaling and prevent B cell proliferation while maintaining functional B cell responses (133).

Pathophysiologic Interpretation and Therapeutic Implications

Production of multiple auto- and alloantibodies is a hallmark of cGVHD, and a variety of auto- and alloantibodies have been found to be associated with the severity of cGVHD (134–137). In mouse cGVHD models of scleroderma and bronchiolitis obliterans, these auto- and alloantibodies are found not only the outcome of dysfunctional B cell activation during cGVHD, but also could be causative to cGVHD pathogenesis (57, 58). Alloantibodies against H-Y minor histocompatibility antigens are significantly associated with cGVHD and disease remission (59). Autoantibodies against platelet-derived growth factor receptor have been found to play a role in the development of skin and lung fibrosis in cGVHD *via* stimulating type I collagen gene expression through the Ha-Ras-ERK1/2-ROS signaling pathway (60). It has been reported that microRNA-17-92 expression is required for alloantibody production and IgG deposition in the skin in cGVHD (138). A recent study found that checkpoint regulator SLAMF3 could modulate the activation thresholds of B cell subsets and SLAMF3 blockade markedly enhanced autoantibody production in cGVHD, thereby revealing a role of SLAMF3 in the negative regulation of cGVHD *via* preventing the expansion of autoreactive B cells (139). Since aberrant activation of B cells contributes to the pathogenesis of cGVHD, approaches directly targeting the key downstream kinases of B cell activation have been developed for cGVHD treatment with promising results. Ibrutinib was designed as a selective inhibitor of Bruton's tyrosine kinase (BTK) and became the first FDA-approved drug for the treatment of steroid-refractory cGVHD in 2017 (140). A small molecule inhibitor of Syk has been found effective in the therapy of cGVHD in mouse models (32, 141). Fostamatinib, a Syk inhibitor drug approved by FDA for the treatment of immune thrombocytopenia, is now under clinical evaluation in patients with cGVHD.

FUNCTIONS OF OTHER APCS IN CGVHD

Among the non-hematopoietic APCs (e.g., epithelial or stromal cells), mTECs play important roles in the induction of T lymphocyte central tolerance and the pathogenesis of cGVHD. Damage of recipient mTECs caused by alloreactive T cells in the donor grafts leads to defective negative selection of donor T cells and release of autoreactive $CD4^+$ T cells into periphery which contribute to the development of cGVHD (71, 77, 142). A recent study has found that transplantation of donor-derived TEC progenitors into cGVHD recipients could restore immune tolerance and ameliorate cGVHD (70). In periphery, non-hematopoietic APCs initiate the initial priming of alloreactive T cells independent of hematopoietic APCs while the latter contribute to the intensification of GVHD (143–145), although most of these studies are based on mouse models of aGVHD. Considering the chronic inflammation and continuing existence of alloreactive T cells in cGVHD, detailed investigation on the role of peripheral non-hematopoietic APCs in pathophysiology of cGVHD is merited.

CONCLUDING REMARKS

While traditional treatments of cGVHD with corticosteroids and other immune suppressive agents are facing more and more challenges, it is of great interest to discover key cellular targets to interfere the pathogenesis of cGVHD. Detailed investigation on APCs in the pathophysiology of cGVHD will provide insights into new potential therapeutic treatments, especially for patients with steroid-refractory cGVHD. Attributed to the broad investigations based on mouse cGVHD models, the functional contributions of different APCs to the pathogenesis of cGVHD have been uncovered which were considered to be promising targets for cGVHD treatment (**Figure 1**). These findings in

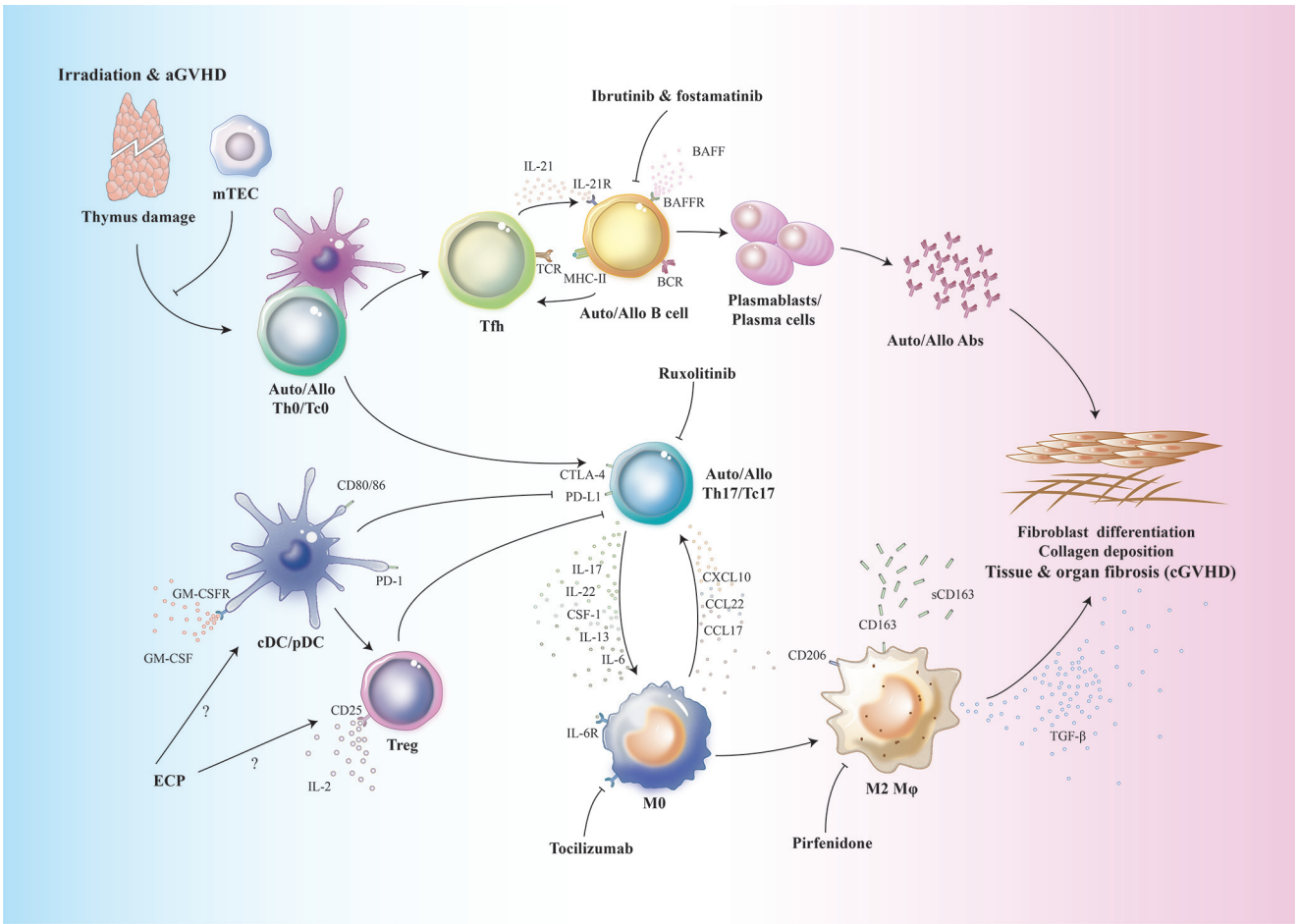

FIGURE 1 | Schematic overview of the functional contributions of APCs to cGVHD. Conditioning regimens such as irradiation, chemotherapy as well as aGVHD cause host thymus damage. Thymic dysfunction contributes to the defective T cell negative selection and release of auto-/alloreactive T cells into periphery. These Th0/Tc0 cells are activated by host or donor DCs and differentiate into auto-/alloreactive Th17/Tc17 and T-follicular helper (Tfh) cells. In germinal center, Tfh cells produce IL-21 which results in activation and expansion of allo-/autoreactive B cells. Elevated levels of BAFF could also contribute to the aberrant B cell expansion. These auto-/alloreactive B cells differentiate into plasmablasts or plasma cells which produce auto-/alloantibodies. Host tissue resident macrophages are eliminated and replaced by donor monocyte derived tissue resident macrophages. These macrophages recruit auto-/alloreactive Th17/Tc17 cells *via* production of chemokines. After migration into target organs, auto-/alloreactive Th17/Tc17 cells further secrete IL-17 to induce more macrophage infiltration. Under the influence of multiple cytokines such as CSF-1, IL-13 and IL-6, donor monocyte derived macrophages are polarized into TGF-β-producing M2 macrophages. The profibrotic cytokine TGF-β, together with auto-/alloantibodies, contribute to the pathogenesis of cGVHD *via* inducing fibroblast differentiation into myofibroblasts which promote collagen synthesis and deposition in target organs and tissues. ECP, extracorporeal photopheresis; Fostamatinib, a Syk inhibitor; Ibrutinib, Bruton's tyrosine kinase inhibitor; Pirfenidone, an anti-fibrotic drug; Ruxolitinib, a selective JAK1/2 inhibitor; Tocilizumab, anti-IL-6R monoclonal antibody.

mouse cGVHD models have been translated into the development of clinical medicines some of which have already showed beneficial results in clinical trials to treat patients with cGVHD (32, 65, 140, 141). However, challenges still remain due to the differences of pathogenesis and kinetics of disease occurrence between mouse models and patients with cGVHD. In addition, there is still lack of effective guidance for selection of optimal therapies for individual patients and none of the drugs available in clinic is effective for all patients with cGVHD. Considering the complexity of cGVHD pathophysiology, comprehensive strategies aiming at multiple APC targets may prove to be more promising in the future.

AUTHOR CONTRIBUTIONS

CH, RJ, XD, and XG collected all the literatures for reviewing and wrote the paper. All authors contributed to the article and approved the submitted version.

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REVIEW
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Transcription Factors Associated With IL-15 Cytokine Signaling During NK Cell Development

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Natural killer (NK) cells are lymphocytes primarily involved in innate immunity and possess important functional properties in anti-viral and anti-tumor responses; thus, these cells have broad potential for clinical utilization. NK cells originate from hematopoietic stem cells (HSCs) through the following two independent and continuous processes: early commitment from HSCs to IL-15-responsive NK cell progenitors (NKPs) and subsequent differentiation into mature NK cells in response to IL-15. IL-15 is the most important cytokine for NK cell development, is produced by both hematopoietic and nonhematopoietic cells, and functions through a distinct delivery process termed transpresentation. Upon being transpresented to NK cells, IL-15 contributes to NK cell development *via* the activation of several downstream signaling pathways, including the Ras–MEK–MAPK, JAK–STAT5, and PI3K–ATK–mTOR pathways. Nonetheless, the exact role of IL-15 in NK cell development has not been discussed in a consecutive and comprehensive manner. Here, we review current knowledge about the indispensable role of IL-15 in NK cell development and address which cells produce IL-15 to support NK cell development and when IL-15 exerts its function during multiple developmental stages. Specifically, we highlight how IL-15 supports NK cell development by elucidating the distinct transpresentation of IL-15 to NK cells and revealing the downstream target of IL-15 signaling during NK cell development.

Keywords: IL-15, signaling/signaling pathways, natural killer cell, development, transcription factor

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INTRODUCTION

NK cells constitute the third most abundant lineage of lymphocytes in the peripheral blood after B and T cells, accounting for approximately 8–15% of circulating cells in humans or 2–6% in mice (1). Similar to $CD8^+$ cytotoxic T lymphocytes, NK cells effectively eliminate virus-infected cells and malignant cells by producing proinflammatory cytokines and directly lysing target cells. NK cell activation is determined by the balance between signals transduced from multiple activating receptors and inhibitory receptors, which interact with their cognate ligand on target cells (2, 3).

Interleukin (IL)-15, a member of the common gamma chain cytokine family, was first described as a T cell growth factor, like IL-2 (4). IL-15 signals through a heterotrimeric receptor consisting of IL-15Rα (CD215), IL-2/IL-15Rβ (CD122) and the common γchain (γc, CD132) (5). Similar to IL-2, IL-15 requires the receptors IL-2/IL-15Rβ and γc to transduce signaling but differs from IL-2 by

virtue of its private binding receptor IL-15Rα, which is incapable of transducing signaling but has high affinity for IL-15 and forms a complex (IL-15–IL-15Rα) in IL-15-expressing cells (4, 6, 7). These IL-15/IL-15Rα complexes have the potential to stimulate neighboring cells that express IL-2/IL-15Rβ and γc *via* a unique mechanism referred to as tanspresentation (8, 9). Since the discovery of transpresentation, increasing evidence has suggested that IL-15 responses are largely mediated by transpresentation at steady state (10, 11).

NK cells primarily develop in the bone marrow (BM), which contains abundant hematopoietic stem cells (HSCs) capable of differentiating toward NK cells through common lymphoid progenitor (CLP) and lineage-restricted progenitor (NKP) cells (12). Multiple internal pathways and external factors contribute to the development of NK cells from HSCs (13). Most importantly, the pleiotropic cytokine IL-15 is indispensable for the development and homeostasis of NK cells as highlighted by their significant deficiency in IL-15-deficient mice. Correspondingly, deficiency in IL-15 or any one of the IL-15 receptor subunits, such as the IL-15Rα, IL-15Rβ, and γc in mice, results in a dramatic paucity of mature NK cells (14–17). Parallel with the role of IL-15 in mice, several studies have demonstrated that the early commitment of NK cells from human $CD34^+$ hemopoietic progenitor cells into NKP cells is dependent on the coordinated function of IL-3, IL-7, c-kit ligand (KL), and flt3 ligand (FL) but not IL-15, whereas IL-15 is involved in the emergence of $CD56^+$ NK cells (18, 19). Furthermore, Huntington et al. demonstrated that human NK cell differentiation that occurs in a linear fashion from $CD56^{hi}CD16^-KIR^-$ to $CD56^{lo}CD16^+KIR^-$ and finally to $CD56^{lo}CD16^+KIR^+$ requires IL-15 in a humanized model (20). Collectively, these findings illustrate that IL-15 signaling is essential for NK cell development and homeostasis in both mice and humans. Interestingly, Sun et al. recently reported that the requirement of IL-15 for NK cell development could be partially overcome by acute mouse cytomegalovirus (MCMV) infection, as IL-12 but not IL-15 primarily drives the anti-viral response of NK cells even in mice lacking the γc (21). Further studies are required to investigate whether this represents an IL-15-independent NK cell development manner.

Due to the critical role of IL-15 in NK cell development, dissecting the signaling pathways that allow IL-15 to control the development and homeostasis of NK cells is fundamental to determine the molecular details of immune regulation. In this review, we provide an overview of the specific IL-15 signaling that transcriptionally regulates NK cell development and maturation.

WHEN DOES IL-15 PROMOTE NK CELL DEVELOPMENT?

IL-15 Is Dispensable for NK Cell Commitment but Promotes Later Development

Mice and human NK cells are generated from HSCs through multiple but sophisticated stages in specific developmental niches with internal and external regulatory pathways governing NK cell development. In brief, NK cell development primarily involves the following two independent and continuous processes: early NK cell commitment to IL-15-responsive NKPs and subsequent phenotypical and functional maturation of NK cells in response to IL-15. Early NK cell commitment to NKP cells is characterized by the acquisition of CD122 (IL-15Rβ), which is a critical subunit of the IL-15 receptor and dimerizes with γc to transduce IL-15 signaling (22, 23). However, IL-15 is not involved in the generation of IL-15-responsive NKPs because the IL-15 receptor is not expressed prior to the NKP stage (24). Recently, pre-NKP cells were identified as the earliest committed NK cell progenitors in murine BM, and these cells reside downstream of CLP and differentiate into NKPs (23). Although pre-NKP cells express undetectable levels of CD122, they are fully committed to the NK lineage both *in vitro* and *in vivo*. Therefore, IL-15 is not necessary for NK cell lineage commitment. Furthermore, mice deficient in γc exhibit an intact NKP compartment (16), and IL-3, IL-7, KL, and FL synergistically drive the differentiation of NKP cells from human HSCs *in vitro* in the absence of IL-15 (25). Conversely, IL-15 is indispensable for the later development of NK cells. The expression of CD122 endows NK cells with the capacity to be responsive to IL-15; thus, these cells can become phenotypically and functionally mature and exhibit survival in response to IL-15 (16).

IL-15 Receptor Expression Varies in Different Stages of NK Cell Development

Intriguingly, the expression of CD122 on NK cells is not static but dynamically changes with NK cell maturation. It has been previously demonstrated that $CD56^{bright}$ NK cells express higher levels of CD122 as well as elevated CD122 transcripts compared with $CD56^{dim}$ NK cells, and thus are intrinsically more responsive to IL-15 (26–29). This observation explains the decreased proliferation capacity in response to IL-15 or dendritic cell (DC) stimulation during NK cell maturation (30, 31) and is consistent with the fact that cytokines, such as IL-2 and IL-15, fail to reverse the proliferation defects of $CD57^+$ terminally matured NK cells (32). Consistent with the observation in human NK cells, CD122 expression is significantly decreased during maturation from mice $CD11b^+CD27^+$ NK cells to $CD11b^+CD27^-$ NK cells and concomitant with decreased proliferation capacity (33). Despite the vitally important role of IL-15 in NK cell maturation, the exact role of decreased CD122 expression during NK cell terminal maturation needs to be further elucidated.

Transcriptional Regulation of IL-15 Receptor Expression at Different Stages During NK Cell Development

Although CD122 (encoded by Il2rb) is critical for NK cell development by transducing IL-15 signaling, the coordinated regulation of CD122 expression by various transcription factors remains elusive. Previous studies have demonstrated that RUNX3 (one of the Runx family transcription factors), T-bet, and Eomesodermin (Eomes) directly bind to the promoter

region of Il2rb and induce CD122 expression (34, 35). However, these transcription factors are not simultaneously functional, but rather function at different stages of NK cell development. In the NK cell development pathway, RUNX3 expression is initiated at the NKP stage. The inactivation of RUNX3 in HSCs partially disturbed the generation of $CD122^{+}$NKP cells *in vitro* but not completely, indicating that other unknown transcription factors contribute to the expression of CD122 during NK cell commitment. In addition, the deletion of RUNX3 in immature NK cells in mice only slightly reduced CD122 expression on NK cells, and the absolute number of NK cells was not significantly affected. These results confirmed that RUNX3 is necessary for the acquisition of CD122 during NK cell lineage commitment but is not essential for the maintenance of CD122 at the later maturation stages of NK cell development (34).

In contrast, T-bet and Eomes are weakly expressed at the NKp stage but highly expressed during NK cell maturation; therefore we speculated that T-bet and Eomes are not firmly involved in the induction of CD122 at the NKp stage but may contribute to the maintenance of CD122 expression during maturation (35, 36). Consistently, mice harboring genomic deletions of T-bet and Eomes lack NK cells, but $CD122^{hi}$ precursors of NK cells were observed (36). In addition, the deletion of Eomes in mice results in significantly decreased CD122 expression at different stages of NK cell maturation (33, 35, 37). Moreover, $Eomes^{+}$ NK cells express more CD122 and proliferate better than $Eomes^{-}$ NK cells, which are called Innate Lymphoid Cells (ILCs) 1 now (38, 39). However, CD122 expression is upregulated in T-bet-deficient NK cells, and this finding may be attributed to increased Eomes expression, which is repressed by T-bet (40). These results indicate that Eomes but not T-bet plays a dominant role in the maintenance of CD122 expression during NK cell maturation. Consistently, although T-bet expression is upregulated during the NK cell transition from the $CD11b^{+}CD27^{+}$ to $CD11b^{+}CD27^{-}$ stage, CD122 expression is progressively decreased, accompanied by a reduction in Eomes expression (33).

In conclusion, the induction of CD122 during early NK cell commitment is dependent on RUNX3, whereas Eomes but not T-bet maintains the expression of CD122 to promote NK cell maturation (**Figure 1**).

WHICH CELLS PRODUCE IL-15 TO PROMOTE NK CELL DEVELOPMENT?

Isolated Expression of IL-15 mRNA and Protein

IL-15 mRNA is constitutively expressed in a broad range of tissues, including hematopoietic cells [monocytes, macrophages, and dendritic cells (DCs)] and non-hematopoietic cells (epithelial cells, fibroblasts, nerve cells, skeletal muscle and keratinocytes) (4). In contrast to the widespread expression of IL-15 mRNA, IL-15 protein is only detectable in a more restricted population at steady state. This discrepancy between widespread IL-15 transcript expression and restricted protein expression is attributed to extensive checkpoints at transcription, translation, and intracellular trafficking, particularly post-transcriptional checkpoints. Multiple 5′-untranslated region (UTR) AUG sequences, a long signal peptide (LSP) (48 amino acid) and a negative regulatory element in the C-terminus of the

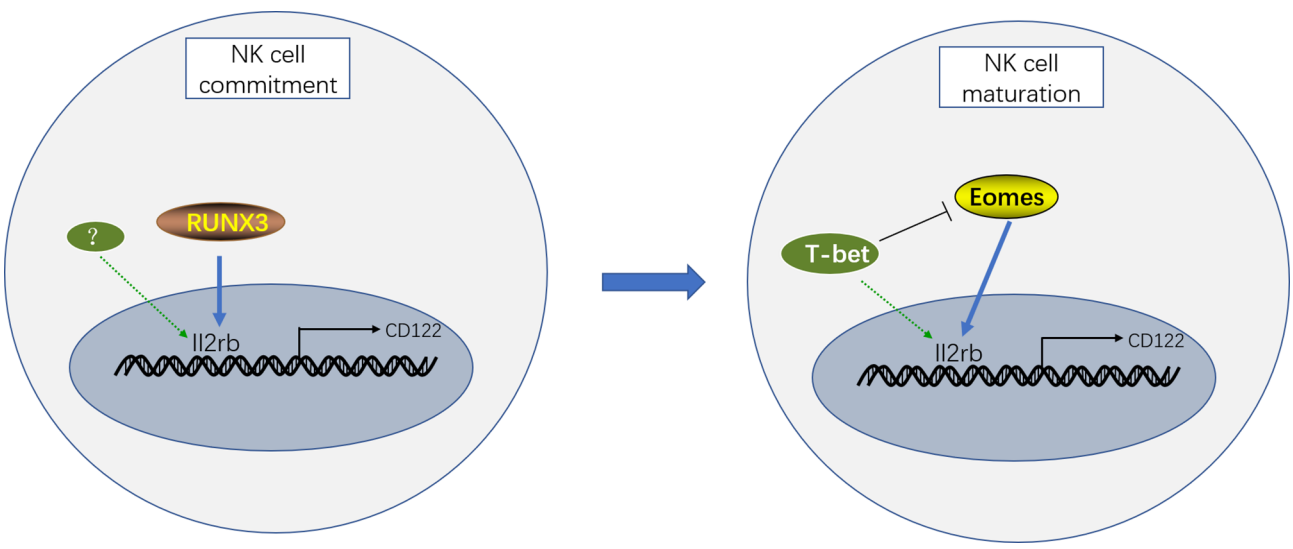

FIGURE 1 | Transcriptional regulation of CD122 expression during different stages of NK cell development. RUNX3 coordinates with T-bet and Eomes to control CD122 expression during NK cell development. Therein, the induction of CD122 expression during early NK cell commitment is determined by RUNX3 and other unknown transcription factors. Moreover, both T-bet and Eomes contribute to CD122 expression during NK cell maturation. However, Eomes but not T-bet plays a predominant role in the maintenance of CD122 expression to promote NK cell maturation.

coding sequence and mature protein all contribute to impede translation (41–44). Surprisingly, a 250-fold increase in IL-15 expression is observed after the removal of those three predominant restraints, further demonstrating the contribution of multiple post-transcriptional mechanisms in limiting IL-15 translation (42). Additionally, there are two isoforms of IL-15 mRNA, differing in their signal peptide, which result in distinct intracellular trafficking, localization, and secretion patterns (44–46). Both isoforms produce mature IL-15 protein. IL-15 with LSP is primarily located in the Golgi apparatus, early endosomes, and endoplasmic reticulum and functions as a secretory signal peptide, whereas IL-15 with a short signal peptide (SSP) (21 amino acid) is not secreted, appears to reside in the nucleus and cytoplasmic components (44). Tight regulation of IL-15 expression is important because of the potent capacity of IL-15 to promote inflammation.

The Production of IL-15 by Hematopoietic and Non-Hematopoietic Cells

Due to the extremely low level of IL-15 protein expression at steady state, even after stimulation, IL-15 is barely detectable by antibodies. However, the establishment of an IL-15 reporter mouse line allows IL-15-producing cells to be visualized by flow cytometry or fluorescence microscopy as well as immunohistochemistry *in vivo* (47–49). Among hematopoietic cells, IL-15 is predominantly produced by monocytes, macrophages, DCs, myeloid cells, and some early hematopoietic cells (**Table 1**) (4, 47). Therein, $CD8^+$conventional DCs are the major DC subsets responsible for IL-15 expression rather than plasmacytoid DCs (47, 48). Moreover, myeloid cells, including neutrophils, basophils, and eosinophils, express high levels of IL-15 *in vivo*, whereas lymphoid lineages, such as T cells, B cells, NKT cells, and NK cells, express minimal to undetectable IL-15 levels. Interestingly, LSK cells ($Lineage^-Sca\text{-}1^+c\text{-}kit^+$), which constitute a heterogeneous population of both long-term and short-term HSCs in BM, uniformly express high levels of IL-15.

Among nonhematopoietic cells, a distinct category of stromal cells together with epithelial cells directs IL-15 expression in primary and secondary lymphoid organs (**Table 1**) (49). In BM, IL-15 is predominantly expressed by $VCAM1^+PDGFR\beta^+CD31^-Sca\text{-}1^-$ mesenchymal stromal cells, which correspond to a distinct subset of CXC chemokine ligand-12 (CXCL12)-abundant reticular (CAR) cells and may function as a developmental niche for NK cells (50, 51). In the thymus, IL-15 is highly expressed in the thymic medulla and medullary thymic epithelial cells with high MHC class II expression, providing a major source of IL-15. In the lymph nodes, IL-15-expressing cells, which include some fibroblastic reticular cells (FRCs) and $gp38^-CD31^-$ stromal cells, primarily reside in the T-cell zone and medulla. In addition, in the lymph nodes, blood endothelial cells (BECs) also express high IL-15 levels. In the spleen, $VCAM\text{-}1^+$ stromal cells are responsible for IL-15 expression.

In contrast to the low expression of IL-15 at steady state, its expression capacity is further strengthened by several inflammatory stimuli, including Toll-like receptor (TLR) ligands and cytokines (47, 52–54). Previously, studies have proven that bacterial lipopolysaccharide (LPS) or the double-stranded RNA mimic Poly I:C initiates TLR signaling to induce IL-15 expression (55). Similarly, IL-15 induction is interferon (IFN)-α receptor (IFNAR)-dependent after viral infection (47). Although IL-15 mRNA is elevated in all DC subsets after inflammatory stimuli, only CD8α+ DCs upregulated IL-15 protein expression, further specifying a DC subset for IL-15 production (47, 55, 56). Moreover, it has been demonstrated that upregulated IL-15 expression also exists in monocytes, macrophages, and tumor-associated neutrophils in inflammatory environments (57–59). In addition, LPS-induced inflammation also greatly increases IL-15 expression in stromal cells, including BECs and lymphatic endothelial cells (LECs), whereas this effect is not significant in other stromal cells (49).

Both Hematopoietic and Non-Hematopoietic Cells Promote NK Cell Development by Producing IL-15

The diverse subsets of IL-15-expressing cells play different but overlapping roles in the development of NK cells in BM and peripherally by producing and transpresenting IL-15 (**Figure 2**). Overall, hematopoietic cells were found to override the importance of non-hematopoietic cells in promoting NK cell development (10, 11). Correspondingly, restricting IL-15Rα or IL-15 expression to hematopoietic cells completely recovered NK cell development at all stages in BM with a slight defect in peripheral mature NK cells, whereas the development of NK cells was only partially rescued in all tissues when IL-15Rα or IL-15 expression was specifically limited to non-hematopoietic cells.

As a critical component of hematopoietic cells, monocytes, DCs, and macrophages contribute to NK cell development by producing IL-15. The indispensable role of monocytes in NK cell development and homeostasis was exemplified by the observation that the interaction between NK cells and spleen monocytes promotes $CD11b^+CD27^+$ NK cell differentiation into $CD11b^+CD27^-$ NK cell in an IL-15Rα- and IL-15-dependent and cell–cell contact-dependent manner (60). Consistently,

TABLE 1 | The production of IL-15 by hematopoietic cells and non-hematopoietic cells.

Hematopoietic cells	Non-hematopoietic cells
Monocytes	In the BM: CXCL12-abundant reticular (CAR) cells
Macrophages	In the thymus: thymic medulla and medullary thymic epithelial cells with high MHC class II expression
DCs: $CD8^+$conventional DCs	In the lymph nodes: fibroblastic reticular cells (FRCs), $gp38^-CD31^-$ stromal cells and blood endothelial cells (BECs)
Myeloid cells: neutrophils, basophils and eosinophils	In the spleen: $VCAM\text{-}1^+$ stromal cells
Early hematopoietic cells: LSK cells	

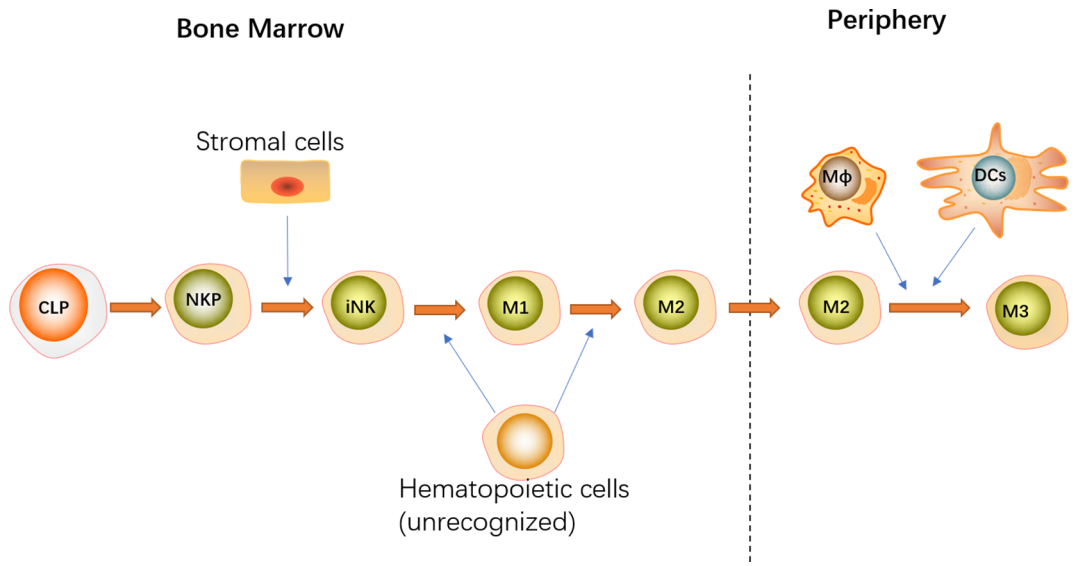

FIGURE 2 | NK cell commitment is represented by the transition from CLPs to NKPs that acquire the most representative hallmark CD122. With the expression of NK1.1 and NKp46, immature NK (iNK) cells are originated from NKPs. According to the expression of CD27 and CD11b, NK cell maturation can be distinguished into four stages: $CD11b^{-}CD27^{-}$(iNK) → $CD11b^{-}CD27^{+}$(M1) → $CD11b^{+}CD27^{+}$(M2) → $CD11b^{+}CD27^{-}$(M3). Stromal cells expressing IL-15α are sufficient for the generation of immature NK cells in the BM. Moreover, some unrecognized hematopoietic cells contribute to differentiate into M1 and M2 NK cells by supplying IL-15. After migration from BM to the periphery, DCs and macrophages transpresent IL-15 for M2 to promote NK cell terminal maturation. Both hematopoietic and non-hematopoietic cells promote NK cell development by producing IL-15 at different stages.

immunobiological studies revealed that monocytes and NK cells reside in close proximity of the red pulp of the spleen (60). Additionally, although IL-15Rα expression on DCs and macrophages is dispensable for NK cell differentiation in BM, it is required for the maintenance of mature NK cells in the periphery given that specific knockdown of IL-15Rα on DCs or macrophages results in a substantial reduction in NK cells in the periphery (61). Moreover, NK cell homeostasis is not exacerbated when IL-15Rα is conditionally deleted from both DCs and macrophages, indicating that DCs and macrophages maintain NK cell populations in the peripheral blood and organs in a similar manner (61). Furthermore, mice with conditional deletion of IL-15Rα in DCs or macrophages exhibit significant deficits in terminally differentiated $CD27^{-}CD11b^{+}$ NK cells, although the subsets of peripheral $CD27^{+}CD11b^{-}$ and $CD27^{+}CD11b^{+}$ NK cells remain intact. Thus, DCs and macrophages were dispensable for NK cell development in the BM and necessary for NK cells' terminal differentiation in the periphery. However, using CD11c/IL-15Rα Tg mice with an IL-15R$\alpha^{-/-}$ background, Castillo et al. revealed that DCs contribute to the development of NK cells in both the BM and peripheral blood and organs. Mice that exclusively expressed IL-15Rα on DCs exhibited partial recovery of NK cells in all tissues, and the greatest reconstitution was noted in the BM (10). Furthermore, IL-15 exclusive transpresentation *via* DCs is insufficient for the maturation of $CD27^{-}CD11b^{+}$ NK cells, which preferentially reside in the peripheral blood and organs. These discrepancies in the function of DCs during NK cell development may be attributed to divergent models. Nonetheless, transpresentation of IL-15 by DCs and macrophages is not responsible for the all IL-15 events attributed to IL-15Rα+ hematopoietic cells, as NK cell deficiency in the BM of mice with IL-15Rα deletion in DCs or macrophages is less apparent than that observed in IL-15Rα-deficient mice (10, 61). Therefore, besides DCs and macrophages, other unrecognized hematopoietic cells in the BM that contribute to NK cell development have not been identified.

Moreover, IL-15 expression by non-hematopoietic cells is more important for NK cell development in BM other than in the periphery, as limiting IL-15Rα expression to non-hematopoietic cells results in more evident NK cell recovery in BM, and this effect is virtually non-existent in the spleen or liver (10). Non-hematopoietic cells expressing IL-15α are sufficient for the generation of immature NK cells but are incapable of NK cell maturation (10). This finding may be attributed to the high expression of IL-15 in CXCL12-abundant reticular (CAR) cells, which are in close contact with NK cells in BM (49–51). In addition to transducing the downstream signaling of CXC chemokine receptor (CXCR4), the engagement of CXCL-12 on CAR cells *via* CXCR4 expressed by NK cells also contributes to NK cell retention in BM, which provides a special IL-15-sufficient niche for NK cell development. *In vivo* and *in vitro* studies demonstrated that the CXCL-12/CXCR4 axis is essential for NK cell maturation and proliferation (50, 51). However, the exact role of IL-15 expression in CAR cells is unidentified. Additionally, consistent with the high expression of IL-15 in fibroblastic reticular cells (FRCs) of lymphoid nodes, the specific ablation of IL-15 in FRCs results in almost complete abrogation

of NK cells in Peyer's patches (PPs) and gut-associated secondary lymphoid organs (SLOs), indicating that FRCs promote NK cell homeostasis *via* the establishment of an IL-15-dependent niche (62).

Furthermore, human spleen-derived fibroblasts are sufficient for the development of functional $CD56^{bright}CD3^{-}$ NK cells *in vitro*, and neutralizing IL-15 signaling or disturbing direct contact significantly abrogates $CD56^{dim}CD3^{-}$ NK cell generation, indicating that fibroblasts express and transpresent IL-15 to support NK cell development (63). However, no *in vivo* studies have demonstrated the role of fibroblasts in NK cell development. In conclusion, although previous studies have uncovered the distinct function of DCs and macrophages in NK cell development, the exact biological role of IL-15 expression in other hematopoietic cells (myeloid cells and early HSCs) and diverse stromal cells that reside in the BM or peripheral blood and organs during NK cell development remains poorly described.

HOW DOES IL-15 TRANSPRESENTATION SUPPORT NK CELL DEVELOPMENT?

Although IL-15 is critical for NK cell development, IL-15 alone only weakly activates its downstream signaling. In fact, the exertion of IL-15 function is dependent on IL-15Rα, which has high affinity to IL-15 (64). With the aid of IL-15Rα, IL-15 is protected from degradation, accumulates on the membrane and in the circulation of mice, and exhibits increased biological activity (65). Accordingly, IL-15-expressing cells must simultaneously express IL-15Rα to supply IL-15 to IL-15-responsive NK cells bearing IL-15Rβ and γc (66, 67). The distinct requirement is further unveiled by the discovery that IL-15 is preassembled with IL-15Rα in a complex in the endoplasmic reticulum/Golgi and subsequently shuttled to the cell surface (8, 68). This cell surface complex is called membrane-associated IL-15-IL-15Rα complex (mIL-15 complex) (**Figure 3**).

Nonetheless, the mIL-15 complex could be cleaved from the surface to form soluble IL-15–IL-15Rα complex (sIL-15 complex) in response to several immune stimuli, including type I interferons (type I IFNs), Poly I:C stimulation, total body irradiation (TBI), Toll-like receptor (TLR) stimulation, virus infections, and activation of the stimulator of IFN genes (STING) pathway (57, 69, 70). It is reported that this process is mediated by A Disintegrin and Metalloprotease (ADAM) 17 protease, whose expression is upregulated on the surface of IL-15 expressing cells after immune stimulus (69). Consistently, *in vivo* evidence demonstrated that the IL-15–IL-15Rα complex exists in two forms, mIL-15 complex and sIL-15 complex, in humans and mice (65, 70). Although the sIL-15 complex was identified several years ago, its biological significance remains controversial. Interestingly, *in vivo* studies revealed that the sIL-15 complex serves as a potent agonist and is approximately 50–100 times more potent at promoting NK cell proliferation than recombinant IL-15 alone (71, 72). Thus, the sIL-15 complex may play an important role in stimulating IL-15 responses. Consistently, Anton et al. (73) demonstrated that low doses of

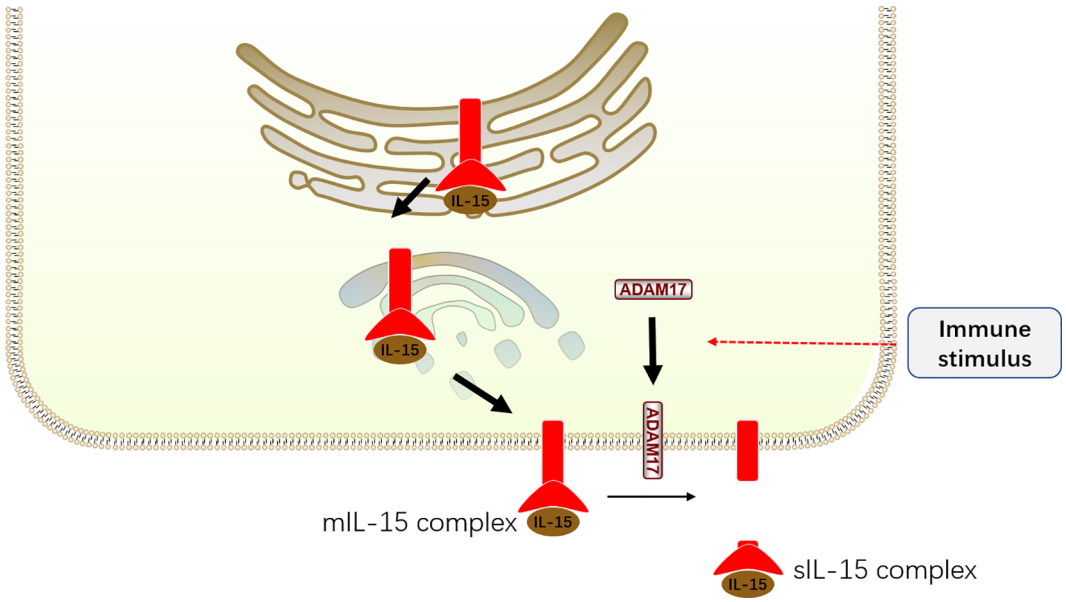

FIGURE 3 | The production of mIL-15 complex and sIL-15 complex. IL-15 preassembles with IL-15Rα in a complex in the endoplasmic reticulum/Golgi and subsequently shuttled to the cell surface, becoming membrane-associated IL-15–IL-15Rα complex (mIL-15 complex). After immune stimulating, the ADAM17 translocates to the cell surface with increased activity. The mIL-15 is cleaved by ADAM17 at the ectodomain of IL-15Rα to induce the formation of soluble IL-15–IL-15Rα complex (sIL-15 complex).

sIL-15 contribute to the phosphorylation of Stat5 in NK cells, whereas higher concentrations of sIL-15 are required to stimulate S6 phosphorylation *in vitro*. However, other studies discovered that the mIL-15 complex mediates NK cell activation rather than the sIL-15 complex present in the supernatants of IL-15-expressing cells cultured *in vitro* or in the serum of mice *ex vivo* (68, 74). This contradiction may be attributed to the different experimental methods and low concentration of sIL-15 complex in the supernatants and serum. Considering the rare detection of sIL-15 complex at steady states and substantial sIL-15 complex produced after immune stimulation, we hypothesize that the sIL-15 complex mediates IL-15 responses during immune activation but not during steady states. However, due to the technological limitation, it is hard to distinguish IL-15 responses mediated by sIL-15 complex from mIL-15 complex.

In contrast to the sIL-15 complex, which functions independently of cell–cell interactions, the mIL-15 complex functions through a distinct delivery mechanism termed transpresentation during cell–cell contact to transduce IL-15 signaling to NK cells *via* the IL-12/IL-15Rβ and γc complex (8, 64). Consistently, although IL-15Rα knockout mice exhibit dramatic defects in NK cell development (15), the specific deletion of IL-15Rα in NK cells has no detrimental effect on NK cell development. However, adoptive transfer of normal NK cells into IL-15Rα-deficient mice results in the abrupt loss of these cells, indicating that IL-15Rα expressed by non-NK cells but not NK cells is required to mediate IL-15 signaling for NK cell development (75, 76).

During transpresentation, the IL-15Rα–IL-15 complex functions through three different mechanisms to transduce IL-15 signaling in NK cells (73, 77) (**Figure 4**). First, presenting cells can directly interact with NK cells *via* the formation of an immunologic synapse where the membrane-associated IL-15Rα–IL-15 complex on presenting cells interacts with the IL-15Rβ-γc receptor at the plasma membrane of NK cells to transduce IL-15 signaling. Consistently, the mIL-15 complex expressed by DCs accumulates at the synapse with NK cells, and the use of an antibody to block IL-15Rα promotes NK cell apoptosis and significantly reduces NK cell survival (78). In addition to the IL-15/IL-15Rα-β/γc interaction, many other receptor–ligand interactions may simultaneously occur at NK cell immunologic synapses, such as interactions between activating receptors or inhibitory receptors and their ligands, separately (78, 79). Interestingly, using a confocal microscopy assay, the mIL-15 complex accumulated in the periphery of activating synapses, whereas the mIL-15 complex was evenly distributed along the entire contact area when the NK cell line made contact with IL-15-expressing cells (79). Nonetheless, the

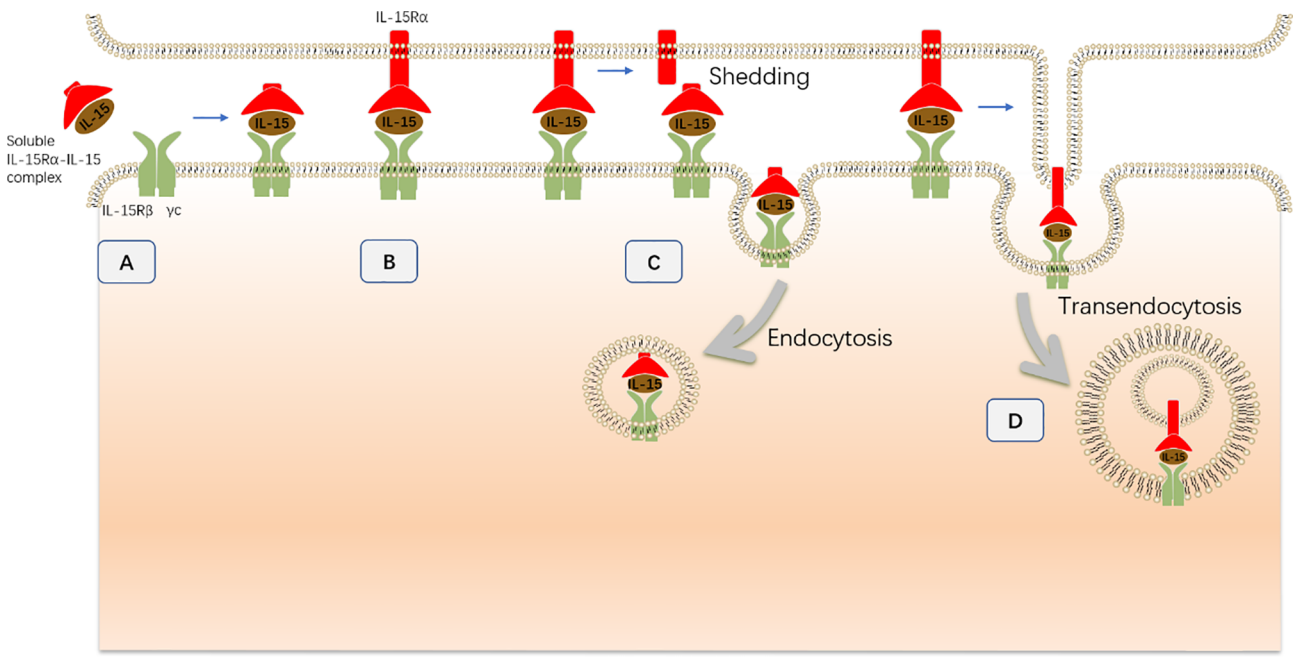

FIGURE 4 | The transpresentation of IL-15 to NK cells during NK cell development. **(A)** The sIL-15 complex (soluble IL-15–IL-15Rα complex), which exists as a soluble extracellular complex in serum, directly interacts with NK cells that express IL-15Rβ–γc chains without transpresentation by presenting cells. **(B)** Presenting cells directly interact with NK cells *via* the formation of an immunologic synapse. Then, the mIL-15 complex (membrane-associated IL-15–IL-15Rα complex) on presenting cells interacts with the IL-15Rβ–γc receptor at the plasma membrane of NK cells to transduce IL-15 signaling. **(C)** During synapse formation, the mIL-15 complex is cleaved from the plasma membrane and then endocytosed by NK cells. The internalized mIL-15 complex co-localizes with the IL-15Rβ–γc receptor in the cytoplasm and contributes to IL-15 signaling activation. **(D)** The intact mIL-15 complex together with the plasma membrane of presenting cells is internalized into NK cells through a distinct process termed transendocytosis without being cleaved. Subsequently, the transendocytosed IL-15Rα–IL-15 complex colocalizes with the IL-15Rβ/γc chain in intracellular NK cell compartments and contributes to IL-15 signaling activation.

regulatory role of these receptor–ligand interactions in IL-15 signaling remains elusive. *In vitro* studies have demonstrated that the interaction between inhibitory receptors, such as KIR2DL1, KIR2DL2/3, or CD94-NKG2A, and their cognate ligands selectively inhibited the phosphorylation of AKT and S6 but not Stat5, and this effect was concomitant with reduced proliferation induced by the mIL-15 complex but not the sIL-15 complex (79).

During cell-to-cell contact, the membrane-bound IL-15Rα–IL-15 complex is internalized by NK cells and contributes to the activation of IL-15 signaling (77). This process is dependent on the proteolytic cleavage of IL-15Rα, which allows the IL-15Rα–IL-15 complex to separate from the presenting cells. In addition, the IL-15Rα–IL-15 complex gradually accumulates in NK cells during the interaction between IL-15-presenting cells and NK cells. After separation from the presenting cells, the previously restored IL-15 complex contributes to the survival and residual proliferation of NK cells in a time-limited manner. In contrast, abrogation of IL-15Rα cleavage results in enhanced and prolonged Stat5 phosphorylation concomitant with increased IL-15 expression in the synapse. This observation further demonstrated that the mIL-15 complex on presenting cells also contributes to the activation of IL-15 signaling during cell-to-cell contact (77). Therefore, mIL-15 complex cleavage and internalization could represent a negative regulatory mechanism that reduces the availability of transpresented-IL-15 and protects NK cell from excessive IL-15 signaling.

However, inhibition of IL-15Rα cleavage did not completely abrogate the entry of the IL-15Rα–IL-15 complex into NK cells, indicating that the IL-15 entry is not exclusively dependent on the shedding of the membrane-associated IL-15Rα–IL-15 complex (77). Indeed, the intact membrane-associated IL-15Rα–IL-15 complex from the presenting cells together with the plasma membrane of presenting cells is internalized into NK cells through a distinct process termed transendocytosis without being cleaved (73). Subsequently, the transendocytosed IL-15Rα–IL-15 complex colocalizes with the IL-15Rβ/γc chain in intracellular NK cell compartments to promote ribosomal protein S6 phosphorylation and NK cell proliferation. Consistently, interference of transendocytosis by silencing the small GTPase TC21, which is a critical component of transendocytosis, substantially inhibits S6 phosphorylation but not Stat5 phosphorylation in NK cells.

WHAT IS THE DOWNSTREAM TARGET OF IL-15 SIGNALING DURING NK CELL DEVELOPMENT?

IL-15-JAK-STAT5 Signaling for NK Cell Development

Upon the engagement of the IL-15Rα–IL-15 complex with the IL-15Rβ/γc receptor, three distinct signaling pathways, including Ras–MEK–MAPK, JAK–STAT5 and PI3K–ATK–mTOR are activated and contribute to NK cell development. The IL-15Rα–IL-15 complex primarily induces the activation of the JAK–STAT5 pathway *via* recruiting Janus kinase 1 (JAK1) and JAK3 (**Figure 5**). Interestingly, JAK1 binding to the IL-2/IL-15Rβ and JAK3 combining with γc is crucial for signal transduction by activating JAK1 and JAK3, which induce the phosphorylation of tyrosine residues in IL-2/IL-15 Rβ (80–82). This model has been further confirmed by the discovery that humans with deletion of JAK3 exhibited similar phenotypes of severe combined immunodeficiency (SCID) as γc-deficient patients (83). Although the specific function of JAK1 and JAK3 varies considerably, genetically engineered mice provide the possibility to determine the distinct roles of individual proteins. While deficiency of Jak1 in mice leads to perinatal lethality (84), a remarkable decrease of immature B220+ NK cells was observed in adult mice with inducible loss of Jak1, indicating that Jak1 is essential for NK cell development (85). These observations were recently validated in mice with conditional deletion of Jak1 in Ncr1-expressing cells ($Jak1^{fl/fl}$ Ncr1Cre), displaying blockade of NK cell development at the NKp and iNK stages in a dose-dependent manner (86). Not surprisingly, JAK3 also plays an important role in NK cell development, coinciding with the finding that Jak3-deficient mice suffer from differentiation block of NK cells at the pre-NKP stage (87). Despite the cooperation of Jak1 and Jak3 in NK cell development, accumulating evidence has proposed that Jak1 plays a dominant role overriding Jak3 during the signal transduction (88, 89). The specific inactivity of Jak3 in human cells lines fails to attenuate STAT5 phosphorylation as anticipated, as two Jak kinases have an equivalent function in signal transduction, whereas remarkable abrogated downstream signaling was found in Jak1-inactive cells lines (88). Furthermore, the knockdown experiments suggested that Jak1 is responsible for the phosphorylation of Jak3 and STAT5 after cytokine receptor activation, and Jak3 contributes to enhance Jak1 activity by phosphorylating it. Likewise, quantitative mass spectrometry analysis also revealed that Jak1 is more important that Jak3 in mature NK cells (90). Although, many studies have addressed the vital roles of Jak1 and Jak3 in signal transduction, molecular interactions between the two Jak kinases and their individual contributions in NK cells remain to be determined.

Although it has long been believed that IL-15 signaling is exclusively mediated by JAK1/3, the role of JAK2 in IL-15 signaling is controversial. Notably, a recent study described that JAK2 phosphorylates STAT5 downstream of IL-15 during NK cell differentiation *in vitro* (91). Accordingly, mice with conditional deletion of Jak2 in HSC exhibited impaired NK cell maturation (92). However, it has been shown that JAK2 is intrinsically dispensable for NK cell development as mice with conditional deletion of JAK2 in NKp46+ cells exhibited intact NK cell numbers and maturation (86). These discrepancies indicate that the absence of JAK2 may extrinsically interfere in NK cell maturation by altering the cytokine milieu.

The discovery of the IL-15-JAK association has contributed to the finding that members of the signal transducer and activation of transcription (STAT) family directly bind to phosphor-tyrosine docking site(s) in the IL-2/IL-15Rβ chain and are then

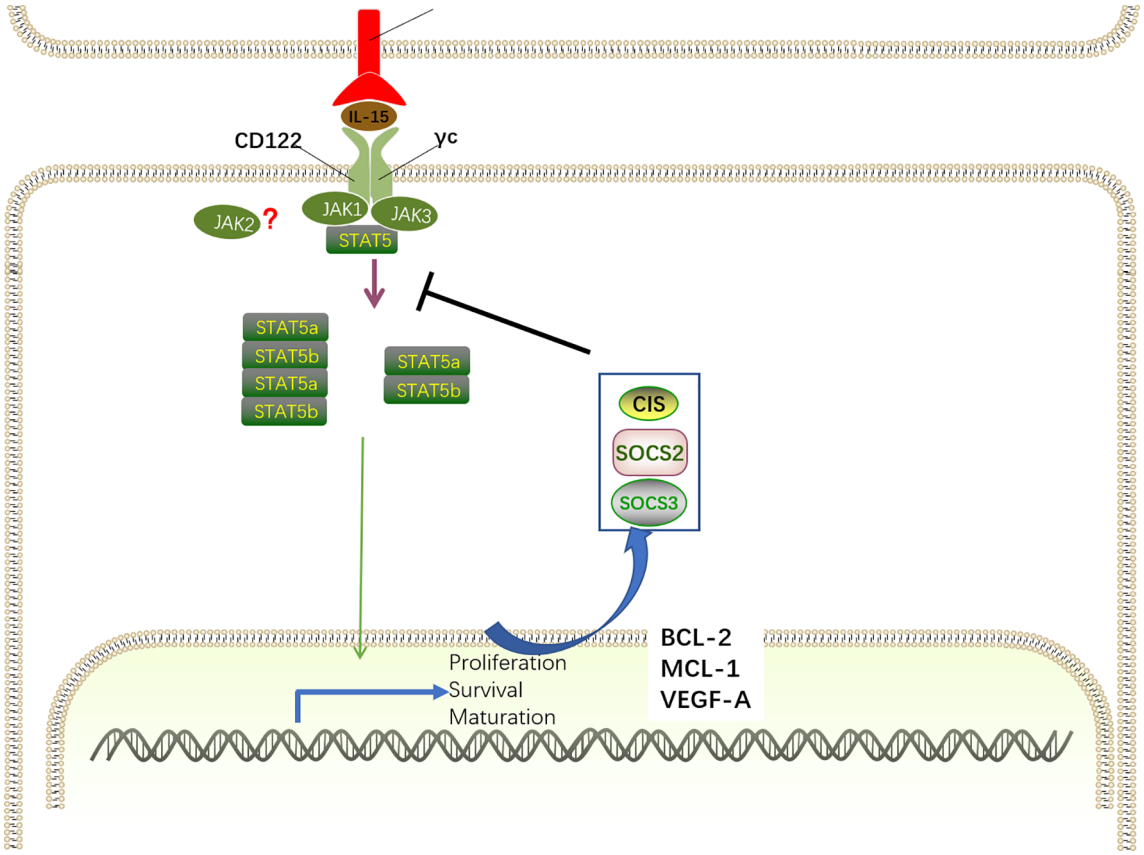

FIGURE 5 | IL-15-JAK-STAT5 signaling for NK cell development. The IL-15Rα–IL-15 complex primarily induces the activation of the JAK–STAT5 pathway *via* recruiting JAK1 and JAK3. For signal transduction, JAK1 binds to the IL-2/IL-15Rβ and JAK3 combines with γc, inducing the recruitment and phosphorylation of STAT5. By oligomerizing into dimers and tetramers, phosphorylated STAT5a and STAT5b translocate into the nucleus to drive the expression of STAT-target genes encoding proteins related with NK cell development, survival, proliferation, and function, including MCL-1, BCL-2, and VEGF-A. Specifically, IL-15–JAK–STAT5 signaling also promotes the transcription of SOCS family members. SOCS proteins comprise a negative feedback loop to retrain the JAK–STAT5-mediated pathway in NK cell development.

phosphorylated by JAK1 and JAK3 on their tyrosine residues (80). Similar with IL-2, IL-15 predominantly induces STAT5 activation, despite the finding that STAT3 and STAT1 can also be activated to a lesser extent (93). STAT5 is comprised by two distinct transcription factors, STAT5a and STAT5b, that have a remarkable degree of sequence homology (approximately 96%) (94). By oligomerizing into dimers and tetramers, phosphorylated STAT5a and STAT5b translocate into the nucleus to drive the expression of STAT-target genes, which is critical for NK cell development, survival, proliferation, and function (95–97). STAT5 dimers preferentially bind to γ-interferon-activated sequence (GAS) motifs, whereas STAT5 tetramers are more flexible given the capacity for various non-consensus GAS motifs (98). Interestingly, Lin and colleagues revealed that STAT5 dimers are sufficient for early NK cell development, proliferation and cytotoxic capacity, whereas STAT5 tetramers are necessary for NK cell maturation and survival through the induction of the anti-apoptotic protein Bcl2 (99).

It is indisputable that STAT5-related transcriptional programs mediated by IL-15 activation are essential for the biological functions of IL-15. The indispensable role of STAT5-mediated transcriptional regulation in NK cell development has been highlighted by the finding that NK cell differentiation was abrogated at the NKp stage in Ncr1-iCreTg mice with conditionally deleted STAT5 (100). Consistently, disrupted NK cell maturation and impaired lytic function were observed in humans with STAT5b mutations (101). Therefore, STAT5 downstream of Jak kinases is essential for transducing IL-15 signaling. Despite the largely redundant functions of STAT5a and STAT5b, their distinct roles have been verified in single knockout mice for STAT5a or STAT5b (102, 103). Deficiency of STAT5b results in more dramatic defects in NK cell development than deletion of STAT5a, indicating that STAT5b plays a dominant role in NK cell development (104, 105). Consistently, transcriptional analysis revealed that the transcripts mediated by STAT5b are more abundant (104). Furthermore, only Stat5b knockout mice exhibit elevated

transcription of VEGFA, an angiogenic factor that is transcriptionally repressed by STAT5.

Moreover, chromatin immunoprecipitation (ChIP) analysis of STAT5 binding sites revealed that STAT5 directly targets a large number of genes encoding proteins related with NK cell development and function, including ID2, EOMES, T-BET, perforin, granzymes, and IFN-γ. Additionally, STAT5 can also bind to Mcl-1 and Bcl-2, correlating with the ability of IL-15 to induce the expression of these genes and sustain NK cell survival (97, 106). Overexpression of BCL-2 enables the survival of STAT5-deficient NK cells but has no influence on proliferation, maturation, or effector functions. However, it seems that Mcl1 is more important in promoting NK cell survival than Bcl-2, as IL-15 stimulation maintains NK cell survival when Bcl-2 was inhibited but not when Mcl1 was inactivated (96).

However, STAT5 is not only correlated with transcriptional activation of gene expression, as the repressive effect of STAT5 binding is also present in NK cells. STAT5 has been shown to bind the Vegf-a gene promoter in NK cells, correlating with suppressed expression of the pro-angiogenic factor VEGF-A in mice and humans (106). *In vitro* studies revealed that STAT5-inactive NK cells showed abundant VEGFA expression, and this effect was also confirmed *in vivo* by increased tumor formation in the absence of STAT5 (106). In line with the observations in mice, tumor-infiltrating NK cells with VEFGA secretion properties promote tumor progression and are associated with poor outcomes in patients (107–109). According to the repressive effects of STAT5 on IL-17 and Bcl6 mRNA expression in T cells (110, 111), further research is essential to deepen our understanding of the distinct roles of STAT5 in NK cells.

IL-15 signaling contributes to the induction of suppressor of cytokine signaling (SOCS) family members, including cytokine inducible SH2-containing protein (CIS), SOCS2, and SOCS3, which comprise a negative feedback loop to retrain the IL-15–JAK–STAT5-mediated pathway in NK cell development (90, 112). Several studies have demonstrated that STAT5 directly targets the genes of these SOCS proteins (99, 113). SH2-containing protein (CIS, encoded by Cish gene) directly interacts with JAK1 to mediate the inhibition of its enzymatic activity and proteasomal degradation, thereby constraining JAK–STAT5 signaling. Consistently, mice with CIS ablation exhibit accumulation of terminally differentiated $CD27^{-}CD11b^{+}$ NK cells in the BM and spleen, which is associated with the hyper-responsive nature of NK cells to IL-15 (114). By directly interacting with JAK2, SOCS2 attenuated JAK2 activity and the corresponding JAK2–STAT5 signaling to negatively regulate NK cell differentiation (91). Increased NK cell differentiation has been observed in the absence of SOCS2 *in vivo* and *in vitro*, whereas the development advantage is reversed after the addition of a JAK2 inhibitor *in vitro*. In contrast to its effect on murine NK cells, SOCS2 has no influence on IL-15-mediated human NK cell differentiation *in vitro* but is essential for human NK cell effector function *via* the regulation of phosphorylated proline-rich tyrosine kinase 2 (Pyk2) (112). These discrepancies may be attributed to different protocols for mouse and human NK cell development *in vitro* or species differences. Although knockdown of SOCS3 in mice has no impact on NK cell development and maturation (90), a recent study revealed that SOCS3 suppressed IL-15-mediated STAT5 phosphorylation, correlating with the desensitization of NK cells to IL-15 simulation, resulting in disrupted NK cell terminal differentiation (115).

IL-15–PI3K–AKT–mTOR Signaling for NK Cell Development

The interaction of IL-15 with its receptor on NK cells also activates the canonical downstream PI3K–AKT–mTOR pathway (**Figure 6**). Phosphoinositide 3-kinases (PI3Ks) are comprised of three subclasses, including class I, class II, and class III (116). The class I PI3Ks, which predominantly transduce signaling triggered by cytokine receptor, are heterodimeric enzymes that include a regulatory subunit (p85α, p50α, p55α, p85β, p55γ, and p101) and a catalytic subunit (p110α, p110β, p110γ, and p110δ). Mice exclusively or simultaneously lacking the PI3K subunits P110 $\gamma$and δ exhibit severely defective NK cell maturation and total numbers (116–119). Consistently, p110 δ mutations in patients impair the development and cytotoxic function of NK cells, leading to severe viremia, whereas rapamycin treatment partially rescues defective NK cells (117). Despite the multiple membranes of PI3Ks, it is unknown which subtypes are required for IL-15 signaling in NK cell development.

PI3K phosphorylates the three positions of the inositol ring of plasma membrane-associated phosphatidylinositol-4,5-bisphosphate [PI(4,5)P2] to generate PI(3,4,5)P3, which interacts with proteins containing pleckstrin homology (PH) domains, including the serine/threonine kinases phosphoinositide-dependent kinase (PDK1) and protein kinase B (PKB; also known as AKT), and localizes these proteins to membranes (120). The interaction between PI(3,4,5)P3 and AKT initiates conformational changes in AKT, allowing PDK1 to phosphorylate AKT at threonine 308 for AKT activation (121).

Subsequently, as an important downstream effector of PI3K/AKT signaling, the mammalian target of rapamycin (mTOR) is activated. mTOR, a serine/threonine protein kinase, includes two components, namely, mTOR complex1 (mTORC1) and mTORC2. Genetic studies have revealed that Raptor and Rictor are important components of mTORC1 and mTORC2, respectively, by defining their downstream substrates (122).

It was proposed that the activation of the IL-15R–PI3K–AKT–mTOR signaling cascade is dose-dependent. Specifically, low IL-15 concentrations only activate the phosphorylation of JAK/STAT5 signaling molecules, whereas the PI3K–AKT–mTOR pathway is further activated after exposure to high IL-15 concentrations (123). PI3K–AKT–mTOR signaling primarily regulates proliferation, differentiation, and maturation as well as NK cell effector function (124). The indispensable role of mTOR in controlling NK cell development was validated in mice with a specific deficiency in mTOR in NK cells in which NK cells almost disappeared in the peripheral organs, and the remaining NK cells

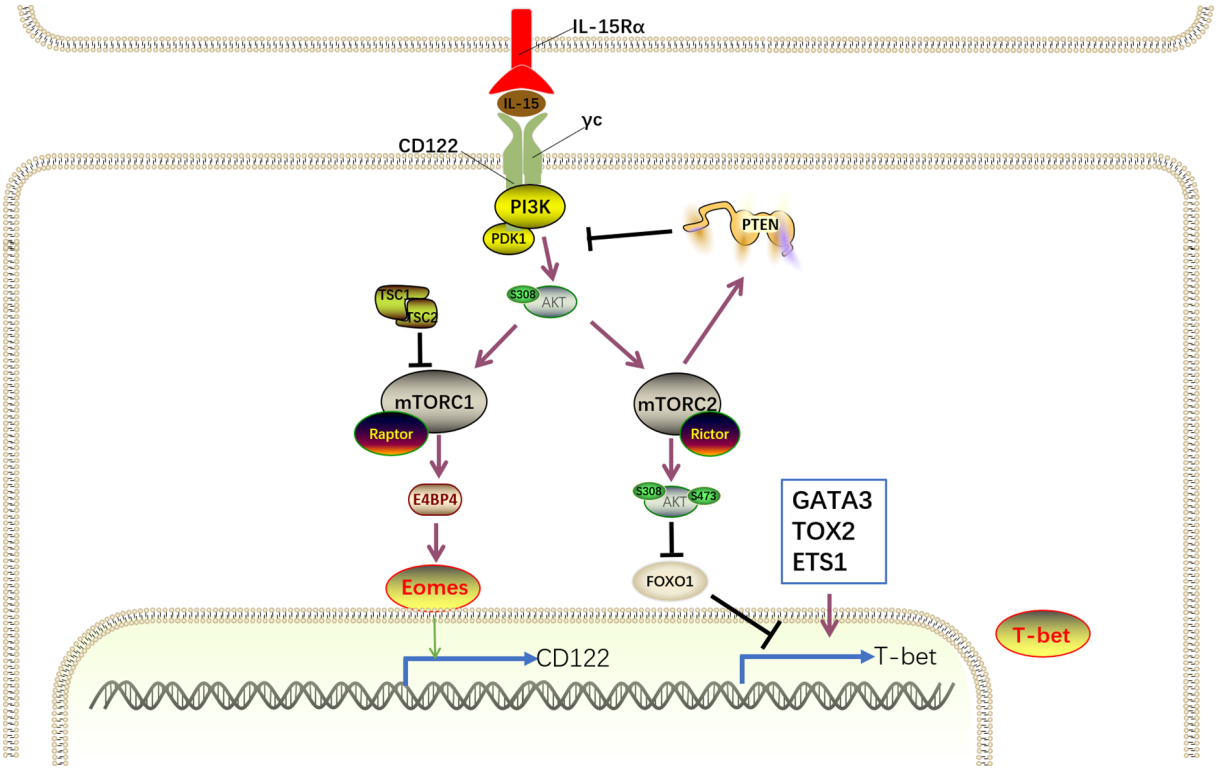

FIGURE 6 | IL-15–PI3K–AKT-mTOR signaling for NK cell development. IL-15 complex interacts with its receptor IL-15Rβ/γc on NK cells to trigger PI3K/AKT pathway autophosphorylation and activation and subsequent activation of mTORC1 and mTORC2. mTORC1 and mTORC2 differentially promote NK cell development in a cooperative and non-redundant manner primarily by divergent induction of corresponding transcription factor Eomes and T-bet. Eomes binds to the il2rb promoter and drives CD122 expression to maintain IL-15 responsiveness, generating a positive feedback loop to amplify the IL-15 signaling. Despite the negative regulation of T-bet expression by FoxO1, several transcription factors, including GATA3, TOX2, and ETS-1, promote T-bet expression. However, the activation of mTOR signaling is tightly modulated by cooperation of TSC1 and PTEN.

in BM were severely blocked at the $CD11b^{-}CD27^{+}$ stage (123). Recent studies have demonstrated that mTORC1 and mTORC2 differentially promote NK cell development in a cooperative and non-redundant manner primarily by divergent induction of corresponding transcription factors, namely, T-bet and Eomes (33, 125). Intriguingly, mTORC1 and mTORC2 also positively or negatively regulate NK cell effector function, respectively. $Ncr1^{iCre}$-mediated ablation of Raptor in mice results in disrupted mTORC1 function, leading to the impaired transition from $CD27^{+}CD11b^{-}$ to $CD27^{+}CD11b^{+}$ NK cells and reduced NK cell function. Conversely, terminal maturation from $CD27^{+}CD11b^{+}$ to $CD27^{-}CD11b^{+}$ NK cells is impeded in mice in the absence of Rictor, which is essential for mTORC2 metabolic signaling. However, Rictor-deficient NK cells display enhanced effector function.

E4 promoter-binding protein 4 (E4BP4), encoded by *Nfil3* (nuclear factor interleukin-3), is the predominant target downstream of mTORC1 (33, 126). Mechanistically, PDK1, a kinase downstream of PI3K, is thought to mediate IL-15-triggered mTORC1 and AKT phosphorylation to drive E4BP4 expression during NK cell development (127). Ectopic expression of E4BP4 rescued NK cell developmental defects in mTORC1-inactiavted and PDK1-deficient mice (126–128). Meanwhile, the absence of PDK1 in NK cells results in attenuated IL-15-triggered mTORC1 activation and significantly decreased E4BP4 expression (127). Moreover, the inactivation of mTORC1 diminishes the IL-15-mediated E4BP4 expression. These results suggest that the IL-15–PI3K–PDK1–mTORC1 signaling pathway is essential for E4BP4 induction. E4BP4 expression is initiated as early as the CLP stage and highly expressed in the iNK and mNK stages. *Nfil3*–/–mice display intact CLP compartment, and the population of NKPs, iNK cells, and mNK cells significantly reduced in the BM, indicating E4BP4 acts as early as CLP stage *via* an IL-15-independent manner and is essential for NK cell commitment. However, $Ncr1^{iCre}$-mediated deletion of *Nfil3* has no effect on NK cell development (129), indicating that Nfil3 is dispensable for NK cell maturation, and other unknown signaling pathways compensate for the absence of Nfil3.

The induction of E4BP4 promotes the expression of Eomes, which binds to the il2rb promoter and drives CD122 expression to maintain IL-15 responsiveness (35). Mice with depletion of

PDK1 or Eomes exhibit significant accumulation of $CD27^+CD11b^-$ NK cells but are devoid of terminally mature $CD27^-CD11b^+$ NK cells, and these findings resemble the findings in Raptor-deficient mice (33, 130, 131). Collectively, the IL-15–PI3K–PDK1–mTORC1–E4BP4–Eomes–CD122 pathway generates a positive feedback loop to amplify IL-15 signaling. However, mTORC1 activation is tightly modulated by Tuberous sclerosis 1 (Tsc1), which exhibits significantly increased expression after long-term IL-15 stimulation and forms a complex with Tsc2 with the aid of AKT (132).

Contrary to the indispensable role of mTORC1 in the early maturation of NK cells, mTORC2 is essential for the terminal maturation of NK cells from the $CD27^+CD11b^+$ to the $CD27^-CD11b^+$ stage. Previous studies have demonstrated that mTORC2 phosphorylates Akt at Serine 473 and augments its kinase activity, leading to the phosphorylation of FoxO1 by Akt (133, 134). Akt-triggered phosphorylation promotes modulator protein to interact with FoxO1, thereby inactivating it by blocking DNA binding and accelerating translocation from the nucleus to the cytosol (33, 135). This model has been further validated in NK cells through the discovery that mTORC2-inactivated NK cells display reduced phosphorylation of Akt^{S473} and FoxO1 (33). In addition, Ingenuity Pathway Analysis (IPA) found a remarkable enrichment of FoxO1 targets in mTORC2-inactivated NK cells. Furthermore, *in vitro* studies reported that IL-15 efficiently induces phosphorylation, and hence inactivation of FoxO1 in developing NK cells, together with the activation of mTOR signaling (136, 137). Based on these results, we speculate that FoxO1 is a direct target of the mTORC2–Akt^{S473} signaling axis that exists downstream of IL-15 signaling in NK cells. In contrast to the high expression of FoxO1 in NKp and iNKs, the level of FoxO1 is significantly decreased in mNKs (136). Apart from FoxO1, FoxO3 is also expressed by NK cells, although it is maintained at relatively low levels throughout NK cell development. Both *Ncr1-Cre -FoxO1*$^{fl/fl}$ and -*FoxO1*$^{fl/fl}$ mice exhibit accumulation of terminally differentiated $CD27^-CD11b^+$ NK cells, indicating FoxO1 and FoxO3 redundantly suppress NK cell maturation (137), contradicting the promoting effects of mTORC2. Owing to the weak expression of FoxO3, it is believed that FoxO1 plays a prominent role in NK cell development. However, in the same *Ncr1-Cre -FoxO1*$^{fl/fl}$ mice, Wang et al. reported a remarkable deficiency of iNK and mNK cells that is attributed to impaired FoxO1-mediated autophagy in iNK cells (136). Therefore, the distinct role of FoxO1 in NK cell development remains to be clearly clarified.

Several studies have demonstrated that the negative regulation of NK cell development by FoxO1 is associated with suppressed T-bet expression (33, 137). In humans, ChIP experiments showed that FoxO1 directly binds to the *Tbx21* promoter, promoting decreased T-bet expression. However, in mice, the recruitment of FoxO1 to the *Tbx21* proximal promoter region by Sp1, which is a FoxO1 protein binding partner, resulted in impaired transactivation of *Tbx21*, leading to disrupted T-bet expression. Consistently, the absence of FoxO1 in NK cells promotes T-bet mRNA and protein expression, whereas T-bet expression is decreased in NK cells with overexpression of FoxO1. In further support of this, Foxo1 and T-bet expression inversely correlate with each other during NK cell maturation. Immature NK cells express high levels of Foxo1, whereas T-bet is present in high amounts in terminally mature NK cells. Furthermore, in contrast to the accelerated maturation of NK cells in $FoxO1^{-/-}$ mice, T-bet deficiency abrogated NK cell terminal maturation (40). Taken together, these results demonstrate that decreased levels of FoxO1 are necessary for NK cell maturation by releasing the negative regulation of T-bet.

Despite the negative regulation of T-bet expression by FoxO1, several transcription factors, including GATA binding protein 3 (GATA3), thymocyte selection-associated HMG box 2 (TOX2) and Ets proto-oncogene 1 (ETS-1), promote T-bet expression (138–141). Thus, inactivation of FoxO1 mediated by IL-15^-PI3K^-mTORC2 signaling coordinated with several transcription factors to promote T-bet expression. T-$bet^{-/-}$ mice exhibited remarkably decreased NK cell populations in the periphery, but the NK cell number was modestly elevated in BM (142). This defect is attributed to the decreased expression of S1P5, which is induced by T-bet and responsible for NK cell egress from BM (143). In the absence of T-bet, NK cell maturation is specifically arrested at $CD27^+CD11b^+$stage, suggesting that T-bet is essential for NK cell terminal maturation (60). It has been proposed that T-bet promotes NK cell maturation by transiently inhibiting Eomes expression (36, 142). Consistent with this, T-bet levels are gradually increased during NK cell maturation, accompanied by the decreased expression of Eomes. T-bet also contributes to the induction of Zinc Finger E-box Binding Homeobox 2 (Zeb2) and B lymphocyte-induced maturation protein 1 (Blimp-1), which are critical for NK cell maturation (36, 40, 144). Thus, the IL-15R–PI3K–mTORC2–AKT–FoxO1–T-bet pathway determines the terminal maturation of NK cells.

In addition, mTORC2 suppresses mTORC1-mediated NK cell effector function by mainly downregulating SLC7A5 expression, which is downstream of STAT5 and regulates mTORC1 activity independent of AKT signaling (125, 145). Therefore, mTORC2 counteracts IL-15-mediated mTORC1 hyperactivation to prevent activation-induced NK cell apoptosis. Inversely, mTORC1 maintains IL-15–CD122–IL-15 signaling to sustain mTORC2 activity.

Phosphatase and tensin homolog (PTEN) directly antagonizes the PI3K–AKT pathway by specifically dephosphorylating PI(3,4,5)P3, which is downstream of PI3K and functions as an activator for downstream signaling proteins, including Vav, Akt, PDK1, and PI(4,5)P2 (146). Consistently, PTEN suppresses PI3K–AKT signaling and MAPK activation in humans, leading to compromised cytotoxic function (147). Conversely, the PTEN signaling pathway is impaired in Rictor-deficient NK cells with an inactive mTORC2 pathway, indicating that mTORC2 promotes PTEN expression to antagonize the PI (3,4,5)P3-mediated activation of mTORC2 (33, 148). Thus, a negative feedback exists between mTORC2 signaling and PTEN expression.

PERSPECTIVES

NK cell development is tightly regulated by the interplay between intracellular transcription factors and extracellular signals, such as cognate ligands, chemokines, and cytokines. Notably, pleiotropic cytokine IL-15 is indispensable for the development of NK cells. Recently, immunotherapy has been applied in anti-cancer and anti-infection treatments. As an important component of immune cell, NK cells have potent cytotoxicity and cytokine production capacity, which allows effective eradication of malignant and infected cells in the absence of graft *versus* host disease (GVHD). Therefore, NK cells are promising for therapeutic utilization. The prerequisite of clinical application is to substantially expand mature NK cells *in vitro*. Correspondingly, understanding of the molecular mechanisms by which IL-15 promotes NK cell development and manipulation of IL-15 for proper NK cell expansion *in vitro* will improve NK cell-based therapeutic strategies (18).

NK cells are the earliest donor-derived lymphocytes recovering after HSCT, whose populations quickly reach donor levels within 1 month (149, 150). The well-established reconstitution of NK cells exhibits a protective effect against leukemia relapse and is associated with improved disease-free survival after HSCT (151–153). Although the level of IL-15 is remarkably high after HSCT, the immature $CD56^{bright}KIR^{-}$NK cells dominate the early reconstruction (149, 150). Investigating the role of IL-15 in NK cell development after HSCT contributes to better prognosis by intervening NK cell maturation.

Given the formidable efficacy in enhancing NK cell development, IL-15 is much more promising than other cytokines in controlling tumor progression and viral infections. Several murine immunotherapy trials have demonstrated that the administration of IL-15 efficiently drove the expansion and activation of NK cells and CD8+T cells *in vivo*, without stimulating the expansion of regulatory T cells which exert an immunosuppressive effect (154–156). However, due to its adverse effects, such as toxicities, hypotension, thrombocytopenia, IL-15 application was constrained (157). More basic research is required to optimize the structure of IL-15 before it can extended to clinical practice.

AUTHOR CONTRIBUTIONS

XW wrote the manuscript. X-YZ outlined the manuscript and made a deep intellectual contribution to the work. All authors contributed to the article and approved the submitted version.

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