IMMUNOLOGICAL CHALLENGES FOLLOWING PEDIATRIC HEMATOPOIETIC TRANSPLANTATION

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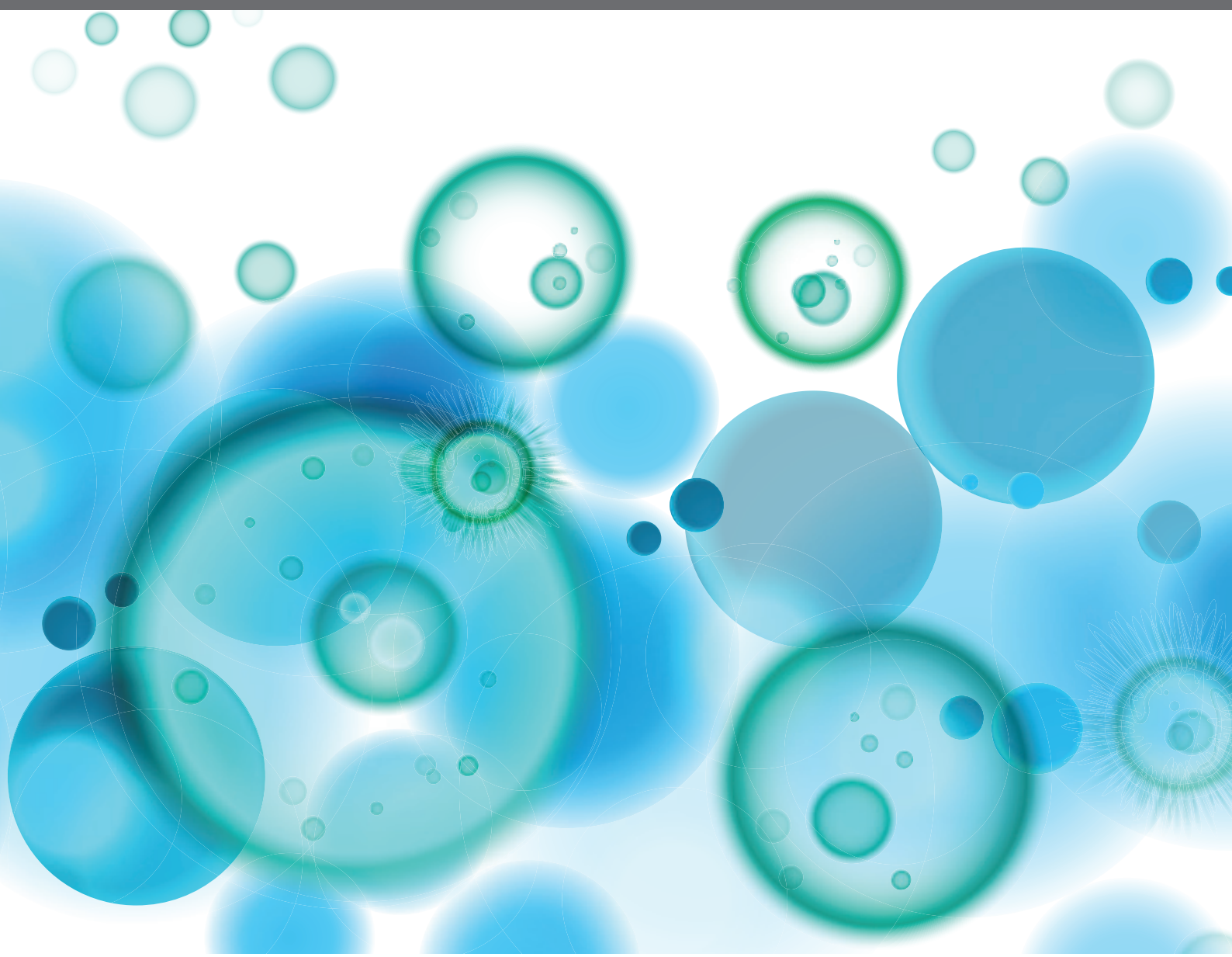

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IMMUNOLOGICAL CHALLENGES FOLLOWING PEDIATRIC HEMATOPOIETIC TRANSPLANTATION

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EDITORIAL
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Editorial: Immunological Challenges Following Pediatric Hematopoietic Transplantation

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Keywords: hematopoietic stem cell transplantation, immunology, pediatrics, non-malignant disease, regulation

Editorial on the Research Topic

Immunological Challenges Following Pediatric Hematopoietic Transplantation

Advancements in hematopoietic stem cell transplantation (HSCT) has made this treatment modality a viable option for hematopoietic based diseases. With the expansion of donor options and the reduced toxicities of preparative regimens, the risk/benefits of HSCT are becoming more and more favorable, making it preferable than to face the complications of a patient's primary disease. Opportunities to improve upon the transplant procedure centrally weighs on improving our understanding of the immune system as most of the primary obstacles for success lie within the immunologic challenges either from the host or the donor. Whether it is overcoming the threats of rejection, the complications of excessive immunosuppression leading to infections, or post-transplant lymphoproliferative disease, (PTLD), or the emergence of immune dysregulation leading to autoimmunity or graft versus host disease, achieving the full potential of this treatment modality rests on our ability to safely eradicate the pre-existing immune system and to establish a competent, regulated one from the donor cells. The pathway to success rests on our ability to sustain 1) Hematopoietic engraftment, 2) Immunologic competence, and 3) Donor cell tolerance (**Figure 1**). Failure to maintain all three will invariably lead to life threatening complications. The collection of manuscripts for this Research Topic spans the full scope of immunological challenges that lay before us, providing insights on what future investigations are needed to overcome them.

Establishing engraftment and minimizing long term toxicity necessitates a thoughtful approach in the selection of the preparative regimen The repertoire of agents for consideration are reviewed in this Research Topic (Hayashi), and although the correct selection invariably differs with the disease of interest, the optimal regimen for most conditions has yet to be defined. Further complicating the issue is the degree of chimerism required to establish a curative outcome for a particular disease. As discussed by Zimmerman and Shenoy, the lack of the necessity for complete donor chimerism to provide efficacy for some diseases gives the clinician flexibility to refine preparative regimens to establish minimum level of donor engraftment to achieve disease control. Challenges remain with our lack of understanding of not only the degree of engraftment needed for each disease but also the variables that ensure stable engraftment in a partial chimera state.

Establishing a new immune system with donor engraftment requires a keen awareness of the essential elements of immune reconstitution along with the vulnerabilities the host experiences to different pathogens at different time points as the new immune system is generated. The elements of

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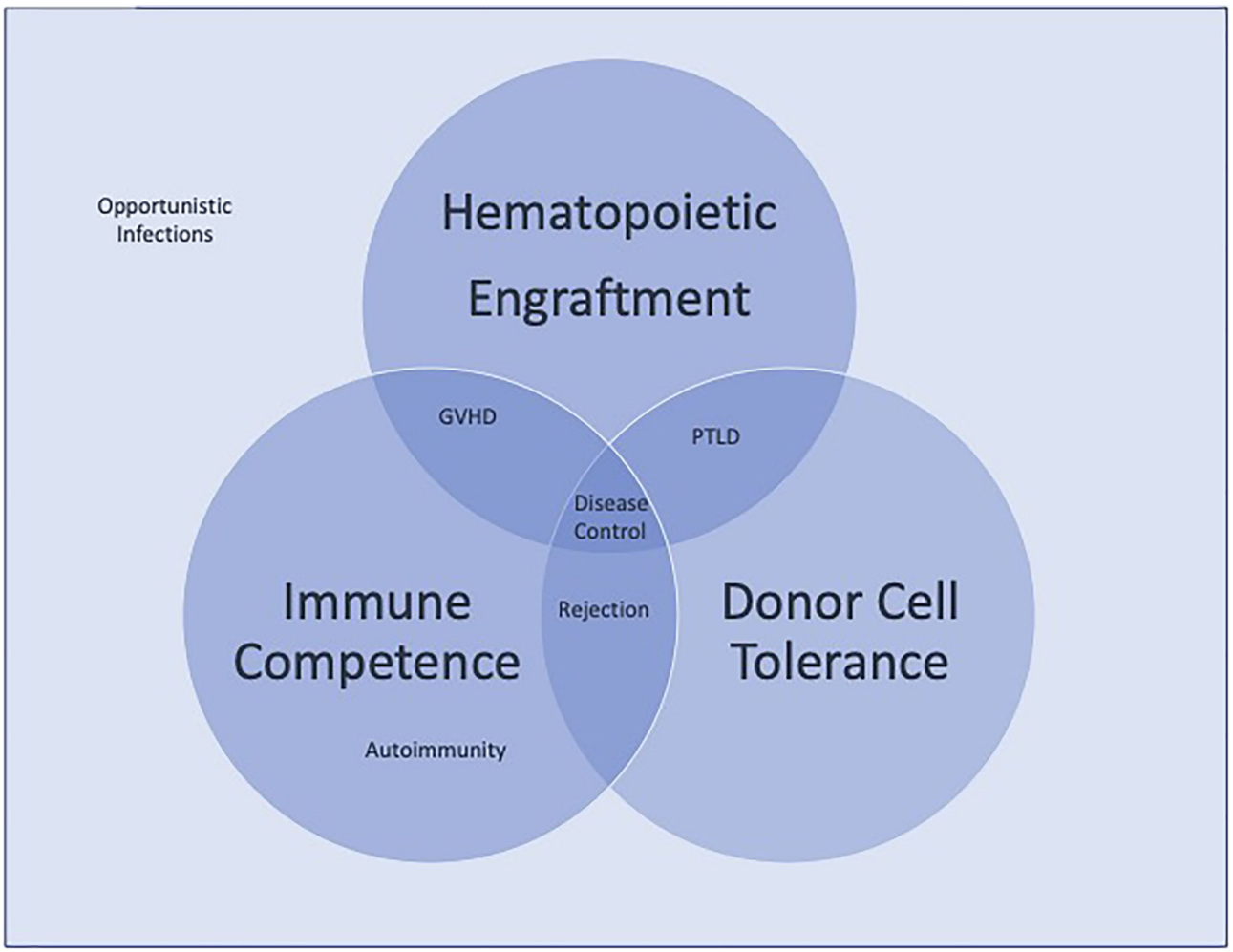

FIGURE 1 | Competing immunologic forces in hematopoietic stem cell transplantation. Disease control requires the balance of hematologic engraftment, immunologic competence, and donor cell tolerance. Failure to achieve all three leads to the clinical complications of transplantation represented.

establishing a robust donor immune system rather than one which leads to infectious risks and rejection was reviewed by Bhatt and Bednarski. A prolonged incompetent immune system leads to susceptibility to viral pathogens and can also lead to additional complications such as PTLD. Our current struggles in promptly establishing immune competence in the post-transplant period incentivizes us to pursue alternative strategies to protect the patient, harnessing our knowledge of effector mechanisms to aid host defenses until sufficient immune reconstitution is achieved. As summarized in Basso et al.'s review, means of generating anti-pathogen effector cells are being developed *via* a variety of strategies and the optimization of such therapies will be a substantial advancement in the battle against infections where effective antibiotics may be lacking. Such efforts can also be utilized to combat Epstein Barr virus driven disease processes such as PTLD (Compagno et al.).

Once donor immunity establishes itself, the threats of rejection and immunodeficiency are supplanted by the threat of dysregulated immunity. As reviewed by Buxbaum and Pavletic, most autoimmune processes are B cell mediated and can be a consequence from residual donor B cells, or donor cells responding to host antigens with dysregulated T cells. In contrast, chronic graft versus host disease is much more complex complication, recruiting all elements of the immune system.

Chronic graft *versus* host disease remains one of the most debilitating and life threatening immune mediated complication of the transplant process. Fully elucidating the mechanisms and identifying targetable elements can provide opportunities to improve outcomes. Rozmus' suggestion that the study of monogenic diseases may give us novel insights in identifying new targets against graft *versus* host disease is a provocative one, and pursuit of investigations along this strategy will hopefully provide new therapeutic opportunities in a disease in need of new treatments. Increasing our understanding of how the elements of the immune system is organized in chronic graft

versus host disease is also of critical importance to formulate thoughtful treatment strategies. Cuvelier et al.'s manuscript that the cells responsible for chronic graft versus host disease differ between adults and children highlights a paradigm that requires further study. This observation suggests that the challenges that we must overcome to understand this disease are even more formidable that what we have traditionally thought; and future efforts must take age related issues into account if we are going to improve transplant outcomes for the pediatric population.

Still, optimism exists, as novel therapies continue to emerge with time. Ringden et al.'s report of their experience using mesenchymal stem cells to treat steroid refractory graft *versus* host disease illustrates the wide scope of therapeutic avenues that are being explored to find impactful therapies for this challenging condition.

Thus, it is clear that there remain many immunologic challenges that need to be overcome to improve the outcomes of HSCT for pediatric non-malignant disease. This collection of reports provides clarity, not only on where we are in this journey, but also highlights potential pathways for success. The pace by which we gain greater command of transplant immunology will dictate the pace in which HSCT becomes the primary therapeutic choice in the treatment of hematopoietic diseases.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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PERSPECTIVE
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Chimerism in the Realm of Hematopoietic Stem Cell Transplantation for Non-malignant Disorders—A Perspective

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Hematopoietic stem cell transplantation (HCT) is a curative intervention in non-malignant disorders (NMD) that benefit from donor-derived hematopoiesis, immunity, and establishment of vital cells or enzyme systems. Stability or reversal of disease symptoms depends on adequacy and long-term stability of donor cell engraftment in the compartment of interest. Unlike hematologic malignancies where complete replacement with donor derived hematopoiesis is desirable for a cure, NMD manifestations can often be controlled in the presence of mixed chimerism. This allows for exploration of reduced intensity conditioning regimens that can limit organ toxicity, late effects, and increase tolerability especially in young recipients or those with a large burden of disease related morbidity. However, the levels of donor chimerism conducive to disease control vary between NMD, need to focus on the hematopoietic lineage necessary to correct individual disorders, and need to be assessed for stability over time, i.e., a whole lifespan. An enhanced ability to reject grafts due to recipient immune competence, alloimmunization, and autoimmunity add to the complexity of this balance making NMD a highly diverse group of unrelated disorders. The addition of donor factors such as stem cell source and Human-Leukocyte-Antigen match extend the complexity such that 'one size does not fit all'. In this perspective, we will discuss current knowledge of the role of chimerism and goals, approach to HCT, and emerging methods of boosting engraftment and graft function, and monitoring recommendations. We draw attention to knowledge gaps and areas of necessity for further research and research support.

Keywords: chimerism, chimerism after allo-HSCT, non-malignant diseases, hematopoeietic stem cell transplantation, bone marrow failure disorders, hemoglobinopathies, immunodeficiencies, metabolic disorder

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INTRODUCTION

Hematopoietic stem cell transplantation (HCT) is a curative option in a variety of inherited and acquired non-malignant disorders (NMD) that present at varying age groups, progress at variable rates, and have a wide range of clinical manifestations. These can vary from chronic supportive care needs and poor quality of life to rapid progression and early mortality. The overall aim of HCT is to correct the pathologic basis of hematologic, immunologic, or enzymatic dysfunction that is the etiology of the underlying disease. HCT results in immunohematologic replacement and thus corrects pathophysiology in NMD such as immune deficiencies and dysregulation, metabolic

disorders, hemoglobinopathies, and bone marrow failure disorders. As identification of genetic abnormalities become more sophisticated, indications for curative transplant are expected to rise. As the number of disorders benefiting from transplant increase, expansion of donor sources that will best serve to mitigate disease manifestations is advantageous. Though HCT is curative, the risk-benefit ratio between disease manifestations and HCT outcomes should be carefully considered. The safety and efficacy of the procedure factors into the decision on how to, when to, and what to monitor post-transplant. Ensuring adequate and stable donor engraftment to effectively suppress disease manifestations or afford a cure in timely manner has a major role in determining outcomes and success when HCT is contemplated for NMD. Successful HCT does not guarantee reversal of non-hematopoietic abnormalities and outcomes are variable depending on disorder, graft source, conditioning regimen, toxicity, and other factors. We will focus on the chimerism aspect of success following HCT for NMD.

CHIMERISM

Full donor chimerism is often unnecessary in NMD provided lineage specific engraftment is adequate to cure or suppress disease manifestations. However, a few scenarios give pause to this conclusion and disease specific assessment of chimerism requirement is necessary. Examples include metabolic neurodegenerative disorders where prompt and high percentage donor engraftment is required to stem neurodegeneration as early as possible and ensure an early rise in protective enzyme levels (1). Patients with inherited marrow failure disorders have a predisposition to myeloid malignancies. The stress of hematopoiesis has been implicated in malignant transformation, a stress relieved by successful HCT. It is not known whether this is adequate protection against transformation to leukemia in the setting of mixed chimerism post-HCT, making a case for regular long-term follow up in all HCT recipients.

Frequency

Chimerism should be monitored at regular intervals after HCT. Though the duration of monitoring is variable, it is recommended that chimerism be monitored for at least 5 years post-HCT. The frequency of monitoring is higher in the first year post-HCT starting from the time of established engraftment, ∼30 days post-HCT. In the presence of complete chimerism, monitoring every 3 months in the first year, 6 months in the second year and yearly for at least 5 years is a general guideline. In the event of unstable or mixed chimerism, intervals between testing should be shorter to determine additional interventions such as immunosuppression, stem cell boosts, or second transplant. Chimerism analysis should be paired with disease specific evaluations such as hemoglobin analysis in hemoglobinopathies, blood counts in marrow failure disorders, immune recovery in immunodeficiency disorders, and enzyme levels in metabolic disorders. In the event of poor marrow function it is important to determine whether the problem is due to lack of engraftment or poor graft function. Since etiology is often immune mediated in the former and a product of the marrow environment or donor source in the latter, the approach to investigation and mitigation vary. In general, a rapid drop of donor chimerism early post-HCT is difficult to halt. A gradual loss of engraftment over time may be more conducive to planning interventions that can help slow or prevent graft loss.

Methods

Chimerism is detected by short tandem repeat (STR) polymerase chain reaction (PCR) analysis that quantifies donor and recipient DNA using individual specific repeats. It is read as a percentage of donor and recipient DNA in the sample, which can be peripheral blood or marrow. This procedure is the most sensitive and accurate method of testing. Pre-transplant samples from both donor and recipient are necessary for reporting post-HCT results. Alternate methods include florescent *in-situ* hybridization (FISH), chromosome analysis for sex chromosomes in the presence of sex discrepancy between donor and recipient, markers of donor hematopoiesis such as change in blood type, and a rise in previously absent enzyme levels in hereditary metabolic disorders. Disease specific testing such as neutrophil oxidative burst in chronic granulomatous disease or CD40 ligand expression in hyper-IgM syndrome can also assist in determining transplant efficacy. The latter tests are cheaper and can be used for screening but are not an accurate prediction of chimerism status.

Lineage Specific Assessment

Most non-malignant disorders require lineage specific chimerism assessment which provides valuable detail despite the cost. Tracking chimerism in this manner allows for prediction of the role of chimerism on disease status and correction of deficit. Lineage specific evaluation is based on positive selection from peripheral blood cells and includes antibody column mediated cell separation into myeloid (CD15, CD33), T lymphoid (CD3), and B (CD19) lineage specificity followed by STR analysis. Additional less frequently used assessments of lineage specific chimerism include NK cells (CD16/CD56) or erythroid lineage cells (CD71) for appropriate disorders. Lower levels of mixed chimerism in non-essential lineages with stable full or adequate donor chimerism in the lineages of interest is capable of providing a cure. Serial tracking is necessary until stability is ensured.

In disorders where single lineage abnormalities cause disease, relevant lineage specific engraftment may be curative. For example, complete lymphoid engraftment with low myeloid chimerism (<50% donor) in Wiskott Aldrich syndrome can reverse the immune deficiency but not the thrombocytopenia. Serial determination of lineage specific chimerism can help predict impending rejection specific to each disease and facilitate earlier intervention (2).

Lineage specific chimerism requires an adequate number of lineage specific cells to determine chimerism levels. In the case of T-cell depleted transplants, reconstitution of the lymphoid compartment may be delayed. This may delay meaningful T-cell engraftment analyses. Similarly in the event of bone marrow suppression from factors such as infection, myeloid chimerism analyses may need to be performed after myeloid recovery.

DISEASE SPECIFIC CHIMERISM

Children transplanted for NMD have a wide range of mixed chimerism where donor cell engraftment ranges from 33 to 78% (3–5). This variability is attributed to disease characteristics, immune competence, conditioning regimen, donor source, and transplant related complications. A retrospective review of an Italian cohort of 101 patients who underwent transplant for NMD found that chimerism remained a dynamic process where 55.4% of patients with early mixed chimerism post-HCT improved to only 12.8% with mixed chimerism at last follow-up (6). However, late graft failure though rare was still prevalent, making the case for continued follow-up. Early complete donor chimerism can correct disease manifestations sooner. However, early alloreactivity from donor lymphocyte engraftment can be associated with a higher incidence of both acute and chronic graft-versus-host (GVHD) disease. Mixed chimerism and gradual donor lymphoid engraftment invites tolerance and has a lower incidence of GVHD (6). A retrospective review of 56 patients transplanted for NMD showed that chimerism assessment on day +14 was significant in predicting 5-year event-free survival (EFS). It was higher in patients with complete or predominant donor chimerism compared to those with low-level mixed chimerism (86.1 vs. 71.4%, $p < 0.001$) (7). Our experience is similar in that a rapid decrease in donor engraftment early post-transplant suggests robust immunologic rejection and is harder to control without consideration of a second transplant.

The extensive variability in NMD makes it worthwhile to summarize chimerism studies by disease groups.

IMMUNE DEFICIENCIES

Immune deficiencies that benefit from HCT are widely variable in the range of immune defects exhibited, some with additional hematologic manifestations. The most common disorders include severe combined immunodeficiency (SCID), Wiskott-Aldrich syndrome (WAS), and chronic granulomatous disease (CGD). The majority present during early childhood and SCID patients may be identified at birth with newborn screening. Immune dysregulation disorders that respond to treatment with HCT include hereditary hemophagocytic lymphohistiocytosis (HLH), immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX), and autoimmune lymphoproliferative syndrome (ALPS). Non-SCID immune disorders are diagnosed at various ages most commonly due to infectious complications or hematopoietic/autoimmune manifestations. HCT provides the opportunity to establish a normal immune system but is a serious undertaking due to the risks associated with treating very young, or patients already exposed to serious infections. HCT is considered successful if patients demonstrate successful immune reconstitution in all lymphoid compartments, normal immunoglobulin levels, vaccine response, and T-cell repertoire. Achieving this in the presence of a dysregulated host immune system can be challenging given the propensity for selective engraftment and partial correction.

Patients with SCID have excellent survival rates post-HCT. Due to T-cell deficiency and the associated inability to reject grafts, for many years, the standard of care for SCID patients was to infuse donor stem cells without conditioning. This led to donor T-cell engraftment while other lineages remained of recipient origin resulting in lifelong dependency on immunoglobulin (IVIG) infusions due to a lack of functional B-cells in some SCID subtypes such as RAG1/2 deficiency.

Recently, retrospective reviews have shown that T-cell reconstitution was poorer with RAG and DCLRE1C mutations than other phenotypes. B-cell engraftment was poorer in IL2RG/JAK3, RAG and DCLRE1C mutation phenotypes especially with mismatched grafts (8, 9). With T-cell replete grafts, if at day +100, recipients had <300 CD3 cells/μL, <50 CD8 cells/μL, <10% CD45RA cells or a T cell repertoire of <13 of 24 families, a second HCT was likely needed (10). Global immune reconstitution has been successfully achieved in typical or leaky SCIDs transplanted after reduced intensity conditioning regimens. In contrast to T-cell engraftment, myeloid and B-cell reconstitution is improved by conditioning strength with myeloablative HCT affording better engraftment than reduced intensity HCT (11). Factors to be considered for toxicity of intensive regimens however include age of the patients, potential for late effects, pre-existing infections, and toxicity due to DNA repair defects (DCLRE1C). Another strategy to improve outcomes may be to avoid conditioning before an initial stem cell infusion if toxicity is imminent. This will need to be followed by a subsequent HCT with conditioning complete immune reconstitution when the recipient can tolerate it better.

All other immune deficiency disorders other than SCID require conditioning for engraftment. While myeloablative regimens have been associated with significant morbidity, reduced intensity regimens have better survival but higher rejection rates (12). Donor chimerism of >30% if stable, can protect against disease reactivation in HLH (13). Stable myeloid chimerism levels of >50% are desirable in conditions such as CGD for functional correction, and platelet count recovery in WAS. If familial donors are considered, the presence of heterozygosity for disease in the donor will need to be considered as it may interfere with full correction of the disease (e.g., HLH, X-linked CGD). Here, carrier donors are best avoided. Some immune dysregulation disorders such as STAT1 or CTLA-4 deficiency may require full donor chimerism for correction. Reducing the intensity or toxicity for safety may have secondary or late rejection rates of 10–15%, (14), autoimmunity with mixed chimerism (15) or delayed myeloid engraftment (16) and need to be tracked. Despite this, success rates continue to improve for these disorders.

METABOLIC AND STORAGE DISORDERS

Metabolic and storage diseases are a heterogeneous group of disorders leading to accumulation of enzymatic by-products in multiple organs with associated toxicities, predominantly neurologic. HCT from unaffected donors can supplement

enzyme following hematopoietic engraftment and migration to affected organs. Timely HCT is important to offset irreversible changes making early engraftment a key determinant of success.

Hurler Syndrome, a lysosomal storage disorder caused by deficiency of the enzyme alpha-L-iduronidase (IDUA) is the most commonly transplanted inherited metabolic disorder. Transplant is considered successful when patients attain normal leukocyte IDUA levels best achieved with full donor chimerism. In a study of more than 200 patients transplanted for Hurler's followed for neurodevelopmental outcomes and growth, factors supporting successful enzyme reconstitution included early transplantation, non-carrier donors, cord blood grafts due to higher inherent enzyme levels, and preparative regimens designed to achieve complete chimerism (1, 17). Speed of HCT for engraftment has no better illustration than early infantile Krabbe disease where donor engraftment following HCT <30 days of age had improved outcomes in domains of mobility, communication and feeding (18).

Leukodystrophies such as adrenoleukodystrophy (ALD), an X-linked disorder resulting in the inability to transport fatty acids into the peroxisome for degradation leads to accumulation in tissues specifically the central nervous system. HCT has been shown to halt neurological disease progression in a significant majority attributed to monocyte engraftment in the brain. It is important to proceed to transplant at diagnosis and with a low Loes radiologic score. Neurologic stability after engraftment takes time and occurs over the span of the first year so delays could accelerate deterioration. ALD patients with 70–100% donor chimerism in the myeloid compartment on day +60 and those with faster recovery of neutrophil counts had better resolution of gadolinium uptake on brain MRI scans post-HCT, a measure of blood brain barrier recovery (19).

Osteopetrosis, characterized by dysfunctional osteoclasts, also responds to HCT. Stabilization of vision and hearing, nasal obstruction and motor deterioration requires transplant in infancy and prompt engraftment is able to rescue 40–70% of patients. Graft failure despite intensive conditioning is the major cause of failure and death after HCT in osteopetrosis. However, donor chimerism in myeloid cells as low has 5–10% prevents death and provides sustained hematopoietic recovery in contrast to many other hereditary metabolic disorders (20).

HEMOGLOBINOPATHIES

Hereditary hemoglobinopathies with severe manifestations can be cured by HCT. The common disorders eligible for HCT are sickle cell disease (SCD) and transfusion dependent thalassemia. Variables influencing donor engraftment in hemoglobinopathy patients include age, HLA alloimmunization due to transfusion history, immune competence, and paucity of matched sibling donors. SCD manifestations are reversed in the presence of successful donor-derived erythropoiesis and normal erythroid precursors may have a survival advantage resulting in abatement of SCD symptoms even in the presence of low lymphoid engraftment. Myeloid lineage chimerism is a good surrogate for erythropoiesis in the absence of red cell chimerism (CD71) evaluation. Lymphoid engraftment can remain low or increase over time but there are no threshold levels necessary to maintain myeloid engraftment (21, 22).

Stable donor chimerism >20–25% paired with a hemoglobin S level <50% is associated with resolution of disease symptoms such as vaso-occlusive episodes and strokes (23, 24). However, hemolytic anemia was detected in SCD patients who had <50% donor cells after myeloablative conditioning and higher engraftment levels (>30%) were better if donors had sickle trait (25). The acceptability of stable mixed chimerism and the presence of mixed chimerism even with myeloablative conditioning (up to 44%) allows the exploration of less toxic regimens for HCT in SCD. Younger patients and donors, those with mismatched donors, low cell dose, and weaker stem cell sources such as cord blood have a higher risk of graft rejection in SCD and should be taken into consideration when fashioning regimens targeting intensity (26).

Thalassemia is cured when a patient no longer requires blood transfusions, regains growth, and restores iron related changes. A cohort of 106 patients with beta-thalassemia major when studied retrospectively revealed that half had sustained mixed donor chimerism with cure. Mixed chimerism was associated with a good transplant outcome and decreased risk of acute or chronic GVHD, a finding that was not noted in SCD. High erythroid lineage engraftment with low level donor chimerism in other lineages is compatible with cure in thalassemia (27). Mixed chimerism though acceptable, needs monitoring for stability over time in patients with hemoglobinopathy irrespective of intensity of conditioning regimens.

TABLE 1 | Classification of non-maligant disorders with associated lineage specific engraftment, and recommended donor chimerism levels for adequate disease mitigation.

Non-malignant disorder	Lineage specificity	Minimum goal for donor Chimerism
Immunodeficiencies		
HLH	NK cell/Lymphoid	>30% (13)
IPEX, ALPS	Lymphoid	>50% (32)
Severe Combined Immunodeficiency	T, B, NK cell	100% (8)
Chronic Granulomatous Disease	Myeloid	>50% (5)
Wiskott-Aldrich Syndrome	Lymphoid/Myeloid	>50% (16)
Hemoglobinopathies		
Sickle Cell Disease	Erythroid/myeloid	20–25% (23)
Thalassemias	Erythroid/myeloid	20–25% (27)
Metabolic disorders		
ALD, Hurlers, Krabbe's	Myeloid	70–100% (1)
Osteopetrosis	Myeloid	>10% (20)
Bone marrow failure syndromes		
SCN, SDS, DBA, FA	Myeloid	100% (30)
	Lymphoid	>50% (31)

BONE MARROW FAILURE SYNDROMES

Bone marrow failure syndromes (BMFS) such as aplastic anemia, severe congenital neutropenia, dyskeratosis congenita, Shwachman-Diamond, Diamond-Blackfan or Fanconi anemias (FA), and congenital amegakaryocytic thrombocytopenia include genetic and acquired pathologies resulting in inadequate hematopoiesis. HCT is curative. Since many are pre-leukemic conditions, full donor chimerism in the myeloid compartment is desirable and mixed chimerism in lymphoid lineages is acceptable. Patients with Fanconi and DNA repair defects are unable to tolerate regimen intensity or alkylating agents. The resulting need for balance between toxicity of intensification and engraftment is delicate. Graft rejection rates can be as high as 20–25% even with myeloablation. Both myeloablative and immunosuppressive regimens have been successful in curing BMFS following successful myeloid engraftment. In FA, with mixed chimerism in the lymphoid lineage, patients can be left with some lymphocytes exhibiting the increased sensitivity to DNA damage and others exhibiting a normal response (28–31). Long-term follow-up post-HCT in all BMFS should include both chimerism evaluation and monitoring for clonal hematopoiesis, a risk that should technically be mitigated with myeloid engraftment.

DISCUSSION

Donor chimerism requirements to achieve disease control are widely variable in NMD and can range from as low as 10% to >90% (**Table 1**). Variables influencing chimerism include age, inflammatory status, immune competence, and transfusion history. Transplant and donor characteristics include donor age, HLA match, stem cell source, cell dose, and intensity of conditioning regimens. In general, reduced intensity regimens are more appropriate for disorders that are conducive to mixed chimerism. As transplant approaches change to accommodate more donors, increase safety and tolerability, and reduce toxicity, chimerism should be tracked and described long-term.

Our understanding of chimerism and adequate interventions for the same continue to evolve. Stable donor chimerism off immunosuppression for over 2 years is unlikely to dwindle. However, continued monitoring is still recommended for occasional late graft rejections as described in thalassemia. These patients are usually identified by gradually dwindling donor chimerism levels in the lineage of interest. The old dogma that T-cell engraftment was necessary to maintain myeloid chimerism has not held true in NMD following the expanded ability to monitor chimerism in a lineage specific manner. While stable mixed chimerism is fully acceptable, dropping chimerism has prompted immune suppression withdrawal in myeloablated or immunoablated recipients whereas continuing immune suppression has been advantageous in low intensity regimens. Donor lymphocyte infusions are generally not of benefit, can induce unwanted GVHD, and should be avoided in NMD. An early rapid drop in chimerism usually requires a second stem cell infusion after reconditioning whereas a gradual decline can be salvageable with immune suppression adjustments. Reduced intensity conditioning does not preclude a second early transplant whereas a time lag is better for toxicity reasons after a myeloablative transplant. The ability to infuse products such as high dose CD34 selected stem cells is valuable in NMD to avert GVHD risks.

CONCLUSIONS

The tracking of lineage specific donor chimerism for stability with time should be routinely incorporated into evaluations post-HCT for NMD. The definition of adequate chimerism for successful HCT varies by disorder and as our understanding of the same matures, our remedial interventions will evolve. The definition and durability of adequate chimerism has direct application to gene-modified therapy that is now under evaluation for many genetic disorders. In both the allogeneic and in the gene-modified autologous HCT setting, chimerism requirements will drive conditioning needs and transplant methods to achieve a cure.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

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

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Immune Reconstitution in Pediatric Patients Following Hematopoietic Cell Transplant for Non-malignant Disorders

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Allogeneic hematopoietic cell transplant (HCT) is curative for pediatric patients with non-malignant hematopoietic disorders, including hemoglobinopathies, bone marrow failure syndromes, and primary immunodeficiencies. Early establishment of donor-derived innate and adaptive immunity following HCT is associated with improved overall survival, lower risk of infections and decreased incidence of graft failure. Immune reconstitution (IR) is impacted by numerous clinical variables including primary disease, donor characteristics, conditioning regimen, and graft versus host disease (GVHD). Recent advancements in HCT have been directed at reducing toxicity of conditioning therapy, expanding donor availability through use of alternative donor sources, and addressing morbidity from GVHD with novel graft manipulation. These novel transplant approaches impact the kinetics of immune recovery, which influence post-transplant outcomes. Here we review immune reconstitution in pediatric patients undergoing HCT for non-malignant disorders. We explore the transplant-associated factors that influence immunologic recovery and the disease-specific associations between IR and transplant outcomes.

Keywords: immune reconstitution, hematopoietic stem cell transplant, non-malignant disorders, hemoglobinopathy, severe combined immunodeficiency, aplastic anemia

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INTRODUCTION

Allogeneic hematopoietic cell transplant (HCT) is a key therapeutic approach for many non-malignant hematopoietic diseases in pediatric patients, including hemoglobinopathies, bone marrow failure syndromes, and immunodeficiencies. Effective reconstitution of donor-derived innate and adaptive immune cell number and function following HCT is critical for promoting donor cell engraftment, restoring protection against infections, and improving overall survival (1, 2).

Recovery of immunity after HCT is influenced by various clinical factors, including primary diagnosis, donor type, stem cell source, graft manipulation, conditioning regimen (i.e., intensity of conditioning, use of irradiation, serotherapy), and pharmacologic prophylaxis, development and treatment of graft-versus-host disease (GVHD) (1, 2). After HCT, establishment of donor immunity is variable and occurs in phases. Innate immune reconstitution (IR) occurs first with

Abbreviations: ATG, anti-thymocyte globulin; BM, bone marrow; CMV, cytomegalovirus; GVHD, graft-versus-host disease; HCT, hematopoietic cell transplant; IR, immune reconstitution; MAC, myeloablative conditioning; MRD, matched related donor; MSD, matched sibling donor; NK, natural killer; PB, peripheral blood; RD, related donor; RIC, reduced intensity conditioning; RTC, reduced toxicity conditioning; UCB, umbilical cord blood; URD, unrelated donor.

neutrophils, monocytes, natural killer (NK) cells, and dendritic cells expected to normalize in the first weeks to month after HCT (1). Adaptive immune system recovery occurs more slowly with B cell and CD8 T cell numbers normalizing between 100 days and 6 months post HCT and thymic-dependent CD4 T cell reconstitution occurring between 6 and 9 months (1). Initial T cell reconstitution occurs through peripheral expansion of CD8 memory T cells from the donor graft or recipient T cells remaining after conditioning (3). These peripherally expanded CD8 T cells are responsive to cytokines and previously encountered viruses; however, they have limited ability to respond to novel antigens (3). The second phase, leading to full T cell reconstitution, relies on lymphoid progenitors undergoing thymic differentiation into naive CD4 or CD8 T cells expressing MHC-restricted, antigen-specific T cell receptors (3). The kinetics of reconstitution of these distinct components of the immune system correlate with post-transplant morbidity related to infections, graft loss and GVHD. Here we review the factors that influence recovery of innate and adaptive immunity in pediatric patients undergoing HCT for non-malignant disorders and the impact of this reconstitution on general and disease-specific outcomes.

TRANSPLANT-ASSOCIATED FACTORS AFFECTING IMMUNE RECONSTITUTION

Stem Cell Source

Peripheral blood (PB), bone marrow (BM), or umbilical cord blood (UCB) stem cells can be utilized for HCT from either related (RD) or unrelated donors (URD). These donor sources vary in cellular composition with PB grafts having 10-fold higher T and B cells than BM grafts and single UCB grafts having 10–100-fold fewer nucleated cells compared to BM (1, 4, 5). The differences in graft composition impact donor IR and infectious complications following HCT. Regarding innate immunity, neutrophil engraftment occurs at approximately 14, 21, and 30 days after a PB, BM, and UCB HCT, respectively (6). Interestingly, NK cell numbers normalize by 1 month post HCT independent of graft source (6). Yet, UCB recipients have been found to have higher numbers of NK cells at 3, 9, and 12 months after transplant (7).

Graft source also impacts reconstitution of adaptive immunity. HCT with UCB has been associated with higher naive and memory B cell numbers at 6 months post HCT compared to BM and PB grafts (8). In contrast, T cell reconstitution is delayed after UCB HCT (7–9). UCB contains antigen-inexperienced naive T cells; therefore, T cell recovery is entirely thymic dependent resulting in profound early lymphopenia (7, 10, 11). Recipients of UCB HCTs have a slower recovery of thymopoiesis than patients receiving BM stem cells as evidenced by a lower thymic-derived naive CD4 T cells at 6 months post HCT (7).

T cell reconstitution also differs between BM and PB recipients. In a single institution randomized trial, patients who received PB grafts had faster lymphocyte recovery, most significantly CD4 T cells, compared to BM graft recipients (4). Consistent with slower IR, BM stem cell recipients had a 2.4-fold higher rate of severe infections and a higher risk of infection-related mortality (4). A larger, phase 3 trial confirmed earlier IR and lower infection risk in patients receiving PB grafts but did not identify any differences in mortality (12). Thus, donor IR after HCT is highly impacted by distinct properties of the different stem cell sources (**Figure 1**).

Alternative Donor Sources

While an HLA matched donor is preferred, less than 25% of patients will have an available sibling donor and the likelihood of identifying a matched URD in the registry is impacted by numerous factors, including ethnicity of the patient (13). Consequently, alternative donors have been increasingly used for HCT with unique implications for post-HCT IR (**Figure 1**).

UCB has been utilized as an alternative donor source and has distinctive IR properties as discussed above. However, there are significant barriers to success of UCB transplants, including graft failure and delayed neutrophil and T cell recovery, resulting in infectious complications (10, 11, 14). Addressing these obstacles has been an active area of investigation (6, 10, 15). UCB has lower total nucleated cell and CD34 + cell dose (per recipient's weight), which has been associated with delayed hematological recovery and graft failure (11). Strategies to improve cell dose for UCB have included double cord blood transplant and *ex vivo* expansion of cord blood units. While IR data on double UCB HCT is limited in pediatrics, in adults, it has not consistently demonstrated an improvement in IR compared to single UCB HCT (10, 16, 17). This may be, in part, related to confounding factors, including the use of T cell depletion (10). Further studies are needed to better address this question. In contrast, recent early phase clinical trials using *ex vivo* cord blood expansion have demonstrated that neutrophil engraftment can be shorted to 9 days from 21 days (15, 18). In regard to T cell recovery, lower doses of anti-thymocyte globulin (ATG) have been associated with faster recovery of CD4 and CD8 T cells after UCB transplant (14, 19, 20). Additionally, use of better HLA-matched cord blood units with higher CD3 T cell counts has been shown to improve immune recovery (21).

The use of haploidentical donors as an acceptable alternative stem cell source has surged with recent studies aimed to reduce the risk of GVHD, sustain donor engraftment, and support earlier IR (13, 22). The kinetics of IR following haploidentical donor HCT depends on conditioning regimen, stem cell source, and graft manipulation strategy utilized. For example, time to neutrophil engraftment varies from a median of 11–12 days after T cell depletion with high dose CD34 + cells to 13 days after GCSF-mobilized haploidentical unmanipulated PB graft to 15 days after unmanipulated haploidentical BM (23). Similar to HLA-matched transplant, monocyte and NK cell recovery is rapid and occurs by day 15 and 30, respectively, after haploidentical HCT (23). Regarding adaptive immunity, patients receiving T cell replete haploidentical grafts have more rapid T cell IR during the first 6 months after HCT compared to patients who received T cell-depleted grafts (23). T cell function and new naive T cell production remain low for 12–24 months after unmanipulated haploidentical HCT (23).

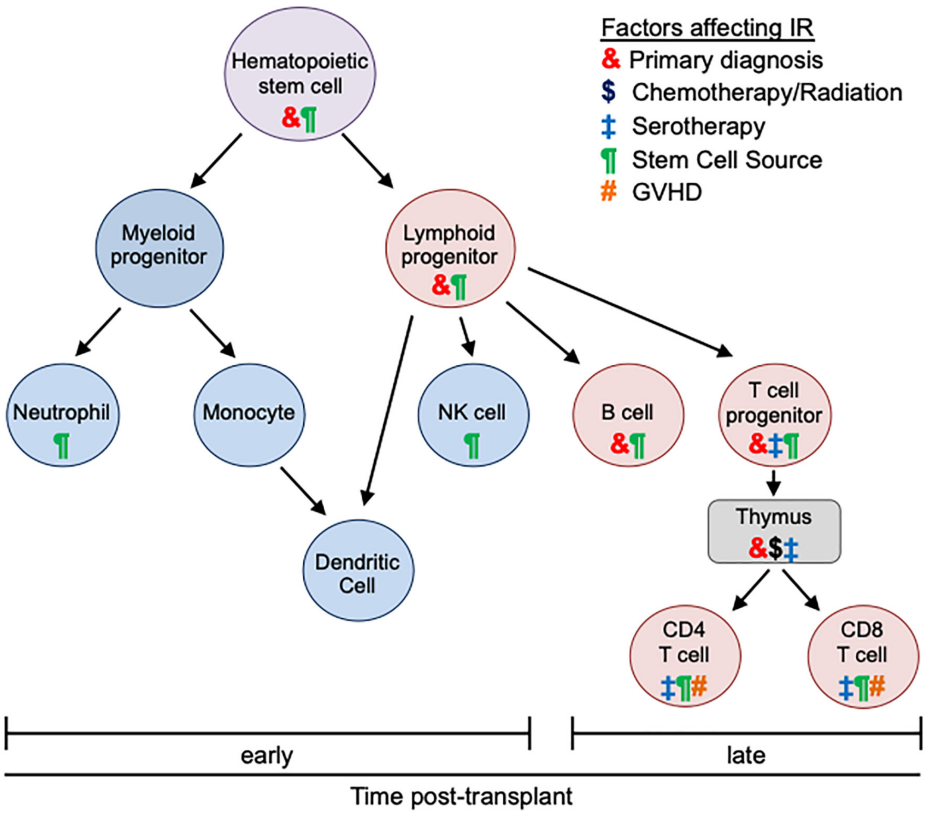

FIGURE 1 | Effects of transplant-related factors on immune reconstitution. Different types of immune cells and their differentiation are depicted. After allogeneic HCT innate immunity (blue) recovers early (within 30 days). Reconstitution of adaptive immunity (red) is later and more variable (often up to 1 year). The kinetics of immune recovery is influenced by primary diagnosis (&), conditioning regimen ($), use of serotherapy (‡), stem cell source (¶), and GVHD (#). Each transplant-associated factor distinctly impacts different immune populations and differentiation stages.

Due to delayed recovery of adaptive immunity and associated infection risks, strategies for *ex vivo* elimination of αβ T cells and CD19 B cells with no pharmacologic prophylaxis for GVHD has been utilized for haploidentical transplant in patients with non-malignant disorders (13, 22). In a study of 23 patients, γδ T cell recovery occurred early (∼1 month post HCT), but αβ T cell and CD19 B cell repopulation was delayed to 9–12 months, respectively (13). Alternative donor sources are often used in patients with non-malignant disorders who have no available familial or registry donor. Improving IR in this patient population remains an active area of investigation.

Conditioning Strategies

IR is also impacted by conditioning regimen, including intensity of chemotherapy, use of radiation, and use of serotherapy (**Figure 1**). In particular, conditioning therapy can damage the thymus and impair its function, which is essential for full T cell reconstitution. For example, cyclophosphamide and radiation induce acute thymic injury with loss of cellularity whereas ATG and alemtuzumab serotherapy significantly deplete thymocytes resulting in prolonged T cell aplasia (3).

Patients with non-malignant disorders often receive reduced toxicity (RTC) and reduced intensity conditioning (RIC) regimens in order to limit the morbidity associated with myeloablative conditioning (MAC). RIC regimens are non-myeloablative while RTC regimens are myeloablative. Both approaches have fewer side effects and organ toxicities compared to traditional MAC. Law et al. reported that following a RTC regimen of alemtuzumab, busulfan, and fludarabine median time to neutrophil recovery was 16 days while time to B cell and T cell reconstitution was 3 and 6 months, respectively (24). A RIC approach with alemtuzumab, fludarabine and melphalan has been used by our group and others (25–28). We recently reported IR and infectious complications in patients after HCT with early alemtuzumab (day -21) (26). NK cell recovery was rapid by day 100 and lymphocyte recovery was dependent on donor source, namely related (RD) versus unrelated donor (URD). Mean CD3, CD4, and CD8 T cell numbers normalized by 6 months after RD HCT and by 1 year in the URD group (26). B cell recovery occurred by day 100 for RD recipients and by 1 year for URD recipients (26). Despite these differences, infections did not differ between the groups (26).

Timing and dose of serotherapy significantly impact IR (20, 29, 30). Admiraal et al. reported on IR in patients with malignant and non-malignant disorders receiving ATG as part of conditioning

(20). They found that successful CD4 IR was related to the area under the curve (AUC) of ATG after donor stem cell infusion (20). Patients who received UCB HCT had delayed IR with an AUC ≥ 20 AU × day/mL while patients who received BM and PB HCT had decreased IR only at an AUC ≥ 100 AU × day/mL (20). Notably, an ATG AUC ≥ 40 AU × day/mL prior to stem cell infusion resulted in a lower incidence of graft failure and acute and chronic GVHD (20). Marsh et al. similarly demonstrated that alemtuzumab level at time of transplant impacts outcomes (30). They found patients with a level <0.15 mg/mL had threefold higher rates of acute GVHD than patients who had levels >0.16 mg/mL at the time of transplant. Alemtuzumab levels above 0.57 mg/mL were associated with delayed T cell recovery and very high levels (4 mg/mL) were associated with mixed chimerism (30). The approach to conditioning is often dictated by primary disease/graft source and requires careful consideration to balance IR with risks of GVHD and graft failure.

DISEASE-SPECIFIC OUTCOMES

Hemoglobinopathies

HCT for pediatric patients with thalassemia and sickle cell disease is potentially curative and the impact of IR on transplant-associated morbidity and outcome has been investigated by several groups. Rajasekar et al. detailed IR patterns in patients with β thalassemia major following MAC and matched related donor (MRD) HCT with BM graft (31). They found that NK cells, monocytes and dendritic cells recovered within 1 month of transplant (31). CD8 T cells and B cells repopulated at 2 and 4 months, respectively, while CD4 T cell recovery did not occur by 1 year post HCT (31). Consistent with this, naive CD4 T cell ($CD45RA^+$) recovery was delayed more than a year and correlated with age, with younger patients having faster recovery (31). Interestingly, multivariate analysis showed that NK cell count correlated with transplant success as patients with NK cells below a median of 142/μL at 28 days post HCT had a significantly higher rejection rate and lower event free survival (31).

In order to prevent graft failure/rejection, *in vivo* T cell depletion is increasingly utilized in patients with hemoglobinopathies (32). An evaluation of IR in children with severe β thalassemia major following matched sibling donor (MSD) HCT found that the addition of ATG led to delayed CD8 T cell recovery at 6 months but no change in CD4 T cell reconstitution, which occurred at 12 months (33). Use of ATG containing conditioning regimens was associated with variable rates of bacterial infection (17–70%) and cytomegalovirus (CMV) reactivation (36–45%) (32, 33). These infectious complications are similar to those in patients transplanted without *in vivo* T cell depletion (32). However, rates of GVHD were lower after ATG-based conditioning (32).

Our group has reported similar outcomes in patients with hemoglobinopathies undergoing HCT with *in vivo* T cell depletion utilizing alemtuzumab (34). Lymphocyte recovery of CD4, CD8, and CD19 occurred by 1 year post transplant and was impacted by duration and intensity of immunosuppression for GVHD prophylaxis/treatment (34). Infection risk was highest in the first 6 months post HCT with bacterial infections and CMV reactivation in 28 and 43% of patients, respectively (34).

Aplastic Anemia

Patients with severe aplastic anemia undergo HCT as first line therapy if a MSD is available or as salvage therapy if they fail immune suppression therapy. A retrospective review of patients who failed immune suppression therapy and received MUD HCT after fludarabine, cyclophosphamide, and alemtuzumab conditioning therapy demonstrated that the majority of children achieved normal lymphocyte subsets by 12 months post HCT (35). Infectious complications included adenoviremia (2.3%), EBV viremia (22.7%), and CMV viremia (22.7%) (35). Our group published a report of 17 patients undergoing HCT with alemtuzumab, fludarabine and melphalan conditioning (36). While NK cells recovered early, T cell (both CD4 and CD8) and B cell recovery was markedly delayed with all populations normalizing by 1 year after HCT (36). Consistent with these kinetics, infection rates were higher in the first 6 months post HCT (36).

A recent study of pediatric and adult patients (median age of 14 years) with aplastic anemia treated with haploidentical HCT utilizing busulfan, cyclophosphamide and ATG reported rapid neutrophil recovery at median of 12 days and monocyte recovery by 30 days after transplantation (37). CD8 T cell recovery

TABLE 1 | Immune reconstitution with and without conditioning for SCID.

Genotype	Immune phenotype	Conditioning	CD8 T Cell	CD4 T Cell	B Cell	References
IL2RG/JAK3	T- B + NK-	No	+	+	–	(38–42, 44, 45)
		Yes	+	+	+	
ADA	T- B- NK-	No	+	+	+	
		Yes	+	+	+	
RAG1/2/Artemis	T- B- NK +	No	–	–	–	
		Yes	+	+	+	
IL7R	T- B + NK +	No	+	+	+*	
		Yes	+	+	+	

*+Indicates reconstitution is likely after HCT. –Indicates unlikely to reconstitute after HCT. *Indicates recipient reconstitution aided by donor cells.*

occurred at 60 days while CD4 T cell repopulation was delayed to 1 year post HCT, resulting in an inverted CD4:CD8 ratio during that time period (37). Interestingly, patients with a lower CD4:CD8 ratio on day 30 post HCT had higher overall survival (37). Younger recipient age, female gender, high mononuclear cell count in the graft, and absence of CMV reactivation were all independently associated with improved IR after transplant (37).

Primary Immunodeficiency

Severe combined immunodeficiencies (SCID) are a heterogeneous group of genetic disorders characterized by a lack of T cell progenitors available to develop within the thymus resulting in failure of T cell maturation as well as impaired cellular and humoral immunity (38). IR following HCT for SCID is variable based on intrinsic factors related to the underlying genetic defect (i.e., timing of developmental arrest) and modifiable factors, such as conditioning therapy (**Table 1**) (38–41). HCT without conditioning from an HLA-matched donor (related or unrelated) or T cell-depleted haploidentical donor allows successful thymopoiesis and T cell IR in SCID patients with IL2 receptor gamma chain (*IL2RG*), Janus-associated kinase 3 (*JAK3*), and adenosine deaminase (*ADA*) mutations (38). However, patients with *IL2RG*- and *JAK3*-mutant SCID transplanted without conditioning have lower (often absent) donor stem cell engraftment and, consequently, do not have donor B cell repopulation (42). In the absence of donor B cell engraftment, patients often require lifelong immunoglobulin replacement. In contrast, patients with interleukin-7 receptor (*IL7R*)-deficient SCID have intact function of B cells, which can produce immunoglobulin with help from donor T cells (38). Notably, without donor stem cell engraftment, patients are at risk of early T cell exhaustion due to limited donor-derived thymopoiesis (38). In *ADA*-deficient SCID, the majority of patients who receive non-conditioned MRD HCT graft engraft donor stem cells and have sustained cellular and humoral IR (43). SCID patients with mutation of *RAG1*, *RAG2* or *DCLRE1C* (*ARTEMIS*) have arrest of thymopoiesis at later developmental stages and require conditioning to achieve recovery of donor immunity (38).

A recent prospective study demonstrated that patients with SCID who received conditioning (RIC or MAC) prior to HCT had significantly higher levels of T, B, and myeloid cell donor chimerism at day 100, which persisted at 1 year post HCT (44). Furthermore, use of conditioning correlated with higher CD4 cell counts and greater likelihood of independence from immunoglobulin therapy at 1 year post HCT (44). There was no difference in overall survival based on receiving conditioning (44). While IR is improved with pre-transplant conditioning, there are significant potential toxicities and optimal conditioning therapy is still not known (38, 44).

In addition to conditioning, many other variables impact IR after HCT in SCID patients. HCT with an URD is associated with better T cell reconstitution whereas HCT with a mismatched related donor has poorer B cell reconstitution (45). IR also varies based on SCID genotype. *RAG1/2* and *DCLRE1C* mutations have poorer T cell reconstitution after transplant (45). In regard to B cell reconstitution, in non-MSD recipients, *ADA*, *IL7R*, *CD45*, and *CD3* genotypes have a higher probability of stopping immunoglobulin replacement therapy compared to *IL2RG*, *JAK3*, *RAG1/2*, and *DCLRE1C* genotypes (45).

Regardless of genotype or conditioning, a CD4 T cell count $\geq$500 cells/cumm at 6 and 12 months post HCT correlates with significantly better long-term overall survival (45). Furthermore, in SCID patients receiving T cell replete grafts, low numbers of total T cells, CD8 T cells, naive CD4 T cells, and polyclonal Vβ diversity at day 100 were all linked to higher risk of death or need for a second transplant at 2 years (44).

DISCUSSION

Reconstitution of the donor-derived immune system is essential for achieving optimal outcomes for pediatric transplant recipients. The timing and extent of recovery of immune cell numbers and function directly impact infectious complications, development and treatment of GVHD, and long-term survival. Innate immunity establishes rapidly after transplant and, generally, is only modestly impacted by transplant-associated variables. In contrast, adaptive immunity recovers with highly variable kinetics that are strongly influenced by numerous factors. Indeed, the timing and characteristics of IR can be adjusted by modifiable factors, including stem cell source and dose, conditioning regimen, and use/timing of serotherapy. The establishment of donor immunity uniquely impacts the post-transplant course based on initial diagnosis and disease presentation. As such, it's critical to not only assess general patterns of IR but to evaluate these within disease-specific contexts.

Newer transplant approaches utilizing alternative donor sources, novel preparative regimens, and innovative graft manipulation strategies will invariably impact recovery of immune function. Additionally, identifying therapies that enhance IR remains an important focus of investigation. Innovative approaches include use of cytokines (IL-7 and IL-22), keratinocyte-growth factor, sex steroid ablation, and adoptive cell therapies (3, 46–51). Cellular therapies, such as viral-specific T cells, provide opportunities to support immune function while awaiting establishment of full IR. Careful evaluation of immune recovery will be essential in determining the impact of these therapeutic advances on transplant outcomes.

AUTHOR CONTRIBUTIONS

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REVIEW
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Autoimmunity Following Allogeneic Hematopoietic Stem Cell Transplantation

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Autoimmune manifestations after allogeneic hematopoietic stem cell transplantation (AHSCT) are rare and poorly understood due to the complex interplay between the reconstituting immune system and transplant-associated factors. While autoimmune manifestations following AHSCT have been observed in children with graft-versus-host disease (GvHD), an alloimmune process, they are distinct from the latter in that they are generally restricted to the hematopoietic compartment, i.e., autoimmune hemolytic anemia, thrombocytopenia, and/or neutropenia. Autoimmune cytopenias in the setting of ASHCT represent a donor against donor immune reaction. Non-hematologic autoimmune conditions in the post-AHSCT setting have been described and do not currently fall under the GvHD diagnostic criteria, but could represent alloimmunity since they arise from the donor immune attack on the antigens that are shared by the donor and host in the thyroid, peripheral and central nervous systems, integument, liver, and kidney. As in the non-transplant setting, autoimmune conditions are primarily antibody mediated. In this article we review the incidence, risk factors, potential pathophysiology, treatment, and prognosis of hematologic and non-hematologic autoimmune manifestations in children after AHSCT.

Keywords: autoimmunity, alloimmunity, hematopoietic stem cell transplantation, allogeneic, immune reconstitution, non-hematologic, autoimmune hemolytic anemia, autoimmune cytopenia

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INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (AHSCT) has the potential to cure refractory hematopoietic malignancies as well as acquired and inherited non-malignant immune diseases, hemoglobinopathies, and inherited metabolic disorders. For inherited disorders, emerging genetic therapies may offer an alternative (1, 2) while immunotherapeutic approaches, including AHSCT, will likely continue to be widely used for malignant diseases. Children are more likely to experience long term survival after AHSCT, but are also susceptible to harmful effects of AHSCT on growth and development of many organ systems (3). As more children undergo ASHCT, identification of biological risks that are unique to this population, and the underlying biological processes is needed.

Reconstitution of the adaptive immune system following AHSCT is primarily mediated through peripheral non-thymic expansion of donor-derived T cells in the host (4, 5). Even in children,

thymic output is significantly reduced in the immediate post-HSCT period due to transplant-related insults. Thus, peripheral (non-thymic) immune tolerance mechanisms appear to be critical during this time of immune recovery and for the emergence of both alloimmune and autoimmune complications of AHSCT. Alloimmunity stems from the donor recognition of host and can be detrimental when it manifests as graft-versus-host disease (GvHD) due to the resultant attack on the recipient tissues (6) or beneficial when directed against the malignant cells, i.e., the graft-versus-leukemia (GVL) effect. GvHD is a common HSCT complication that has acute and chronic forms. Both have well-characterized clinicopathologic features involving the gastrointestinal tract, liver and skin, with additional organ involvement in chronic GvHD (7). Chronic GvHD (cGvHD) typically affects tissues that form the physical and immune barrier between the host and potential infectious pathogens and thus are enriched with immune cells, i.e., skin, eyes, pulmonary tract, mouth, gastrointestinal tract, and genital tract. Autoimmunity after HSCT affects tissues that are often targeted by idiopathic autoimmune diseases (AD).

Outside of the AHSCT setting the pathophysiology of autoimmunity is multifactorial and the exact timing and type of the inciting event is usually unknown. In the AHSCT setting, the timing of the autoimmunity initiating cascade starts with the donor cell infusion. AHSCT adds a number of potentially detrimental effects that can skew the reconstituting immune system toward AD and represents a unique clinical model for AD research (8, 9). Whether GvHD and AD after HSCT have shared pathophysiology is an active research question. Both are driven by donor immune reaction, in the former the targets are host, while AD is directed against the donor hematopoietic compartment, or non-hematopoietic targets that are common to the donor and host. Children experience lower rates of GvHD (3) but those that undergo AHSCT for non-malignant indications appear to have a higher risk of hematologic autoimmune manifestations (9). Despite non-hematologic autoimmune-like manifestations being less frequent in children than adults they too have a higher incidence in the setting of non-malignant AHSCT (10). Although isolated ADs following AHSCT are rare they are observed in higher frequency in patients with GvHD (8, 9).

A recent comprehensive review of the literature on hematologic AD in children identified non-malignant indication for AHSCT, the use of unrelated transplant donor, omission of total body irradiation in the conditioning regimen, presence of cGvHD, and the use of peripheral or cord blood stem cell grafts as significant risk factors (9). These provide important clues about potential pathophysiology of AD. AD after AHSCT is characterized by impaired immune reconstitution that may stem from either incomplete lymphodepletion prior to HSCT, possibly leaving partially intact the antigen presenting compartment, or permitting donor B cell expansion concomitant with significant T cell depletion of the graft and/or peri-transplant use of immunosuppression that preferentially suppresses donor T cell reconstitution. As a result, an imbalance in T and B cell immunity may lead to an impaired peripheral tolerance, facilitating immune dysregulation that allows emergence of autoimmunity after AHSCT (8, 9, 11, 12). In hematological AD after AHSCT, the reaction direction is most consistent with donor immune recognition of donor antigens. In cases of non-hematologic AD after AHSCT, donor immune recognition of shared donor-host antigens is likely, but residual tissue resident antigen presenting cells may be of host origin even when full donor chimerism is confirmed in circulating immune cells. Thus, the potential for non-hematologic AD to be of host against host direction cannot be eliminated at the present time. Future studies may enable delineation of immune cell chimerism in tissue and thus may provide clarity on the ontogeny of the immune reaction in non-hematologic AD after AHSCT.

The goal of this article is to comprehensively review hematologic and non-hematologic AD after AHSCT in children, summarized in **Table 1**. Furthermore, features of adult AD following HSCT and corresponding ADs observed outside of the HSCT setting are described with the aim of improving the combined understanding of the underlying biology, risk factors, and identifying potential interventions or changes to existing HSCT platforms that may need to be implemented.

HEMATOPOIETIC AUTOIMMUNE MANIFESTATIONS FOLLOWING AHSCT

Incidence

The most common autoimmune manifestations following AHSCT in pediatric and adult recipients are hematologic, i.e., autoimmune cytopenias (AICs) (8, 9). AICs are classified based on the affected lineage/s and include autoimmune hemolytic anemia (AIHA), immune thrombocytopenic purpura (ITP), Evans syndrome (AIHA and ITP), autoimmune neutropenia (AIN), and tri-lineage autoimmune cytopenia (AIHA with ITP and AIN) (12–14). While AIHA is the most commonly diagnosed AIC following AHSCT, accurate reporting of ITP in this setting is challenging because there are several alternate potential transplant related causes of thrombocytopenia that have to be excluded prior to making the diagnosis and laboratory confirmation of AIHA is more readily obtained compared with detection of anti-platelet antibodies, which are not uniformly observed in ITP (8, 9, 14). Of note, in the setting of AHSCT for acquired aplastic anemia, ITP incidence reportedly exceeds that of AIHA (14), and is the same as that of AIHA in autologous HSCT for ADs (15). Meanwhile, the incidence of AIHA in the general population is lower than in the post-AHSCT setting and far less common in children compared to adults (16, 17).

Despite AICs being rare following AHSCT with an estimated incidence of ∼3% in adults (14, 18–26) and ∼5% percent in children (9, 13, 26–31), they occur with much greater frequency in certain AHSCT settings. The highest AIC rates, over 50%, were reported in very young infants that received unrelated cord blood (UCB) grafts for inherited metabolic disorders (12) and those who received AHSCT for Wiscott-Aldrich syndrome (WAS) (32), with antithymocyte globulin (ATG)-containing conditioning used in both studies. Several additional case series that demonstrated higher than average AIC incidence of 20–35% (10, 11, 33) also involved children undergoing AHSCT for non-malignant indications following intense lymphodepletion.

TABLE 1 | Incidence, risk factors, associated clinical features, and proposed mechanisms for autoimmune disease after AHSCT.

Disease	Incidence	Risk factors and clinical features	Proposed mechanism
Autoimmune cytopenia/s, including AIHA, ITP, Evans syndrome, AIN, and tri-lineage autoimmune cytopenia	Rare, but common in subsets of pediatric ASHCT compared to adult recipients	Non-malignant transplant indication	Skewing of immune reconstitution toward unregulated B cell proliferation and auto-antibody production due to impaired peripheral tolerance in the absence or reduced function of T cells
		Unrelated donor	
		Lack of TBI	
		cGvHD	
		peripheral or UCB stem cell source	
		additional risk factors in adult HSCT: T cell depleted grafts, ATG and alemtuzumab in the peri-transplant setting, GvHD	
Autoimmune thyroid disease, including Hashimoto thyroiditis and Graves' disease	Rare, except for one pediatric study reporting 25% rate	Non-malignant transplant indication	Unchecked autoantibody production against thyroid antigens In adults, transmission of autoantibody in the graft has been described
		T cell depleted graft and/or ATG or alemtuzumab peri-transplant	
		Lack of TBI	
		Immune recovery (albeit dysfunctional)	
Guillain-Barre syndrome	Rare, 10 pediatric cases described	Malignant indication for AHSCT	Potentiation of Ara-C neurotoxicity Possible molecular mimicry of PNS antigens by viral antigens
		Associated with infection or viral reactivation	
		Antecedent use of high dose Ara-C (practice discontinued after this association was identified)	
Myasthenia Gravis	Exceedingly rare, 2 pediatric cases reported	Non-malignant transplant indication	Unchecked autoantibody production against acetylcholine receptor
		Acute and chronic GvHD	
		Manifested upon tapering of immunosuppression	
		Generalized severe presentation	
		No association with thymoma	
Transverse myelitis	Exceedingly rare, 1 pediatric case and several adult cases	Non-malignant transplant indication	Unchecked inflammatory milieu
		Unrelated donor	
		Lack of TBI	
		Peri-transplant use of alemtuzumab	
Other CNS manifestations, including vasculitis, white matter lesions and atrophy	Exceedingly rare, in children and adult recipients	Unrelated donor	Lymphocytic infiltration of CNS vasculature or parenchyma by immune cells of donor origin
		Manifested upon tapering of immunosuppression	

(Continued)

TABLE 1 | Continued

Disease	Incidence	Risk factors and clinical features	Proposed mechanism
Vitiligo	Exceedingly rare, 8 pediatric cases reported, similar incidence in adult recipients	One pediatric series with non-malignant indication for AHSCT and no GvHD Another subset of patients with a malignant indication for HSCT and a GvHD association	Unchecked autoantibody production against melanocytes
Autoimmune hepatitis	Exceedingly rare, 2 pediatric and one adult case described	Possibly associated with GvHD Responded to immunosuppression	Portal eosinophilia and plasma cell infiltration of donor origin
Rheumatologic diseases, including arthritis, spondyloarthropathy, vasculitis, phospholipid antibody syndrome	Exceedingly rare in children with 2 possible young adult cases reported, more common in adults undergoing AHSCT for autoimmune disease	Both pediatric patients received AHSCT for a malignant indication	Predisposition toward autoimmunity that may be potentiated by ASHCT

One such study reported a combined 50% rate of hematologic and non-hematologic AD in children following AHSCT for chronic granulomatous disease (CGD) following conditioning that included alemtuzumab (10).

Risk Factors

The following significant risk factors for the development of AIC in children undergoing HSCT have been recently confirmed: non-malignant primary diagnosis, the use of an unrelated donor, lack of total body irradiation (TBI) in the conditioning regimen, chronic GvHD, and the use of peripheral or UCB stem cell source (9). Similar risk factors have been identified in the adult AHSCT literature with the strongest association seen between AIC and the use of unrelated donors, T cell depleted grafts, conditioning regimens that include ATG and alemtuzumab, and GvHD (8). These studies provide important clues about the pathophysiology of AIC following AHSCT since randomized clinical trials and pre-clinical modeling to understand mechanisms of AIC are lacking.

Proposed Pathophysiology of AIC After AHSCT

As stated above, non-malignant disease and the use of unrelated donor grafts are most strongly (9) and consistently (10–12, 27, 28) associated with the development of AIC in children. The proposed pathophysiologic mechanism that could explain their combined role in the emergence of autoimmunity after HSCT is that AD may be driven by an impairment in peripheral immune tolerance due to the lack of functional T cells, in particular T regulatory cells (T_{regs}) with resultant inability to suppress B cell expansion after HSCT (11), **Figure 1A**. In the post-transplant time frame when AICs typically emerge, the thymus has yet to recover from transplant related insults. Thus, peripheral tolerance mechanisms are likely dominant in keeping autoimmunity in check. Peripheral tolerance is mediated by T cells, which are expected to be preferentially eliminated in conditioning regimens used in unrelated donor HSCT that include ATG and alemtuzumab. Alemtuzumab targets CD52, which is expressed on T cells with greater density than other lymphocytes (34, 35), and can be particularly effective at inhibiting CD4 + T cell recovery compared to other T cell types (36). Both drugs have long lasting *in vivo* effects and would be expected to provide sustained T cell suppression in the post-HSCT period. T cell depletion is also commonly performed on haploidentical grafts, which too have been associated with greater propensity for AIC (33). Unregulated polyclonal B cell expansion would be more likely in the absence of T cell immunoregulatory signals combined with the anticipated pro-inflammatory viral stimuli commonly encountered in the immediate post-transplant period, such as CMV, EBV, and HSV infection or reactivation (23). In patients with cGvHD, another established risk factor for AIC after AHSCT (30), B cell alloantibody production is a common feature that stems from the inability of T_{regs} to dampen alloimmunity (37). Furthermore, cGvHD has been shown to respond to adoptive T_{reg} transfer in multiple pre-clinical and clinical studies (38, 39) and demonstrated the

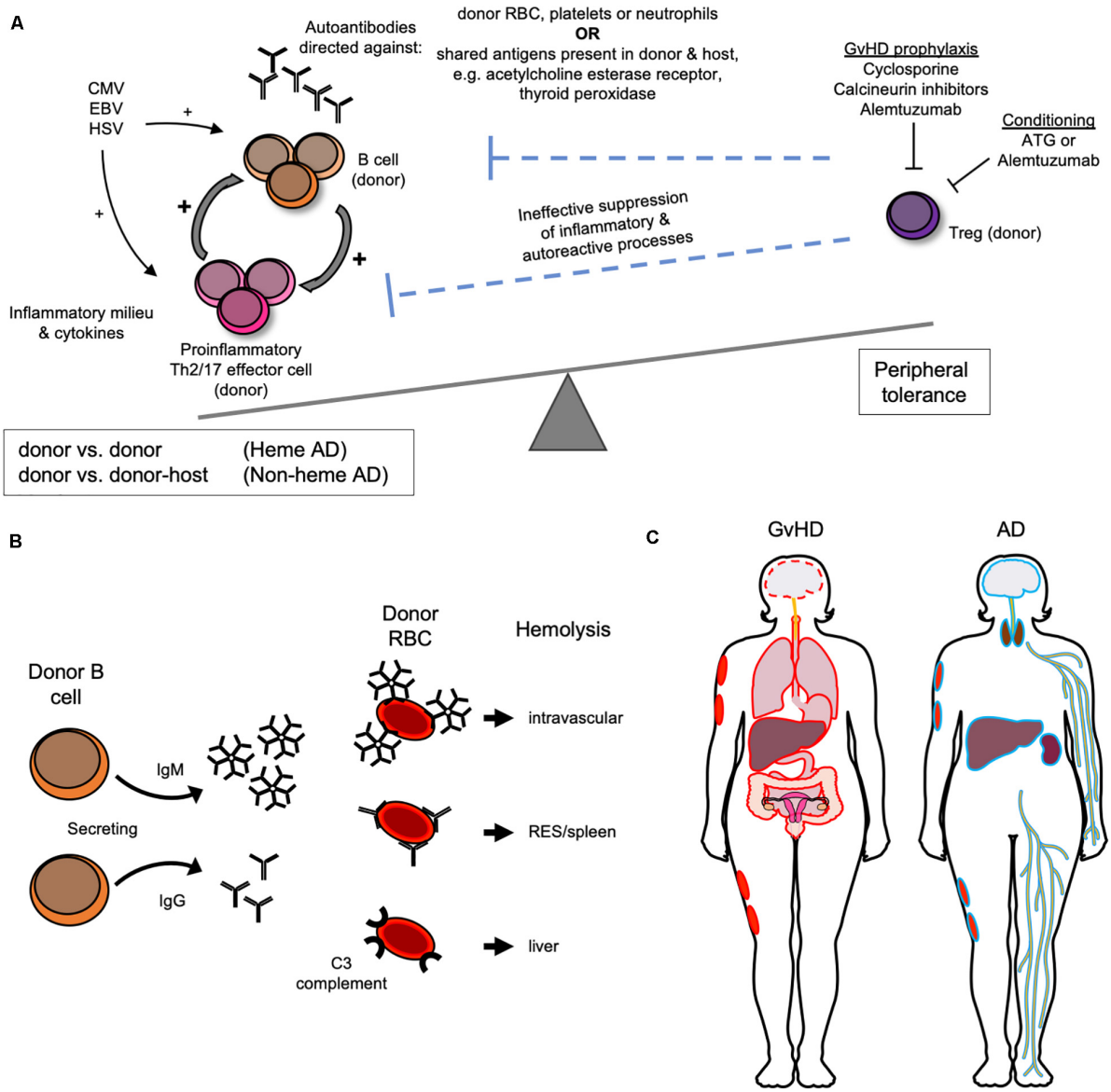

FIGURE 1 | Biological and clinical features of autoimmune manifestations following AHSCT. **(A)** Proposed pathophysiology for the development of autoimmune manifestations after AHSCT as a result of donor T regulatory (T reg) cell impairment. **(B)** Donor immune reactions directed against donor red blood cell (RBC) antigens mediate autoimmune hemolytic anemia after AHSCT. **(C)** GvHD versus "autoimmune" non-hematologic tissue/organ targets outlined in red and blue, respectively.

ability to prevent AIHA in animal models (17). Additionally, T_{reg} impairment is implicated in idiopathic AIHA (17, 40). Immunophenotyping of patients with AIC post AHSCT has confirmed low circulating CD4 and CD8 T cell numbers, low T_{reg} numbers (11, 26, 41), as well as Th2 skewing (13). The latter is a shared feature of idiopathic and AD associated AIHA (17, 40), and animal models of the former. Th17 polarization has also been implicated in the pathogenesis of idiopathic AIHA (40), but has yet to be confirmed in AHSCT-associated AD. Cyclosporine (CSA), the most common form of GvHD prophylaxis used in the multiple case series with higher AD rates presented above, would also be expected to have a greater impact on T rather than B cell subsets, in particular on IL-2 dependent expansion of T_{regs} (42). Of note, cyclosporine-, and calcineurin-based immunosuppression and incomplete lymphodepletion are associated with AICs after both solid organ transplantation (SOT) (41, 43–46) and non-malignancy HSCT and could point to shared biological mechanisms. Supporting this notion is the observation in the AHSCT AIC where withdrawal of CSA followed by anti-B cell directed therapy with rituximab or anti-CD38 resulted in clinical responses (11, 47). Finally, decades ago cyclosporine was shown to induce autologous GvHD-like reaction purportedly via disruption of peripheral tolerance (48).

While the pathophysiology of AICs is not fully understood, it does appear that AIHA is primarily driven by donor immune reactions against donor erythrocytes (9, 23) (**Figure 1B**). Donor chimerism was not uniformly reported in studies of AIC after AHST, but was usually full donor at the time of AIC diagnosis when reported (13, 23, 24, 31, 41), which implicates a donor against donor process. Thalassemia HSCT is characterized by higher AIC incidence, which could implicate prior transfusion and resultant alloimmunization playing a role in AIHA via the host versus donor response. Although if true, this would also be expected with other non-malignant indications, such as sickle cell disease, which have not been identified as risk factors for AIHA after AHSCT. Other AICs after AHSCT, ITP, and AIN, are also antibody mediated and evidence suggests that they too are donor against donor (12, 13, 41).

Distinguishing AIHA From Major and Minor ABO Mismatch Hemolysis

While ABO mismatched AHSCT can be associated with delayed engraftment and other complications, it is often unavoidable in the HSCT setting (49) where HLA matching is prioritized over ABO and the two are not genetically linked (49). AIHA following AHSCT is distinct from ABO mismatch driven hemolysis, which can arise from either major or minor ABO mismatch between the donor and host (49). The ABO mismatch driven hemolysis can present with (1) immediate intravascular hemolysis mediated by host ABO antibodies directed against donor RBCs in the graft; (2) delayed hemolysis from residual host cells reacting to RBCs produced by the engrafted donor marrow, and (3) pure red cell aplasia (PRCA). Immediate intravascular hemolysis is more common when marrow is used as an HSCT source due to potential transfer of donor RBC in the stem cell graft, which can be minimized with RBC removal prior to graft infusion. Additionally, the timing of this clinical presentation readily distinguishes this alloimmune process from AIHA (49). Delayed hemolysis from residual host cells reacting to RBCs produced by the engrafted donor marrow typically occurs later in the post-HSCT period and mediates delayed engraftment commonly observed with ABO mismatched HSCT that typically occurs ∼5 weeks after graft infusion compared to ∼3 weeks that is routinely observed with ABO matched HSCT or peripheral blood stem cell grafts. Delayed hemolysis can present months after HSCT if full chimerism is not yet established and requires chimerism studies to distinguish it from AIHA. PRCA results from destruction of erythroid progenitors within the marrow by residual anti-donor antibodies. It may present in the same time frame as AIHA, within 6 months of HSCT, and have a similar clinical presentation despite the mechanisms between these entities being distinct, i.e., with ABO mismatch being host antibody driven and with AIHA being donor antibody driven (23) reactions against donor RBC.

AIHA Diagnosis

Diagnosis of AIHA is established by performing a direct anti-globulin test (DAT), also called direct Coombs test, which detects *in vivo* coating of erythrocytes with antibodies (16). In the DAT, non-specific anti-human globulin will agglutinate RBCs coated by all antibody isotypes, IgA, IgM, IgG, etc. (16, 40) with agglutination more likely to be detected at warm testing temperatures when IgG antibody subtypes (IgG1, IgG2, IgG3, and IgG4), and/or C3 (complement) are present on the RBC and cold testing temperatures when IgM is bound. IgG mediates extravascular hemolysis via the reticuloendothelial system that is mainly splenic (**Figure 1B**). The pentameric antibody IgM is most efficient at fixing complement both in laboratory testing and *in vivo* and can cause significantly greater *in vivo* RBC destruction than other isotypes mainly via intravascular hemolysis. Meanwhile, C3-mediated hemolysis predominantly occurs in the liver. The majority of AIHA after AHSCT is warm type, followed by cold, then mixed (41). The type is important to ascertain because it can guide treatment, with cold agglutinin disease being less likely to respond to splenectomy since the RBC destruction would not be expected to occur in the reticuloendothelial system. If the DAT is positive, monospecific anti-IgG, anti-IgM, and anti-C3 antisera are used to further define the autoantibody. In cases of AIHA following AHSCT when monospecific testing was reported, IgG was commonly detected in combination with C3, with some cases of IgG, and IgM co-occurring. IgG when present was eluted to test for specificity. When looking at available studies, there was a suggestion that co-occurrence of warm and cold AIHA may have a more severe course (11), but no consistent pattern of severity of hemolysis or likelihood of response to treatment was clearly apparent for a particular type of antibody in post-AHSCT AIHA.

Treatment and Prognosis of AIHA After AHSCT

Post-AHSCT AIHA is most commonly treated with intravenous immunoglobulin (IVIG) or steroids, rituximab monotherapy, plus a variety of other approaches. Only a third of the cases are steroid responsive. Rituximab has a reported ∼60–80% response rate. Other combinatorial immunosuppressive approaches have been described, including azathioprine, cyclosporine, 6-mercaptopurine, mycophenolate mofetil, danazol, cyclophosphamide and vincristine, bortezomib, alemtuzumab, sirolimus, and second stem cell infusion (9). A treatment strategy of reducing immunosuppression that is more heavily directed against T cells (i.e., CSA and calcineurin inhibitors) and instead using anti-B cell directed therapies in a subset of patients resulted in resolution of AIHA once T cell reconstitution was achieved (11). While prognosis appears to be marginally better in children than in adults, mortality in some cases did occur (9) and overall higher mortality in AHSCT recipients with AIHA compared to those without was reported (13). Also, AHSCT associated AIHA appears to be more treatment refractory (9) compared to non-AHSCT associated cases with the latter having ∼80% of response rate to corticosteroids within 3 weeks, splenectomy having a 70% response rate, and rituximab having a 60% response rate. Several recalcitrant cases of post-AHSCT AIC, including AIHA, have been recently reported to respond to daratumumab (47, 50, 51), which targets CD38 that is expressed on plasmablasts and plasma cells.

Other Hematologic Autoimmune Manifestations

Immune thrombocytopenic purpura after ASHCT appears to have slightly higher incidence in children than adult recipients (9, 52), but is quite rare in both, limiting understanding of its pathophysiology and prognostication. Idiopathic (53) and post-AHSCT ITP (54) have been associated with T_{reg} dysfunction. In both clinical settings, ITP diagnosis is that of exclusion. Antibodies against platelets were not consistently obtained across the reported case series and reviews of ITP occurring after AHSCT, but when testing was reported, ∼75% of the clinically determined cases were associated with detectable direct and indirect anti-platelet antibody (12, 28). The antigenic specificity of the antibody was even less frequently reported, but when available appeared to be directed against similar thrombocyte antigens as non-HSCT associated ITP, i.e., platelet membrane glycoproteins IIb-IIIa or Ib-IX. As already described for AIC in general, donor chimerism was most often full donor at the time of diagnosis, confirming that the post-AHSCT ITP is frequently autoimmune, i.e., donor against donor. Passive transfer of donor ITP has been described in the adult AHSCT literature (31, 52), but not in the pediatric setting. For post-AHSCT ITP and Evans syndrome, systemic corticosteroids and IVIG were the typical first line treatment, with a majority of the patients eventually receiving multiple lines of therapy, including rituximab, which resulted in a few complete responses (30, 31), daratumumab (47, 51), vincristine, cyclophosphamide, azathioprine, 6-mercaptopurine, alemtuzumab, plasma exchange, stem cell boost, splenectomy, rapamycin, romiplostim, and eltrombopag (28). It appears that ITP after AHSCT is often chronic, recalcitrant to treatment, and can result in mortality (15, 28, 31, 55).

Autoimmune neutropenia after AHSCT is also an antibody driven process, although in most AIC studies confirmatory testing of direct or indirect anti-neutrophil antibodies was not reported, and when reported was infrequently positive (12, 14, 48). Similar treatment approaches for AIN have been reported as for AIHA, ITP, and Evans syndrome, but again, given the rarity of this AHSCT complication, prognostication is not appropriate. Acquired hemophilia, i.e., development of factor VIII inhibitors, has been reported in the setting of autologous HSCT for AD (15, 20, 48, 56, 57), but not in the setting of adult or pediatric AHSCT. Thrombotic microangiopathic manifestations following AHSCT as a result of high ADAMTS 13 inhibitor levels have been described (8) albeit not in children.

NON-HEMATOLOGIC "AUTO" IMMUNE DISEASES AFTER AHSCT

Non-hematologic manifestations after AHSCT that are potentially autoimmune in mechanism, involving the thyroid, central and peripheral nervous systems, integument, liver, and kidney are far less common in children than in adults and have not been extensively described or reviewed in the setting of AHSCT (**Figure 1C**). Whether these conditions are autoimmune or alloimmune is an ongoing research question because immune effectors are of donor origin but are directed against targets that are common to the donor and host, e.g., acetylcholine esterase receptor, thyroid peroxidase, etc. These conditions are antibody mediated, which is also the case outside of the AHSCT setting when they are truly autoimmune. Such ADs have also been reported in the setting of dysregulated immunity associated with immunosuppression after SOT where the autoimmune reaction is that of host against host. In contrast, GvHD is a common, well-recognized, and better described immune driven complication of AHSCT that is mediated by donor immunity against non-hematopoietic host organs and tissues. Donor against host directed antibodies are implicated in GvHD pathophysiology. While myasthenia gravis (MG) and peripheral neuropathies are not formally part of cGvHD diagnostic criteria, they are considered "other" or "associated features" of cGvHD when present concomitant with classical GvHD manifestations and are observed in the setting of cGvHD. Whether non-hematologic thyroid, peripheral and central nervous system (PNS and CNS) manifestations and vitiligo should be considered "other GvHD" or referred to as autoimmune is not clear at this time. Since in the adult HSCT literature they are most often described as autoimmune, in this review they will be referred to as such for consistency.

Autoimmune Thyroid Disease

Thyroid ADs after AHSCT, mainly Hashimoto thyroiditis and Graves' disease, are mediated by antibodies against thyroid antigens and have been described primarily in the setting of autologous and allogenic HSCT for AD in adults (8, 15, 22, 48, 58–60), and AHSCT for non-malignant indications in children (10, 55, 61–63). For the reported series in children, common features were T cell depletion (graft *ex vivo*, ATG or alemtuzumab peri-transplant), non-malignant indication (all cases described here), lack of TBI (in all cases summarized here), and an incidence of 1–25%. The highest Autoimmune Thyroid Disease (AITD) incidence was described in a cohort of 24 boys that received matched sibling or unrelated donor AHSCT after alemtuzumab conditioning (10), with 5 cases of Graves' disease and one case of Hashimoto thyroiditis, which represents an unusually high rate. Of note, non-AHSCT AITD has also been associated with alemtuzumab treatment in multiple sclerosis (62). Multiple reports discussed in this review of autoimmune hematologic manifestations in children did not observe AITD and three pediatric case series that described AITD had also reported on hematologic AD occurring at higher rates than AITD (55). In the pediatric series that reported the highest AITD rate, above average incidence of AIC of 20% was also observed, as was the rate of CNS and PNS manifestations with 8% and 4%, respectively. Thus, this series appears to have had unique features resulting in significantly higher rates of AITD, an otherwise rare complication of AHSCT. In both children and adults, AITD diagnosis after AHSCT was established with appropriate antibody detection and treatment approaches were supportive, i.e., thyroid suppression for hyperthyroidism and replacement hypothyroidism. Unlike hematologic AD, there were no deaths attributable to AITD in the pediatric studies. In adult AHSCT

reports of AITD, possible transmission of donor autoantibodies in the graft has been reported (8, 48, 64–66) while in children this etiology does not appear to play a role. Interestingly, one study noted that recovery of CD4 T cell counts was coincidental with onset of AITD, similar to the setting of immune restoration in patients with HIV in whom AITD has also been reported (62). In many of the pediatric post-AHSCT AITD cases reviewed here, onset was typically later than that of AICs, perhaps suggestive that immune recovery, albeit dysfunctional since it resulted in a manifestation of autoimmunity, may play a role in the emergence of AITD in the post-AHSCT setting.

Intriguingly, another autoimmune mediated endocrinopathy, type 1 diabetes mellitus, has not been described in the pediatric studies reviewed here for hematological and non-hematological AD after AHSCT or otherwise, while insulin resistance after AHSCT is commonly described in adults and children after AHSCT (67–69).

Neurological Autoimmune and Graft-Versus-Host Disease Manifestations After AHSCT in Children

Neurological manifestations of autoimmunity after AHSCT and cGvHD have been reported to affect central and peripheral nervous systems (8, 70). Neurological manifestations are not included in the definitive cGvHD diagnostic criteria, but nonetheless are considered "associated" with cGvHD when involving the PNS: peripheral nerve, including Guillain-Barre syndrome (GBS), neuromuscular junction, i.e., MG, and muscle. Of the latter, myositis and polymyositis are deemed the only "distinctive" neurological manifestations of cGvHD (70); however, these entities are not described in this review due to paucity of reports on these manifestations in pediatric AHSCT setting. MG and peripheral neuropathies are considered "other" GvHD or "associated with GvHD" in the presence of classical cGvHD manifestations in other organs. For CNS manifestations to be regarded as "definitively" cGvHD, they have to occur with classic cGvHD manifestations in other organs, other causes have to be excluded, and presence of imaging, CSF, and biopsy proven evidence of alloreactivity and proven response to immunosuppression are necessary (70). Notably, most antibody driven neurological entities after ASHCT manifest in the setting of full donor chimerism, hence the cGvHD or autoimmune processes again arises.

PNS Manifestations

Guillain-Barre syndrome is a rare complication of AHSCT in children and adults (8, 48, 71, 72), and in the latter more likely in allogenic than autologous HSCT (72). We found 10 reported cases of pediatric GBS after HSCT in the literature (10, 71, 73–75). Of those, 8 were in the setting of HSCT for a malignant indication and 2 for CGD (10). In the former, 2 cases were reported following autologous HSCT (71, 74), and 2 were associated with infection, bacterial sepsis, and parainfluenza 1. No evidence of GvHD had been described in all 10 cases, although 4 occurred within the immediate post-transplant window, hence potential association with GvHD would not be evaluable (73, 75). In adult recipients, GBS has been associated with GvHD (70) and with infection/reactivation of CMV, HSV, and HHV6 (8, 71, 76, 77) as well as antecedent infections (70). A strong association between GBS in the first week after AHSCT and the use of antecedent high dose Ara-C was observed in two reports involving 4 children (73, 75), and resulted in 3 fatalities (75). The remaining patients responded to treatment with systemic corticosteroids, IVIG, plasmapheresis, and rituximab.

Two cases of MG after HSCT have been reported in children, both in the setting of non-malignant indication and mismatched sibling AHSCT (78, 79). In both instances, MG was generalized (non-focal), was associated with cGvHD and manifested as immunosuppression was being tapered, with one patient having also experienced acute GvHD and the other engraftment syndrome. Acetylcholine receptor antibodies were present in both cases. One patient was treated with pyridostigmine, atropine, AZA, thymectomy and plasmapheresis, and was eventually responsive to thalidomide (78). The other patient was found to be initially ANA positive prior to the development of MG manifestations and presented with severe generalized MG that required intubation and eventually resolved after treatment, which consisted of MMF, IVIG, methylprednisolone, pyridostigmine, cyclosporine, plasma exchange, and finally a course of rituximab. Notably, outside of the HSCT setting, MG is quite rare in children and when it occurs in pre-pubertal setting is often ocular and remains so without generalization. In such settings it is also less likely to be antibody positive and has a favorable prognosis, including reported spontaneous remissions (80). In the adult HSCT literature, 23 cases of MG have been reported after allogeneic and autologous HSCT (8, 48, 70), with a majority of the patients having acetylcholine receptor antibodies and few with musculoskeletal receptor antibodies. The majority were associated with cGvHD and most presented upon discontinuation or tapering of cGvHD immunosuppression. Treatment was similar to idiopathic MG, i.e., pyridostigmine, acetylcholine esterase inhibitors, and immunosuppression. In contrast to MG in adults in the non-HSCT setting there was no observed association with thymoma (8). Of note, MG in the setting of immunosuppression (ATG or alemtuzumab) after renal transplantation has been reported (81).

Myositis and polymyositis although rare after AHSCT are associated with cGvHD (70), but have not been well described in children.

CNS Manifestations

CNS immune manifestations that are cGvHD related have been rarely reported in adult HSCT literature and can be ascribed to cerebrovascular, stroke-like presentations, encephalopathy with resultant seizures, and demyelination processes (70, 82–84). Isolated cases of immune CNS disease after HSCT have been reported in children (85–88), and only one of these presented as transverse myelitis (TM) that was not associated with GvHD, but was associated with AIC and GBS (10), while several cases of TM after HSCT have been reported in adults (8, 89). Isolated optic neuritis has also been described in the adult AHSCT literature (70), but has not been reported in

the pediatric AHSCT setting. A case of CNS granulomatous angiitis/vasculitis was described in an 18-year old recipient of mismatched unrelated graft, in association with weaning of immunosuppression for resolving acute GI GvHD. The patient was found to have generalized CNS atrophy on MRI, which was obtained due to progressive spasticity and seizures. The patient's cognitive dysfunction worsened further to progressive encephalopathy with concurrent skin cGvHD onset that manifested 5 months after HSCT (87). Interestingly, short tandem repeat (STR) analysis of a micro dissected section of her inflamed arteriole confirmed that the lymphocytic infiltrate was of donor chimerism. The patient improved following very high doses of steroids, stabilized, but eventually lost her graft and died 2 years after HSCT. Similarly, in adult patients with CNS-GvHD, infiltrating lymphocytes were of donor origin (70, 83) suggesting that CNS-GvHD can be appropriately classified as a GvHD manifestation. Two case reports have described diffuse white matter lesions in children with GvHD and a case of cortical atrophy associated with cGvHD (85, 86). While CNS immune manifestations after HSCT in children are extremely rare, they are primarily associated with GvHD and tapering of immunosuppression and likely represent alloimmune rather than autoimmune processes.

Skin Autoimmune GvHD-Associated Manifestations After AHSCT

Vitiligo is a rare manifestation in the AHSCT setting, which is mostly observed with concomitant acute or chronic skin GvHD. A total of 8 pediatric vitiligo cases after HSCT have been described in the literature in several case series combining adult and pediatric patients (55, 90–93). Cathcart et al. described one pediatric case of extensive vitiligo developing 4 years after mismatched sibling T cell depleted HSCT for malignancy, interestingly without concomitant chronic GvHD, albeit with a history of resolved acute skin and liver GvHD (92). In the same series, nine adult cases of post-HSCT vitiligo were described. All had been transplanted for a malignant indication and were associated with acute or chronic GvHD. Another case series reporting on vitiligo after AHSCT for malignant indications included a pediatric patient with extensive vitiligo after AHSCT for ALL associated with skin and GI GvHD (91). Sanli et al. described six cases that were observed in a single transplant center in 421 consecutive patients (93). One of these 6 cases occurred in a 19-year-old who developed vitiligo 6 months after matched sibling HSCT for CML, and which was associated with liver cGvHD, alopecia areata, and subsequently oral and lichenoid skin GvHD. Finally, five additional cases of vitiligo in children were reported, one in association with lichenoid skin cGvHD after AHSCT for aplastic anemia (94) and four in the setting of peripheral blood stem cell grafts for hemoglobinopathy (55) without an association with GvHD or other AD. Vitiligo has been associated with autoimmunity outside the HSCT setting (95) and can be mediated by antibodies directed against melanocytes (55, 96), and thus could represent an autoimmune process. Nevertheless, alloimmunity cannot be excluded in this clinical entity as half of the reported pediatric cases of vitiligo after AHSCT were associated with concomitant classic skin cGvHD.

Other Rare Autoimmune Manifestations After HSCT

Whether autoimmune-like hepatitis (AIH) occurring after HSCT truly represents an autoimmune manifestation versus drug-associated hepatitis, i.e., by cyclosporine, is difficult to ascertain since only two pediatric cases (55, 97) and one adult case have been reported (98). The liver biopsy in all three cases showed portal eosinophilia and plasma cell infiltration, with chimerism of the lymphocytic infiltrate demonstrated to be of donor origin in the adult patient. In one pediatric case after AHSCT for a metabolic disorder there was no concurrent or prior history of GvHD (55) and in the other AIH was associated with GvHD (97), as was the case reported in the adult. All three cases were steroid responsive. In the adult patient, azathioprine was used in combination with steroids and in one pediatric case ursodiol was combined with steroid (97). This was the only patient that had been on cyclosporine prior to AIH diagnosis, upon which it was discontinued. It was not clear from the description of the case whether the other pediatric patient had been on cyclosporine after HSCT.

Whether the kidney is a target of autoimmunity after AHSCT or a manifestation of GvHD-associated alloimmunity is not clear. Reports of membranous nephropathy and minimal change disease have been described in the post-HSCT setting, most commonly observed in association with cGvHD and particularly upon weaning of immunosuppression (99). However, a few cases have occurred in the autologous HSCT setting without GvHD (100), with two such reports in children (101, 102). The pathophysiology of these renal manifestations appears to be mediated by antigen-antibody complexes deposited in the glomerular subendothelium as a result of either kidney antigens being targeted or indirect injury from deposition of the complexes targeting antigens exogenous to the kidney (99). The pediatric cases of immune-mediated nephropathy after HSCT had been reportedly treatment responsive to systemic immunosuppression with corticosteroids, which are also used outside of the HSCT setting for these clinical entities.

Rheumatologic diseases possibly autoimmune in etiology have been described after autologous and allogeneic HSCT, including rheumatoid arthritis, psoriatic arthritis, spondyloarthropathy, vasculitis, and antiphospholipid antibody syndrome, more commonly after AHSCT for autoimmune indications (8, 15, 48). In one report, two young adult patients had been described as having possible autoimmune arthritis (103). A 24-year-old man who underwent AHSCT for a T cell lymphoma and antecedent history of arthritis (concomitant ANA titers negative) developed acute symmetrical polyarthritis involving the proximal interphalangeal (PIP) and metacarpophalangeal (MCP) joints, wrists, and knees 1 month after discontinuation of post-HSCT immunosuppression. This was associated with a high ANA titer. The patient experienced spontaneous resolution of symptoms 6 months later. The other patient was a 2l year-old woman who received matched sibling HSCT for AML, with resolved

acute skin GvHD skin and ongoing lung cGvHD. She developed bilateral shoulder and unilateral hip and knee pain, with arthrocentesis finding of coagulase-negative Staphylococcus. Despite appropriate antibiotic treatment, synovitis now involving bilateral knees and several PIP joints persisted and was then treated with anti-inflammatory medications (not specifically stated) and intra-articular injection of the knee with a corticosteroid, which resulted in complete resolution of the symptoms. Whether the arthritis was immune- or infection-driven in the latter case is not entirely clear. Otherwise, autoimmune arthritities have been almost exclusively described in the setting of HSCT for autoimmunity (15, 22), which indicates that a predisposition toward autoimmunity in combination with the transplant factors likely plays a pathophysiological role in such manifestations.

FUTURE DIRECTIONS AND SUMMARY

Identification of risk factors for autoimmune manifestations in children and adults undergoing ASHCT as well as comprehensive diagnostic characterization of these rare cases are imperative to advance the understanding of the biological mechanisms behind these complex set of conditions. Many AD manifestations remain poorly understood due to lack of prospective studies and pre-clinical models. The former would be difficult to implement due to rarity of these complications, but subsets of patients have been identified to have higher inherent risks for developing AD (8, 9), which could facilitate such efforts. Such studies should include the collection of clinically well annotated samples of blood and tissue. Innovative study designs adapted to rare entities and development of novel minimally invasive biological sampling techniques suitable for pediatric patients are imperative to move this area of research forward. Emerging diagnostic approaches could provide further mechanistic insights into pathophysiology of manifestations. For example, single cell sequencing approaches are now able to capture TCR diversity, and as analytical methods advance valuable insights into antigenic targets, i.e., TCR specificity, may be identified (104). Patient outcomes could be improved by selection of targeted treatments (if targets are known), which would potentially be more effective and less toxic than general immunosuppression (105). For example, unregulated B cell expansion had been implicated as a potential mechanism of AD and the use of anti-B cell agents has demonstrated clinical efficacy in steroid refractory cases (28, 48). T_{reg} dysfunction is a common feature of GvHD (106) and AD after AHSCT and perhaps *in vivo* T_{reg} expansion is a strategy that could be attempted in the setting of AD after ASHCT. Janus tyrosine kinase (JAK) inhibitors were developed for ADs outside of the AHSCT setting (107) and have elicited clinical responses in patients with steroid refractory GvHD (108), which indicates they may also be efficacious in AD after AHSCT. In conclusion, AD likely stems from T and B cell dysfunction in the context of pro-inflammatory post-AHSCT milieu in which immunoregulatory processes are impaired. As risk factors for the development of AD after ASHCT are better characterized and the underlying biology is better understood, patients and families can be appropriately advised about the risks, changes to the existing BMT platforms can be implemented, and therapeutic targeting of underlying biology can be explored.

DISCLOSURE

The views expressed here are of the authors and do not represent views of the NIH or the US government.

AUTHOR CONTRIBUTIONS

NB and SP contributed to the conception of the article. NB wrote the manuscript. SP contributed to the manuscript revision. All authors contributed to the article and approved the submitted version.

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Management of PTLD After Hematopoietic Stem Cell Transplantation: Immunological Perspectives

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Post-transplant lymphoproliferative disorders (PTLDs) are life-threatening complications of iatrogenic immune impairment after allogeneic hematopoietic stem cell transplantation (HSCT). In the pediatric setting, the majority of PTLDs are related to the Epstein–Barr virus (EBV) infection, and present as B-cell lymphoproliferations. Although considered rare events, PTLDs have been increasingly observed with the widening application of HSCT from alternative sources, including cord blood and HLA-haploidentical stem cell grafts, and the use of novel agents for the prevention and treatment of rejection and graft-vs.-host disease. The higher frequency initially paralleled a poor outcome, due to limited therapeutic options, and scarcity of controlled trials in a rare disease context. In the last 2 decades, insight into the relationship between EBV and the immune system, and advances in early diagnosis, monitoring and treatment have changed the approach to the management of PTLDs after HSCT, and significantly ameliorated the prognosis. In this review, we summarize literature on the impact of combined viro-immunologic assessment on PTLD management, describe the various strategies for PTLD prevention and preemptive/curative treatment, and discuss the potential of novel immune-based therapies in the containment of this malignant complication.

Keywords: epstein-barr virus, T cell immunity, virological monitoring, prophylaxis, preemptive treatment

INTRODUCTION

Post-transplant lymphoproliferative disorders (PTLD) are heterogeneous lymphoproliferative diseases that stem from the unchecked proliferation of neoplastic lymphoid or plasmacytic cells in the setting of immunosuppression after transplantation (1–3).

PTLD in the hematopoietic stem cell transplantation (HSCT) setting are almost exclusively related to Epstein-Barr virus (EBV) infection; they usually develop between 3 and 6 months post-transplant, when virus-specific T cell immunity has not yet reconstituted, and are generally of donor origin. Although recipient-derived PTLDs have been described, they occur mainly in patients with poor graft reconstitution.

This review outlines our current understanding of the interplay between the virus and the immune system in the pathogenesis of these disorders after HSCT, and how our knowledge has improved current approaches to the management of PTLD in this clinical setting.

INTERACTIONS BETWEEN EBV AND THE HOST

EBV is a human γ-herpesvirus that infects more than 90% of the individuals worldwide (4–6). The virus enters the organism through the oropharyngeal route, and, in healthy subjects, causes a self-limiting primary infection. In normal, seropositive individuals, virus neutralizing antibodies control the spread of infectious virus particles and EBV-specific, HLA class I restricted, CD8+ cytotoxic T lymphocytes (CTL) specific for the early lytic cycle proteins kill cells entering the lytic cycle before they are able to release infectious virus particles (7).

The virus is B lymphotropic, and persists in resting memory B cells for the lifetime of the host in a non-pathogenic state that is invisible to the immune response (8). Initially, EBV infects naïve B cells in tonsillar lymphoepithelium, driving their activation through the expression of nine latent proteins (EBV nuclear antigens, EBNAs 1, 2, 3a, 3b, 3c, and LP and membrane antigens LMP 1, 2a and 2b), two small non-translated RNAs and about 40 microRNAs that constitute the EBV growth program (9). CTL directed to EBV latent cycle antigens prevent the outgrowth of cells latently infected with the virus (7).

Thence, the virus biology parallels that of normal mature B lymphocytes. EBV-infected naïve B-cells migrate to germinal centers in lymph nodes, lymphoid tissue present in mucosa, or the spleen. In germinal centers, normal B-cells undergo activation-induced somatic hypermutation and class switch recombination of the antigen-binding variable region of immunoglobulin genes. Within the germinal center, EBV-positive B cells shift to a more restricted virus transcription program, the default program (EBNA1, LMP1, and LMP2a expression), that helps rescue them into the memory compartment where the virus persists (6). Expression of viral proteins provides EBV-infected naïve B-cells with a selective advantage in the germinal center, and stimulates maturation into memory B-cells, which are the presumed reservoir of EBV (10).

Memory cells latently infected with EBV in the peripheral blood are in the latency program, and do not express any of the known latent proteins, unless they undergo division, in which case they express EBNA1, essential for the maintenance of the viral episome in dividing cells (8, 9). The frequency of infected memory B cells in a healthy carrier is stable over time, although it varies among different individuals, and has been calculated to be around 0.5×10^6, with only 1% residing in the peripheral blood (10). The virus is no longer pathogenic to the host, as the genes that drive cellular proliferation and may lead to neoplastic disease are no longer expressed. Likewise, the virus is safe from immune surveillance, as immunogenic viral protein expression, which serves as a target for the immune system is absent.

INTERPLAY BETWEEN EBV AND THE IMMUNE SYSTEM: PATHOGENESIS OF PTLD AFTER HSCT

EBV is considered an oncogenic virus, because of its association with tumors. EBV has latent proteins that can drive cellular proliferation, at least in B lymphocytes, such as LMP1 and LMP2, and these likely play a causative role in tumor development through inappropriate or deregulated gene expression (4, 8). HSCT recipients have impaired T-cell mediated immunity due to the pre-transplant conditioning regimen, immunosuppressive agents for prophylaxis of graft-vs.-host disease (GVHD) and GVHD itself (11–16). The reduced numbers of EBV-specific CTLs facilitate uninhibited growth of EBV-infected cells (17, 18). However, only a small number of EBV-positive patients develop PTLD after HSCT or other conditions of immunosuppression (11), and advanced PTLD is an oligoclonal rather than polyclonal disease, suggesting that other rare events contribute to the pathogenesis of the disorder. Thus, in order to have PTLD development, the growth program must be erroneously expressed in a B cell that cannot exit the cell cycle, and immunosuppression must prevent the elimination of these rare cells. At this stage, the disease may still be controlled by intervening on the immune status. Indeed, PTLD patients in early stages of disease may regress in response to the reduction of immunosuppression (19, 20) or after donor lymphocyte (DLI) (21) or EBV-specific CTL infusion (18, 22), strongly pointing to the essential role played by the underlying state of immunosuppression. However, in the absence of T cell immunity, such as it is often observed after T-cell depleted HSCT, proliferating cells acquire additional genetic or epigenetic damage, and these new cell clones may become unresponsive to immune surveillance (23).

A similar pathogenetic mechanism may be hypothesized in the rare cases of EBV-positive PTLD of T-cell origin. It has been postulated that some T cells may express CD21, the EBV receptor on B cells, and thus may allow viral entry (24).

RISK FACTORS FOR EBV-PTLD AFTER HSCT

Development of PTLD after HSCT is mainly associated with T-cell depletion of the graft before transplantation and the type/duration of immunosuppression employed to prevent and treat graft-vs.-host disease, and the degree of mismatch between recipient and donor (1, 2, 25–28). Consequently, PTLD is more often observed in T-cell depleted HSCT from haploidentical donors.

Among *ex-vivo* approaches, elective T cell depletion methods are associated with a greater increase in PTLD risk (26), as donor

Abbreviations: PTLD, Post-transplant lymphoproliferative disorders; HSCT, hematopoietic stem cell transplantation; EBV, Epstein-Barr virus; CTL, Cytotoxic T lymphocytes; a/cGVHD, acute/chronic graft-vs.-host disease; ATG, antithymocyte globulin; Mab, monoclonal antibody; CBT, cord blood transplantation; PTCy, post-transplant cyclophosphamide; PCR, polymerase chain reaction; IS, immunosuppression; UD, unrelated donor.

EBV-targeted cytotoxic T cells are removed from the inoculum, thus compromising specific immune surveillance. However, the use of lymphocyte depletion strategies that target both T and B cells, such as *in vitro* alemtuzumab (26) or combined depletion of αβ T-cell and CD19 B-cells (29), have a lower risk of PTLD, by delaying potential EBV-infected B cell proliferation until recovery of functional T cell immunity. This observation supports the concept that an imbalance between EBV-infected B cells and EBV-specific T cells favors neoplastic outgrowth of EBV-positive B cells. Likewise, *in vivo* depletion of T-cells using antithymocyte globulin (ATG) is associated with a higher risk of developing PTLD than the use of broad lymphocyte-targeting alemtuzumab monoclonal antibody (Mab) (26, 30, 31). Rabbit ATG was suggested to be more likely to cause profound lymphodepletion than horse ATG (32). However, a recent study in the setting of pediatric and adult haploidentical HSCT show comparable rates of EBV DNAemia and PTLD (33). The effect of ATG seems dose-dependent, as high-dose ATG had a 2.3-fold higher risk of PTLD than low-dose (30, 34).

The degree of HLA matching between donor and recipient correlates with the development of PTLD. HSCT from a HLA-mismatched donor has been observed to be associated with a higher risk of PTLD than the use of a HLA-identical donor (26, 27). Although a certain degree of mismatch between recipient and donor may impair EBV antigen recognition by HLA-restricted donor T cells, the risk associated with HLA mismatch is mainly due to the *in vitro* and/or *in vivo* T-cell depletion strategies employed in mismatched transplants to facilitate engraftment and prevent GVHD: the combination of different depletion approaches results in additive risk (26).

The incidence of PTLD with regard to the different types of donors ranges from 1% in HSCT from matched related donors to 4% for matched unrelated and 11% for mismatched unrelated donors (20). Among different stem cell sources, cord blood was associated with a greater risk of PTLD (30), due to low numbers and naiveté of infused T cells that likely delay early immune reconstitution, although there is no evidence of delayed virus-specific immune recovery in pediatric CBT recipients beyond the first 100 days post-transplant (35). Moreover, there is *in vitro* evidence that CB lymphocytes may mediate a sizeable immune response directed against autologous EBV-infected cells, exerted by both NK cells and CD4+ T lymphocytes (36). The development of graft engineering strategies and pharmacologic GVHD prevention protocols, together with optimal conditioning regimens, have significantly ameliorated the outcomes of haploidentical HSCT, and this progress has led to a widespread use of the procedure (18, 29, 37–46). Interestingly, despite the high degree of mismatch and the procedures employed to facilitate engraftment and prevent GVHD, the incidence of PTLD with the newest platforms for haplo-HSCT, either T-cell and B-cell depleted (29, 43) or T-cell repleted with post-transplant cyclophosphamide (PTCy) (44–46), are unexpectedly low. In the case of PTCy, the incidence is <3% (47), possibly due to the destruction of EBV-infected B cells, together with an immune reconstitution that is hypothesized to be faster than that observed after the use of ATG (48).

Among other risk factors relevant for pediatric HSCT recipients, a higher incidence of PTLD has been observed in recipients of allogeneic HSCT conditioned with a reduced-intensity regimen (49), and the development of acute or chronic GVHD (20, 26, 27, 30), due to a delay in the reconstitution of functional specific immunity. Finally, EBV serology mismatch, in particular EBV-seronegative patients receiving grafts from seropositive donors, are also at increased risk for PTLD development (25, 27).

Some studies have suggested that significant factors could be combined within a prognostic model. Three single-center risk factor scoring systems have been published, but their use in common clinical practice is limited and needs to be validated (25, 27, 30).

EPIDEMIOLOGY, CLINICAL PRESENTATION AND DIAGNOSIS

EBV-PTLD is a severe complication that occurs in 1–3.5% of HSCT recipients (20, 50), although incidence rates may exceed 10% in patients with established risk factors (2, 20, 27, 28, 50). An expansion in the indications for HSCT from alternative donors, including haploidentical family members, and the use of novel T-cell depletion strategies, together with improved diagnostic protocols, have led to the observation of an increased incidence of PTLD in the last 2 decades (20). However, greater awareness of the disorder has fueled studies that have addressed PTLD preemptive/preventive strategies, and facilitated patient management.

Patients with PTLD after HSCT generally present with fever, lymphadenopathy, tonsil enlargement or discrete organ lesions, although the disease may manifest as a systemic process that mimics fulminant sepsis syndrome (2, 28). Primary central nervous system (CNS) localization of PTLD is rare, and generally burdened with a dismal prognosis (20), partly due to the challenges associated with limited drug penetration across the blood-brain barrier. In order to overcome this peculiar feature, intrathecal drug delivery has been proposed (51).

The diagnosis of EBV-PTLD is based on symptoms and/or signs consistent with PTLD, together with the quantitative determination of EBV-DNAemia or detection of EBV in a specimen from the involved tissue (1, 2), and imaging studies, such as computed tomography (CT) or positron emission tomography CT (PET-CT). Definitive diagnosis of EBV-PTLD requires biopsy of sites suspected for EBV disease and histological examination. EBV detection requires identification of viral antigens or *in situ* hybridization for the EBER transcripts. The histological WHO 2016 classification includes six morphological types of PTLD: plasmacytic hyperplasia, infectious mononucleosis-like, florid follicular hyperplasia, polymorphic, monomorphic (B-cell or T-/NK-cell types), and classical Hodgkin lymphoma PTLD (52).

EBV-PTLD may be diagnosed at the probable or proven level (53). Probable EBV disease is defined as the presence of symptoms and/or signs of lymphoproliferative disease in the absence of tissue biopsy, but without other documented causes,

together with significant EBV DNAemia, measured in any blood specimen. Diagnosis of proven EBV-PTLD requires detection of EBV nucleic acids or EBV-encoded proteins in a tissue sample.

EARLY IDENTIFICATION OF PATIENTS AT RISK OF PTLD

The development of EBV-PTLD after HSCT represents a life threatening event; mortality is still relevant, at 30 and 40% of diagnosed cases (20, 54). The onset of PTLD is preceded by a pre-clinical phase denoted by increased EBV DNA levels in the peripheral blood. Indeed, it has been demonstrated that, irrespective of baseline characteristics, the post-transplant monitoring of peripheral EBV viral load after HSCT is effective in predicting risk of EBV-PTLD (18, 55–61).

Thus, according to international guidelines, prospective monitoring of EBV DNA should be started within the first month after HSCT, and continued on a weekly basis at least until the fourth post-transplant month (1). The frequency and duration of EBV DNAemia screening should be based on the risk profile of the transplanted patients (62).

EBV DNA analysis is an indispensable tool for early diagnosis and the application of preemptive strategies to avoid progression of early-stage PTLD to oligoclonal/monoclonal disease (18, 63). However, even with the available data there is not a defined EBV-DNA threshold for prompt initiation of preemptive therapy (1), as EBV PCR assays are not standardized (63), and evidence has been obtained in cohorts with heterogeneous clinical characteristics using different peripheral blood specimens. Thresholds for assays using mononuclear cells, plasma, or whole blood in the reported studies range from 1,000 to 40.000 copies/ml according to the source, and data on the best specimen source are inconclusive (1, 59–61, 64, 65). Moreover, probable/proven PTLD has been described in a proportion of patients with EBV DNA levels below commonly adopted intervention thresholds (66, 67). Thus, it seems rational to adopt validated center-specific cut-off values, tailored on the specific cohort characteristics, and employ the rate of EBV DNA level increase, that is an indicator of EBV-infected B cells, as a predictor of when to start preemptive interventions. Regarding peripheral blood specimen choice, a recent study including 121 pediatric and adult HSCT recipients evaluated the kinetics of EBV DNA, assessed with a molecular method approved by regulatory agencies, in paired whole blood and plasma samples during episodes of post-transplant EBV infection, and found that plasma had a low sensitivity for identifying PTLD, thus suggesting a preferential use for whole blood in the post-transplant management of infection (64). Some studies indicated that plasma measurement may be useful in the follow-up after treatment, but these studies included high numbers of solid organ transplant recipients, and data are yet not conclusive (60).

EBV DNA analysis is not a precise predictor of PTLD development, and tailoring screening on the basis of a whole cohort is not always practical, feasible, or successful. As the other central factor determining progression to PTLD is the lack of a protective immune response, it seems reasonable to associate DNAemia screening with analysis of immune reconstitution. This approach has been used successfully for other viral infections in HSCT or solid organ transplant patients (13, 68–75), and has been proposed by some groups also in the setting of EBV infection and PTLD after HSCT (18, 76–82). Although studies are largely descriptive and based on the use of different technologies, the results suggest that numbers and function of virus-specific T cells inversely correlate with viral DNA levels and risk of disease, whereby strong cellular immune responses are associated with containment of viral replication or EBV-infected B cell outgrowth. The key obstacles to the introduction of EBV-specific T cell quantification into clinical practice is the definition of reliable cutoffs for clinical decision making for the different assays, and the absence of controlled interventional clinical trials.

PREVENTION OF PTLD AFTER HSCT

There are two possible approaches for prevention of EBV-PTLD after HSCT: prophylaxis and pre-emptive therapy (53, 83, 84). Prophylaxis of EBV disease includes any intervention applied to an asymptomatic patient to prevent EBV DNAemia. Pre-emptive therapy includes any intervention given to a patient with EBV DNAemia to prevent EBV disease.

Prophylaxis

In the setting of HSCT, there are two strategies to prevent EBV DNAemia. The first is based on interventions on the graft or the patient prior to HSCT, in order to decrease the risk of EBV-infected B cell outgrowth. As we have already seen, in the case of T-cell depleted HSCT, the use of *in vitro* or *in vivo* methods that deplete B cells as well as T cells reduce the risk of PTLD by temporarily removing the EBV reservoir and potential EBV-transformed B lymphoblasts, at least until functional immune reconstitution is achieved (28, 29, 43, 44, 47). If no T-cell depletion is employed, but the risk of PTLD is high due to the use of ATG and/or the presence of HLA mismatches between donor and recipient, peri-transplant B-cell depletion by rituximab may be considered (85). The efficacy of peritransplant rituximab was suggested by observations in adult patients receiving anti-CD20 monoclonal antibody close to HSCT, as part of their treatment for B cell malignancies (85), and was tested in a study from the European Group for Blood and Marrow Transplantation (EBMT) as part of the conditioning regimen for pediatric and adult patients with severe aplastic anemia (86). Based on these studies, peritransplant rituximab has been employed in pediatric recipients of αβ T-cell/B-cell depleted haploidentical HSCT, and the combination of *in vitro* and *in vivo* B cell depletion succeeded in counteracting the risk of PTLD given by T-cell depletion, ATG and HLA mismatch (29, 87). Relevantly, rituximab role in controlling acute and chronic GVHD may also favorably impact PTLD development (29, 85–87).

Regarding the use of ATG to prevent rejection and GVHD, given that the increased risk of PTLD is dose-dependent, in pediatric allogeneic HSCT a lower therapeutic dose may be administered. Indeed, a recent multicenter randomized trial has shown that 15 vs. 30 mg/kg rabbit ATG was equally effective in

TABLE 1 | Results of published trials using EBV-specific T cells to prevent or treat EBV infection and PTLD.

References	Pt n.	EBV stimulation (other targeted viruses)	Clinical design	Clinical and virologic effects on EBV and PTLD	GVHD
HSCT donor-derived, single-VST					
Rooney et al. (22) and Heslop et al. (94)	113	EBV-LCL	Prophylaxis	11/13 pts achieved CR, none PTLD	8/51 pts aGvHD; 13/108 cGvHD (11 limited, 2 extensive)
Doubrovina et al. (95)	14	EBV-LCL	PTLD Treatment	10 pts achieved CR, 4 pts progressive disease	None
Gustafsson et al. (96)	6	EBV-LCL	Pre-emptive	5 pts had EBV-DNA decreased, 1 pts died of PTLD	None
Lucas et al. (56)	1	EBV-LCL	PTLD treatment	CR	Limited skin aGvHD
Imashuku et al. (97)	1	EBV-LCL	PTLD treatment	No response	None
Comoli et al. (18)	4	EBV-LCL	Preemptive or PTLD treatment	3 pts achieved CR, 1 pt had decreased EBV-DNA level without PLTD	None
Moosmann et al. (98)	6	Peptide mix from lytic and latent EBV antigens	PTLD treatment	3 pts had CR, 3 pts had no response	None
Icheva et al. (99)	10	Recombinant EBNA1 protein or EBNA1 peptides and direct selection	Pre-emptive or PTLD treatment	7/10 pts achieved CR	1 grade II aGVHD
Jiang et al. (100)	15	DCs pulsed with EBV-LCL lysate	PTLD treatment (+ rituximab and/or CHOP)	7/8 pts achieved CR	5 pts (33%) aGVHD (1 gr. I, 3 gr. II, 1 gr. III) 2 (13%) limited cGVHD
Velvet et al. (101)	2	unknown	CNS-PTLD treatment	1 pt achieved remission	None
HSCT donor-derived, multi-VST					
Leen et al. (102) and Melenhorst et al. (103)	26	EBV LCLs transduced with Ad5f35-pp65 (ADV, CMV)	Prophylaxis/preemptive	6/6 pts with EBV cleared infection;	2 grade I aGVHD
Leen et al. (104)	14	EBV LCLs transduced with Ad5f35 vector (ADV)	Prophylaxis	11 pts treated as prophylaxis remain negative	3 grade I aGVHD
Dong et al. (105)	3	DCs pulsed with EBV IE1 and LMP2 peptides (CMV)	Prophylaxis/preemptive	1 pt cleared viremia; 1 pt treated as prophylaxis remains negative	1 grade I aGVHD
Gerdemann et al. (106)	10	DCs nucleofected with plasmids encoding for EBV LMP2 and BZLF1 (ADV, CMV)	Preemptive/PTLD treatment	3/4 pt: complete virologic responses	1 skin rash due to GVHD or BKPyV infection
Papadopoulou et al. (107)	11	Peptides pool from immunodominant antigens (ADV, CMV, PyVBK, HHV6)	Prophylaxis/preemptive	3 pts treated as prophylaxis remain negative; 4/4 pts cleared EBV viremia	1 grade I aGVHD
Ma et al. (108)	10	Ad5f35-EBNA1/LMP (ADV, CMV, VZV)	Prophylaxis	no EBV reactivation	1 grade II aGVHD 1 grade III aGVHD
Third-party donor-derived single-VST					
Haque et al. (109)	33	EBV-LCL	PTLD treatment	14 pts attained EBV CR, 3 pts had PR, 16 pts no response at 6 m	None
Barker et al. (110)	5	EBV-LCL	PTLD treatment	4 pts attained EBV CR, 1 pts progressive disease	None

(Continued)

TABLE 1 | Continued

References	Pt n.	EBV stimulation (other targeted viruses)	Clinical design	Clinical and virologic effects on EBV and PTLD	GVHD
Uhlin et al. (111)	1	Peptide-HLA multimer selection	Preventive and PTLD treatment	CR after 9 m, recurrence then response to 2nd infusion	None
Prockop et al. (112)	33	EBV-LCL	PTLD treatment	CR or PR was achieved in 68% of HSCT recipients. For patients who achieved CR/PR or SD after cycle 1, 1y OS was 88.9%	1 grade I skin aGvHD
Third-party donor-derived multi-VST					
Leen et al. (113)	50	LCLs transduced with Ad5f35-pp65 (ADV, CMV)	PTLD treatment	6/9 pts with EBV attained CR or PR;	6 grade I aGVHD 2 grade II aGVHD
Tzannou et al. (114)	38	EBV LMP2 + EBNA1 + BZLF1 peptide pools (ADV, CMV, PyVBK, HHV6)	Preemptive/PTLD treatment	3/3 pts with EBV attained CR;	2 grade I aGVHD de novo; 4 grade I-III recurrent aGVHD

preventing acute and chronic GVHD, but was associated with lower relapse and non-relapse mortality in pediatric patients receiving UD HSCT for hematologic malignancies (88). An alternative GVHD prophylaxis, that may have a direct impact on PTLD development due to its anti-tumor activity, is the use of mTOR inhibitors (89, 90).

Post-transplant prophylactic administration of agents, such as antiviral drugs, rituximab and EBV-CTLs has been proposed. Treatment of latent EBV with antivirals has been unsuccessful, as latently infected B cells do not express the EBV thymidine kinase enzyme (1, 84).

In a large retrospective study, prophylactic post-transplant rituximab significantly reduced the risk of EBV DNAemia (91); however, no statistically significant impact on PTLD incidence, treatment-related mortality, and overall survival in comparison to a pre-emptive approach was shown. Post-transplant rituximab is associated with cytopenias (92) and delayed B cell recovery with an increased risk of infections (93), that seem less evident with peritransplant use. Thus, prophylactic rituximab post-HSCT ought to be employed with caution (1). The use of prophylactic EBV-CTLs, pioneered by Rooney et al. in high-risk, pediatric unrelated-donor HSCT recipients, has been highly successful, and devoid of side effects (22, 94) (**Table 1**). None of the 101 patients who received CTLs as prophylaxis developed PTLD compared with 11.5% of controls. As the donors were EBV-seropositive, even in the absence of circulating EBV one may hypothesize that the efficacy of this treatment was due to stimulation by EBV present in patient tissues or donor B cells, or just cross-stimulation of low-affinity T cells present in the infused product by other antigens. Current use of EBV-CTLs is, however, limited to a few selected centers.

Preemptive Therapy

The mainstay of pre-emptive therapy for EBV PTLD after HSCT is anti-CD20 antibody rituximab, given at increase in EBV-DNA load especially in patients lacking T-cell reconstitution (18, 63, 86, 115), due to its acceptable toxicity and widespread availability (1, 84). A retrospective study reviewed the results of more than 300 patients described in reported case series, and found that successful prevention of PTLD was observed in almost 90% of treated patients (84). Pre-emptive rituximab is employed at the dose of 375 mg/m^2, once weekly until EBV DNAemia is found negative. Dose number should be assessed on the basis of EBV DNAemia monitoring and on the patient's specific immune recovery, but 1–4 doses are generally sufficient. However, it has been shown that, in certain circumstances, clearance of EBV DNA from peripheral blood may not reflect a long-lasting response (67). Limitations of this approach are selection for CD20-negative clones (18), and the fact that rituximab acts on the EBV reservoir, rather than on restoration of the cellular immune response to EBV, which is central to the long-term control of EBV mediated B-cell proliferation (18, 72).

Among the strategies that boost specific immune reconstitution and immune surveillance, reduction of maintenance immunosuppression (IS), whenever feasible in the absence of GVHD, should be employed in association with anti-CD20 Mab therapy (1). Data on IS reduction employed

alone are too limited to derive any useful indications (19). The use of donor EBV-specific T cells in unrelated donor (UD) or haplo-HSCT in a pre-emptive approach has been very successful (18, 96, 116–119), with long-lasting EBV viral load clearance in more than 90% of patients, and responses observed also in patients with increased viral load after rituximab treatment (18) (**Table 1**). In HSCT recipients, EBV-CTL therapy enhances virus-specific immune responses, and allows establishment of a memory T cell response, observed for as long as 9 years after T cell administration (117). No major toxicity was observed (118), and the reported rate of new-onset GVHD was around 1% (119). When the donor is not available, or is EBV-seronegative, or to increase access to T cell treatment, the use of third-party CTLs has been advocated (109). The first reported study used banked EBV-specific CTLs to treat PTLD after solid organ or HSCT, matching by low resolution HLA typing and screening for absence of alloreactivity, obtaining 50% responses in established PTLD and no GVHD development (109). Since then, a number of studies have further explored this option and refined matching criteria by evaluating activity against viral epitopes through the shared HLA allele (110–114, 119) (**Table 1**). A recent study treated 33 HSCT recipients with third-party CTLs, obtaining a 68% remission rate, with a 89% overall survival. Interestingly, patients in progression after the first cycle benefited from a change in CTL donor (9% survival after repeated cycles with same donor CTLs vs. 60% survival after donor switch) (112).

CONCLUSIONS

Prognosis of EBV PTLD after HSCT is still suboptimal. Because of the relatively low incidence of this complication, and its particular situation related to the post-HSCT period, there is limited evidence on the best treatment strategy for established disease failing first line treatment.

Thus, therapeutic strategies with high efficacy and minimal toxic effects for HSCT patients at high risk of PTLD are a clinical need. Knowledge on the interplay between the virus and the host immune system (120) has allowed the design of tailored management approaches, based on longitudinal combined virological and immunological testing, and the development of novel cellular therapeutic agents burdened with little toxicity, and therefore suitable for employment in pre-emptive therapeutic strategies. Limitations to the pre-emptive approach are related to the difficulty in establishing viral load cut-off values for the start and discontinuation of therapeutic interventions, and standardized and cellular immunity assays with validated thresholds, together with limited availability of cellular therapies. These hurdles may be overcome by a general effort in standardization, which has already begun, and by local management. So far, the use of EBV-specific T cells has been limited to the few academic centers with infrastructure resources to produce advanced cellular therapies. Recently, excellent cell therapy clinical results, together with the development of new methodologies to obtain rapid manufacture of third-party T cells, have fuelled considerable interest from the Pharmaceutical industry to bring to the market third-party cellular therapies including EBV CTLs. Further efforts are required to design the most appropriate clinical trials to rapidly identify efficient combinatorial approaches, and to invent new and sustainable reimbursement modalities for novel therapies.

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Harnessing T Cells to Control Infections After Allogeneic Hematopoietic Stem Cell Transplantation

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Dramatic progress in the outcome of allogeneic hematopoietic stem cell transplantation (allo-HSCT) from alternative sources in pediatric patients has been registered over the past decade, providing a chance to cure children and adolescents in need of a transplant. Despite these advances, transplant-related mortality due to infectious complications remains a major problem, principally reflecting the inability of the depressed host immune system to limit infection replication and dissemination. In addition, development of multiple infections, a common occurrence after high-risk allo-HSCT, has important implications for overall survival. Prophylactic and preemptive pharmacotherapy is limited by toxicity and, to some extent, by lack of efficacy in breakthrough infections. T-cell reconstitution is a key requirement for effective infection control after HSCT. Consequently, T-cell immunotherapeutic strategies to boost pathogen-specific immunity may complement or represent an alternative to drug treatments. Pioneering proof of principle studies demonstrated that the administration of donor-derived T cells directed to human herpesviruses, on the basis of viral DNA monitoring, could effectively restore specific immunity and confer protection against viral infections. Since then, the field has evolved with implementation of techniques able to hasten production, allow for selection of specific cell subsets, and target multiple pathogens. This review provides a brief overview of current cellular therapeutic strategies to prevent or treat pathogen-related complications after HSCT, research carried out to increase efficacy and safety, including T-cell production for treatment of infections in patients with virus-naïve donors, results from clinical trials, and future developments to widen adoptive T-cell therapy access in the HSCT setting.

Keywords: multipathogen infection, T cell immunity, T-cell therapy, pathogen specific T cells, Allo-HSCT

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INTRODUCTION

Dramatic progress in the outcomes of allogeneic hematopoietic stem cell transplantation (allo-HSCT) from alternative sources in pediatric patients has been registered over the past decade, providing a chance to cure the children and adolescents in need of a transplant (1–4). Despite encouraging results, infections are still important causes of morbidity and mortality in immunosuppressed patients following HSCT (5). Viral reactivations predominantly develop within the first 6 months after HSCT. Double-stranded DNA viruses contribute to substantial morbidity, with herpesviruses, adenovirus (AdV) and polyomaviruses BK (BKPyV) and JC (JCPyV) as the clinically most relevant infections (5–14). In addition, respiratory viruses and fungal infections are also associated with dismal outcome (15–18).

If the development of single opportunistic infections may have severe consequences in transplant recipients, it has been demonstrated that persistent detection of multiple DNA viruses is frequent after allogeneic HSCT, and had a dose-dependent association with increased mortality (19). Indeed, cumulative viral load AUC in the first 100 days post-HSCT was consistently and independently associated with increased risk for early and late overall mortality and non-relapse mortality (NRM). The effects on NRM do not appear to be direct, as only a small portion of patients succumbed to viral disease. Rather, viremia may cause indirect effects due to increased production of proinflammatory and immunomodulatory cytokines that contribute to the pathogenesis of HSCT complications (20, 21).

In recent years treatment of viral complications after HSCT has improved in part because of the introduction of new antivirals, and in part from the preemptive use of antiviral agents at the onset of viremia. The latter is successful thanks to the widespread use of surveillance by molecular detection methods (22, 23). Likewise, the ability to recognize invasive fungal disease while in the early stages, by means of imaging and peripheral blood antigen measurement, coupled with assessment of antifungal immune responses, allowed for prompt treatment and amelioration of outcome (24). Despite advances in prophylactic and preemptive pharmacotherapy, anti-pathogen therapeutics are limited by toxicity, in particular myelosuppression and renal injury, and to some extent by a lack of efficacy in breakthrough infections (25).

The development of infections in the post-transplant period principally reflects the inability of the absent/depressed host immune system to limit pathogen replication and dissemination; loss of T cell function is central to this effect (26–28). T-cell reconstitution is a key requirement for effective infection control following HSCT, and factors that influence the speed of T-cell recovery also impact the risk of infection in this period (27). A high degree of HLA mismatch between donor and recipient reduces the efficacy of immune surveillance due to poor epitope recognition, and increases the risk of inducing alloimmune responses, thus requiring stronger immunosuppression to prevent and treat graft-vs.-host disease. Likewise, delayed immune recovery is associated with T-cell depletion of the graft before transplantation.

Given the central role of pathogen-specific T cells in infection surveillance, immunotherapeutic strategies to accelerate reconstitution of pathogen-specific immunity and to hasten T cell recovery after HSCT represent a compelling alternative to drug treatments (14, 23, 27, 29–36). Moreover, preventive strategies may be expanded toward the use of virus-specific T cell assays to help identify patients at risk and to tailor therapeutic intervention (23, 37–40).

Here, we discuss the clinical achievements of T-cell therapy for infections, describe the impact of technical developments on clinical applicability, and give indications on future directions to broaden access.

CELL THERAPY FOR INFECTIONS AFTER HSCT

Donor Lymphocyte Infusions

The use of donor lymphocyte infusions (DLI) derived from seropositive stem cell donors is an effective salvage therapy for viral infections in HSCT recipients prior to T-cell recovery, but the risk of potentially severe acute or chronic graft-vs.-host disease (GVHD) is a concern (41). In order to reduce the risks derived from alloreactivity associated with DLI, non-specific T cells transduced with a retroviral construct containing suicide genes, to induce susceptibility to drug mediated lysis in case of development of alloreactive response, have been employed with success (42). The use of DLI modified with the iCasp9 cell-suicide system in a small cohort of children transplanted for acute leukemia demonstrated the potential advantages in terms of rapid and consistent cell removal in case of GVHD development (43).

Pathogen-Specific T Cells: Production Protocols

An alternate strategy consists in delivering infectious antigen-specific T cells selected by cell culture or by sorting. A major breakthrough was achieved by the adoptive transfer of virus-specific cytotoxic T lymphocytes (CTL) reactivated from the peripheral blood of HSCT donors as prophylaxis/treatment against CMV disease or EBV-positive post-transplant lymphoproliferative disease in patients given T-cell depleted, HLA-disparate, unrelated HSCT (32, 33). This approach has been successful in preventing and treating CMV and EBV infectious complications after T-cell depleted haplo-HSCT, both in the pediatric and adult setting, while limiting the risk of inducing GVHD (27, 30).

Initially, protocols for production of virus-specific T cells (VSTs) were all based on complex procedures of stimulation and *in vitro* expansion, leading to a final product of polyclonal T cells

Abbreviations: allo-HSCT, allogeneic hematopoietic stem cell transplantation; AdV, adenovirus; BKPyV, polyomavirus BK; JCPyV, polyomavirus JC; CMV, Cytomegalovirus; HHV6, human herpes virus 6; EBV, Epstein-Barr virus; PTLD, Post-transplant lymphoproliferative disease; HC, hemorrhagic cystitis; PML, progressive multifocal leukoencephalopathy; UD, unrelated donor; NRM, non-relapse mortality; DLI, donor lymphocyte infusions; CTL, Cytotoxic T lymphocytes; a/cGVHD, acute/chronic graft vs. host disease; VSTs, virus-specific T cells; CI: calcineurin inhibitors.

with broad specificity. One of the main advantages of *ex vivo* differentiation is the ability to overcome the hurdle of obtaining substantial numbers of VSTs from donors with low-frequency memory T cells for a given antigen, and the ability to reduce alloreactivity by continuous stimulation with viral antigens. This is counterbalanced by production times, that can be as long as 3–8 weeks, limiting its usefulness in patients with urgent clinical need and running the risk of inducing cellular exhaustion. The latter does not seem to be a major obstacle, however, as donor gene-marked EBV-specific T cells cultured for 4–6 weeks were able to reconstitute T cell memory in HSCT recipients, and were detected as late as 9 years after administration in patients with viral reactivation (44). The availability of synthetic peptide pools, novel techniques, and progress in culture reagents and vessels has allowed reduction in production time, bringing it to < 2 weeks (45–47).

A valid alternative to cell culture is direct selection of pathogen-specific T cells by using viral peptide HLA class I multimers conjugated to magnetic beads (48), or stimulation with viral peptides followed by the IFN-gamma capture assay with magnetic beads (34, 49, 50). The latter has an important advantage over multimers, as it allows selection of CD4+ in addition to CD8+ virus-specific T cells, guaranteeing sustained long-term immune protection (51). Direct selection allows rapid production of VSTs, but it is generally feasible only for pathogens inducing an ample memory T cell pool, such as for CMV or EBV, and requires a leukapheretic procedure to obtain starting cellular material. In addition, it is not an option for virus-naïve subjects.

Pathogen-Specific T Cells: Clinical Results for EBV, CMV, ADV, and Aspergillosis

Since the early clinical trials for EBV and CMV, the prophylactic, preemptive and curative use of T cell therapy for infection has expanded, due to the reported high rates of response and low toxicity (**Tables 1**, **2**). The efficacy of virus-specific adoptive cellular therapy has been difficult to assess, due to the difficulties of running large prospective multicenter clinical trials, and heterogeneity of reported studies in study design, cell product characteristics and treated cohorts. However, prophylaxis/preemptive treatment of EBV PTLD after HSCT has shown more than 95% response rate in the 107 patients treated with cultured single VSTs (23, 33, 44, 52, 53, 85). Treatment of overt disease was successful in over 80% of the patients treated for PTLD (52, 54–56, 85) or CMV viremia or disease (32, 35, 58–62), with little toxicity almost exclusively limited to a 1–10% rate of GVHD. The rate of GVHD was generally lower in patients treated for EBV infection/disease, probably due to a prevalence of CD8+ T cells in the infused EBV-specific CTLs, compared to a larger portion of CD4+ T cells present in CMV-specific products. Directly selected cellular products employed in more recent studies have proven equally effective in reconstituting post-transplant immunity, but rates of clinical responses were slightly lower, reportedly 60% in patients with PTLD (50, 57) and 70% in patients treated for CMV (48, 49, 63, 65, 73) or ADV (34, 66–68, 86) viremia or disease. Moreover, the incidence of new onset or exacerbation of GVHD was higher at 15%, likely due to residual, potentially alloreactive, T cells in the product. Clearly, as head-to-head controlled studies with cell products obtained by culture vs. direct selection have not yet been performed, the reported efficacy and safety rates of the different strategies may be confounded by the variety of protocols and clinical settings.

Attempts at reconstituting cellular immune responses to fungal antigens, and controlling invasive aspergillosis (IA) in HSCT recipients have been also successful. Pioneer work showed the feasibility to expand T cell clones directed to aspergillus conidia and devoid of alloreactivity, that were employed to treat IA in 10 recipients of haplo-HSCT (35). Emergence of circulating pathogen-specific T cells were associated with control of Aspergillus antigenemia and infectious mortality.

Pathogen-Specific T Cells: Preliminary Clinical Results for PyVs and HHV6

Cell therapy has been employed also for the treatment of other infections, such as polyomaviruses and HHV6. Although very preliminary, initial experiences with BKPyV-specific cells are promising (36, 69), as 13 of 14 patients treated for BKV-associated hemorrhagic cystitis within a clinical trial of third-party banked multivirus-specific T cell therapy in allogeneic HSCT experienced complete resolution of gross hematuria within 1–2 months (36). Of the two patients treated for virus-related nephropathy, one responded to treatment by ameliorating renal function. In 50% of the treated patients, an increase in BKPyV-specific immune response was observed. The main side effects were recurrence or new onset of GVHD in 16% of the whole study cohort and transient hydronephrosis and a decrease in renal function in one patient who received VSTs as treatment for BKPyV HC. The latter, associated with a concomitant bacterial urinary tract infection, could have also been due to lysis of infected cells in renal tubular cells.

Four patients, reported in two studies, were treated for JCPyV PML (14, 74). One pediatric HSCT recipient received donor JCPyV-specific T cells, that was associated with reconstitution of specific viral immunity, clearance of viral DNA from the cerebrospinal fluid (CSF) and disease control with remarkable neurological improvement, in the absence of immune reconstitution syndrome (14). Three patients were treated with third-party allogeneic BKPyV-specific T cells, based on reported observations of a certain degree of cross-reactivity between PyV BK and JC due to high homology (74). The CBT recipient fully recovered. In the other two patients, viral load was cleared or reduced in CSF, with the patients showing neurologic improvement with residual deficit in one case, and disease progression in the other. Two of the patients had immune reconstitution syndrome. Phase I or I/II trials are currently underway.

Two patients with HHV6 infections were treated with T cells specific for U11, U14, and U90 within a clinical trial of third-party banked multivirus-specific T cell therapy in allogeneic HSCT (36). One patient was treated for HHV6 encephalitis and the other for HHV6 viremia with fevers and symptoms of bone marrow suppression, including neutropenia. Both patients showed decreased viral load and normalization of clinical disease.

TABLE 1 | Published trials using single pathogen-specific T cells.

Virus	Pt n.	Antigen	LTC stimulation	Clinical effects	GVHD	References
HSCT donor-derived						
EBV	113	LCLs	*in vitro* culture	11/13 pts achieved CR, none PTLD	8/51 pts aGvHD; 13/108 cGvHD (11 limited, 2 extensive)	(33, 52)
EBV	6	LCLs	*in vitro* culture	5 pts had EBV-DNA decreased, 1 pts died of PTLD	None	(53)
EBV	14	LCLs	*in vitro* culture	10 pts achieved CR, 4 pts progressive disease	None	(54)
EBV	1	LCLs	*in vitro* culture	No response	None	(55)
EBV	4	LCLs	*in vitro* culture	3 pts achieved CR, 1 pt had decreased EBV-DNA level without PLTD	None	(23)
EBV	15	LCLs	DCs pulsed with LCL lysate; *in vitro* culture	7/8 pts achieved CR	5 pts (33%) aGVHD (1 gr. I, 3 gr. II, 1 gr. III) 2 (13%) limited cGVHD	(56)
EBV	6	Lytic and latent EBV antigens	Peptide mix stimulation; direct selection	3 pts had CR, 3 pts had no response	None	(57)
EBV	10	EBNA1	Recombinant protein or peptides; direct selection	7/10 pts achieved CR	1 grade II aGVHD	(50)
CMV	14	CMV virions	Fibroblasts infected with CMV strain; CD8 T cell cloning	All pts reconstituted CMV-specific immunity	3 grade I or II aGVHD	(32)
CMV	8	CMV lysate	PBMCs cultured in the presence of virus lysate	6 pts cleared infection after 1 or 2 doses; 1 pt NR; 1 pt NE	None	(58)
CMV	16	Inactivated CMV virions	DCs pulsed with lyophilized CMV antigen; *in vitro* culture	All pts reconstituted specific immunity; 8/16 pt did not require antivirals	1 grade I aGVHD	(59)
CMV	25	CMV lysate	PBMCs pulsed with CMV lysate; T cell colony expansion	7/25 pts developed CMV antigenemia; 5/25 pts developed CMV disease (3 CR, 2 NR)	1 grade I GVHD	(35)
CMV	9	CMV pp65 peptide	DCs pulsed with pp65-derived peptide; *in vitro* culture	6/9 pts developed CMV reactivation; no CMV disease	3 grade III GVHD (1 fatal)	(60)
CMV	7	CMV pp65 and IE1 peptides	PBMCs pulsed with CMV peptide mixes; *in vitro* culture	5/7 had increased antiviral immunity in PB	None	(61)
CMV	16	CMV pp65 peptides	PBMCs pulsed with 15-mer CMV peptide mixes; *in vitro* culture	14/16 pts cleared viremia	None	(62)
CMV	9	CMV pp65 or IE1	Peptide-HLA tetramer selection	8/9 cleared CMV infection	2 grade I or II aGVHD	(48)
CMV	18	CMV pp65 protein	PBMCs pulsed with protein; direct selection	15/18 pts had reduction or clearance of viremia	1 cGVHD	(63)
CMV	18	CMV pp65 protein or peptides	PBMCs pulsed with protein/peptides; direct selection	1/7 pts treated prophylactically reactivated 11/11 pts treated preemptively cleared CMV	5 grade I, 3 grade II- III aGVHD; 6 cGVHD	(49)
CMV	6	CMV pp65 peptides	PBMCs pulsed with peptides; direct selection	6/6 pts cleared viremia	None	(64)
CMV	2	CMV pp65 peptides	PBMCs pulsed with peptides; direct selection	2/2 pts attained CR	None	(65)
AdV	9	Type C AdV antigen	PBMCs pulsed with antigen; direct selection	5/6 evaluable pts attained viral clearance	1 aGVHD exacerbation	(34)
AdV	30	AdV hexon protein	PBMCs pulsed with antigen; direct selection	21/30 pts had clinical/virological response	1 grade I GVHD	(66)
AdV	8	AdV hexon peptides	PBMCs pulsed with peptide mix	8/8 pts cleared viremia; 1 pt subsequently reactivated due to GVHD therapy	1 grade IV GVHD	(67)
AdV	11	AdV hexon peptides	PBMCs pulsed with peptide mix; direct selection	10/11 pts cleared viremia and/or AdV disease	1 grade I, 1 grade III aGVHD; 1 ext. cGVHD	(68)

(Continued)

TABLE 1 | Continued

Virus	Pt n.	Antigen	LTC stimulation	Clinical effects	GVHD	References
BKPyV	1	BKPyV VP1 and LT	PBMCs pulsed with Peptides; direct selection	1 pt cleared infection and had CR	None	(69)
JCPyV	1	JCPyV VP1 and LT	PBMCs pulsed with overlapping peptides; *in vitro* culture	1 pt cleared infection and had CR	None	(14)
Aspergillus f.	10	Fungal conidia	PBMCs pulsed with conidia; T cell colony expansion	9/10 pts attained CR	None	(35)
Third-party donor-derived						
EBV	33	LCLs	*in vitro* culture	14 pts attained EBV CR, 3 pts had PR, 16 pts no response at 6 m	None	(70)
EBV	5	LCLs	*in vitro* culture	4 pts attained EBV CR, 1 pts progressive disease	None	(71)
EBV	33	LCLs	*in vitro* culture	CR or PR was achieved in 68% of HSCT recipients. For patients who achieved CR/PR or SD after cycle 1, 1 y OS was 88.9%	1 grade I skin aGvHD	(72)
EBV	1	EBV peptides	Peptide-HLA multimer selection	CR after 9 m, recurrence then response to 2nd infusion	None	(73)
CMV	5	CMV pp65	Peptide-HLA multimers selection	4/5 pts attained viremia clearance	None	(73)
JCPyV	3	BKPyV VP1, VP2, VP3, ST and LT peptides	PBMCs pulsed with overlapping peptides; *in vitro* culture	2/3 pts cleared infection and CR (1 with sequelae)	1 IRIS	(74)

Experience With Multivirus-Specific T Cells

Most of the cell therapy experience regards treatment of CMV and EBV infections. However, patients with multiple infections have a worse outcome (19), and in the pediatric population or in recipients of haplo-HSCT, the impact of other viral infections, such as adenovirus or HHV6, has important implications for overall survival (8, 87). Thus, the possibility to produce in a single process VSTs specific for multiple viruses is crucial for progress in the field. Proof of principle studies have been conducted, that demonstrated feasibility and preliminary efficacy of controlling viral reactivation after allogeneic HSCT by multivirus-specific VST of HSCT donor or third-party origin, obtained by *ex-vivo* stimulation with virus-transduced EBV lymphoblastoid cell lines (75–77, 82, 84), dendritic cells nucleofected with plasmids encoding for viral proteins or pulsed with viral peptides (78, 79, 81), or directly with 15-mer peptide pools from immunogenic viral proteins (36, 80) (**Table 2**).

Prophylactic or curative administration in a total of 82 patients treated with HSCT donor-derived cells and 96 third-party donor cells showed responses in the range of 80–95 and 70–100%, respectively (**Table 2**). Clinical benefit could be demonstrated also in patients treated for multiple coincident infections (36). Although clinical responses have been registered for all targeted viruses, evidence of T cell expansion in the peripheral blood of treated patients is mainly seen for viruses with large memory cell pools, such as CMV and EBV, while, due to the small size of their memory compartment, immune responses to AdV or HHV6 do not seem to be boosted unless a reactivation is underway. Indeed, antigenic competition that will ensue when engaging multiple target antigens within the same culture, will determine a preferential expansion of T cells recognizing the immunodominant specificities of viruses with large memory cell pools. This will impact on the composition of multivirus-specific T cell products, as T lymphocytes directed to certain non-immunodominant targets, as well as to viruses with low-frequency memory T cells, will be underrepresented, and it may also ultimately impact on efficacy.

CURRENT LIMITATIONS OF T CELL THERAPY FOR INFECTIONS

There are several hurdles that concur in limiting the use and the clinical efficacy of pathogen-targeted T cell therapy. First of all, production of pathogen-targeted T cells have been so far mostly confined to a relatively small numbers of academic centers with required Good Manufacturing Practice (GMP) expertise and facilities, that have limited ability to provide widespread access to these therapies. Moreover, for some patient categories, such as recipients of HSCT from pathogen-naïve donors, expansion of dedicated T cell products may not be feasible.

In addition, the appropriate timing and schedule for T cell delivery, as well as T cell dose or optimal cell product composition, have not been yet established, due to the presence of many different confounding variables, such as transplant ad infectious disease setting, use of *in vitro* or *in vivo* T-cell depletion, and immunosuppressive regimens. These issues will have to be addressed in future controlled comparative trials.

TABLE 2 | Published trials using multivirus-specific T cells.

Virus	Pt n.	Antigen	LTC stimulation	Clinical effects	GVHD	References
HSCT donor-derived						
AdV, CMV and EBV	26	AdV5; CMV pp65; EBV-LCL	LCLs transduced with Ad5f35-pp65	6/6 with EBV cleared infection; 5/6 with AdV cleared infection; 10/11 CMV cleared infection and 1 pt progressed despite VSTs/pharmacotherapy	2 grade I GVHD	(75, 76)
AdV and EBV	14	AdV5; EBV-LCL	LCLs transduced with Ad5f35 vector	11 pts treated as prophylaxis remain negative; 2/3 pts with AdV cleared infection	3 grade I GVHD	(77)
CMV and EBV	3	CMV pp65; EBV IE1 and LMP2	DCs pulsed with peptides	2 pts cleared infection; 1 pt treated as prophylaxis remains negative	1 grade I GVHD	(78)
AdV, CMV and EBV	10	AdV5 Hexon and Penton; CMV IE1 e pp65; EBV LMP2 and BZLF1	DCs nucleofected with plasmids encoding for viral antigens	8/10 pt: complete virologic responses	1 skin rash due to GVHD or BKPyV infection	(79)
AdV, BKPyV, CMV, EBV and HHV6	11	AdV5 Hexon; BKPyV LT + VP1; CMV IE1 + pp65; EBV LMP2 + EBNA1 + BZLF1; HHV6 U11 + U14 + U90	Peptides pool from immunodominant antigens	3 pts treated as prophylaxis remain negative; 94% response rate (15 Cr and 2 PR) in 8 pts with 18 viral infections/reactivations	1 grade I GVHD	(80)
AdV, CMV, EBV, and VZV	10	AdV5; CMV pp65; EBV EBNA1 and LMP; VZV vaccine	Ad5f35-pp65, Ad5f35-EBNA1/LMP, commercial VZV vaccine	6 pts with CMV reactivation, only 1 receving antiviral therapy; no EBV, AdV or VZV reactivation	1 grade II GVHD 1 grade III GVHD	(81)
AdV, CMV and EBV	3	AdV5; CMV pp65; EBV-LCL	LCLs transduced with AdV5-pp65 vector	1 pt cleared infection. 2 pts treated as prophylaxis remains negative	None	(82)
CMV, AdV and EBV	7	Various source antigens	T cell culture; in 1 case, streptamer selection	2 pts with EBV attained CR; 5 pts with CMV:2 CR, 2 PR and 1 failure	1 grade I GVHD 1 grade II GVHD	(83)
Third-party donor-derived						
AdV, CMV and EBV	50	Ad5, CMV pp65, EBV-LCL	LCLs transduced with Ad5f35-pp65	6/9 pts with EBV attained CR or PR; 14/18 pts with AdV attained CR or PR; 17/23 pts with CMV attained CR or PR	6 grade I GVHD 2 grade II GVHD	(84)
AdV, CMV and EBV	4	Various source antigens	T cell culture	1/2 pts with EBV attained CR or PR; 1 pt with AdV cleared infection; 1 pt with CMV reactivation required specific pharmacotherapy.	None	(83)
AdV, BKPyV, CMV, EBV and HHV6	38	AdV5 Hexon; BKPyV LT + VP1; CMV IE1 + pp65; EBV LMP2 + EBNA1 + BZLF1; HHV6 U11 + U14 + U90	Peptide pools from immunodominant antigens	3/3 pts with EBV attained CR; 8/10 pts with AdV attained CR or PR; 20/21 pts with CMV attained CR or PR; 19/21 pts with BKV attained CR or PR 3/3 pts with HHV6 attained CR or PR;	2 grade I GVHD *de novo*; 4 grade I-III recurrent GVHD	(36)

Finally, HSCT recipients treated with steroids or calcineurin inhibitors (CI) for GVHD are among those at highest risk of infectious complications. However, in these patients cell therapy has the least chance of success, as steroids have a direct cytopathic effect, and CI impair T cell expansion potential. Recently, preclinical studies have demonstrated the feasibility of producing pathogen-specific single or multivirus-specific T cells resistant to steroids (88), or to CI (89, 90), by genetic modification, and clinical studies are underway to assess safety and preliminary efficacy.

IMPROVING ACCESS TO CELL THERAPY

Manufacturing VST From Antigen-Seronegative Donors

Pediatric recipients who reactivate viral infections after HSCT from virus-naïve stem cell donors are at high risk of developing complications. It has been shown that it is possible to prime tumor- or virus-specific responses by delivering viral antigens presented by professional antigen-presenting cells in the presence of activating/homeostatic cytokines (91, 92). Stimulation by

dendritic cells pulsed with EBV LCL, or stimulation with EBV-LCL, either with subsequent selection of CD25-positive T-cells, or in the presence of cytokines, such as IL-7 and/or IL-12, have all been described (92–94). The latter approach was demonstrated effective when employed to expand EBV-CTL that were successfully infused *in vivo* to treat a disseminated PTLD, unresponsive to multiple courses of rituximab and chemotherapy, in a pediatric recipient of unrelated HSCT from a EBV-seronegative donor (95).

Multivirus (CMV, EBV, and adenovirus)-specific T-cells have been activated and expanded from CB, by stimulation with DC or LCL pulsed with a CMV-pp65 overlapping peptide library, in the presence of IL-7, IL-12, and IL-15. The primed cells were only able to recognize atypical pp65 epitopes, but when administered to CBT recipients mediated CMV-directed activity in one patient experiencing viral reactivation (82).

Third-Party Banked VST

Donor-derived VST infusions are not always feasible in clinical practice, due to impossibility to obtain starting material from the donor, as in UD or CB transplantation. Moreover, rapid disease progression may not allow the time required for dedicated production. A practical approach to overcome these issues is to employ banked, HLA-typed VST obtained from healthy donors, selected for a candidate recipients on the basis of the most closely matched line with specific activity against a given pathogen through one or more shared HLA epitopes.

Theoretically, third-party VST could have short persistence *in vivo*, with limited clinical benefit, as the partial HLA disparity may induce allorecognition by recipient T cells. Alloresponses by infused third-party cells may, in turn, cause GVHD. So far, results have been encouraging, with only one report of bystander-induced liver GVHD (96). Seminal data were obtained in solid organ-transplanted patients with EBV PTLD: the response rate in the 33 patients enrolled in the first phase II trial, that included 6 HSCT recipients, was 52% at 6 months (70). Since then, third-party VST have been effectively used also in the setting of HSCT (36, 62, 71–73, 83, 84, 96), demonstrating that the approach is feasible without inducing a higher rate of GVHD than HSCT donor-derived VST, while producing significant clinical responses. A recent study demonstrated safety and efficacy of third-party rapidly-generated single-culture donor VST that recognized 12 antigens from EBV, AdV, CMV, BKPyV, and HHV6 in 38 patients enrolled in a phase II trial. Importantly, clinical benefit could be demonstrated also in seven patients treated for multiple coincident infections (36). VST banks recently created include products characterized for epitope specificity and HLA restriction elements, to further refine selection of the best VST for each patient.

CONCLUDING REMARKS

It is likely that VSTs will have an increasing role as therapeutics in the prevention and management of viral infections after HSCT, due to high rate of response and limited toxicity profile observed in reported studies. However, many issues are still open, and will need to be addressed in future studies, such as the most suitable predictive markers for response, the identification of patients at risk of treatment failure, optimal treatment schedule in different clinical settings, and choosing adequate end-point for future clinical trials.

The majority of subjects treated to date with cell therapy for infections have received dedicated donor T cells, but this approach may not be best suited for widespread cost-effective access, since these are personalized medicines that are produced on-demand through a complex and costly supply chain. The development of new methodologies to obtain rapid manufacture of third-party T cells, refinement of strategies to allow adequate selection of the "best VST" for each candidate patient and the possibility to widen applicability to setting beyond HSCT, has prompted considerable interest from the industry to bring to the market third-party cellular therapies. This process will allow to benefit patients through better T cell therapy access.

AUTHOR CONTRIBUTIONS

SBa, FC, PZ, GG, SBo, EB, JB, MS, CDF, ML, MZ, and PC all participated in writing the manuscript. SBa, MZ, and PC co-edited the final version of the manuscript. All of the authors have read and approved the final manuscript.

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"Age Related Differences in the Biology of Chronic Graft-Versus-Host Disease After Hematopoietic Stem Cell Transplantation"

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It is established that pediatric hematopoietic stem cell transplant (HSCT) recipients have a lower rate of chronic graft-versus-host disease (cGvHD) compared to adults. Our group has previously published immune profiles changes associated with cGvHD of clinically well-defined adult and pediatric HSCT cohorts. Since all analyses were performed by the same research group and analyzed using identical methodology, we first compared our previous immune profile analyses between adults and children. We then performed additional analyses comparing the T cell populations across age groups, and a sub-analysis of the impact of the estimated pubertal status at time of HSCT in our pediatric cohort. In all analyses, we corrected for clinical covariates including total body irradiation and time of onset of cGvHD. Three consistent findings were seen in both children and adults, including elevations of ST2 and naive helper T (Th) cells and depression of NK_{reg} cells. However, significant differences exist between children and adults in certain cytokines, B cell, and T_{reg} populations. In children, we saw a broad suppression of newly formed B (NF-B) cells, whereas adults exhibited an increase in T1-$CD21^{lo}$ B cells and a decrease in T1-$CD24^{hi}CD38^{hi}$ B cells. Prepubertal children had elevations of aminopeptidase N (sCD13) and ICAM-1. T_{reg} abnormalities in children appeared to be primarily in memory T_{reg} cells, whereas in adults the abnormalities were in naïve T_{reg} cells. In adults, the loss of PD1 expression in naïve T_{reg} and naïve Th cells was associated with cGvHD. We discuss the possible mechanisms for these age-related differences, and how they might theoretically impact on different therapeutic approaches to cGvHD between children and adults.

Keywords: chronic graft-versus-host disease, children, adolescent, adults, puberty, immune profile

BACKGROUND

Pediatric hematopoietic stem cell transplantation (HSCT) recipients have a lower rate and possibly different presentations of chronic graft-versus-host disease (cGvHD) compared to adults (1). It has long been hypothesized that greater thymic function in children is the primary reason for the lower rate cGvHD, yet little human evidence after HSCT supports this hypothesis. Previous evaluations of cellular and plasma markers of cGvHD in adults, by our group and others, have identified 3 primary cellular populations that characterize cGvHD, including $CD21^{lo}$ B cells, NK_{reg} cells, and naïve T cells; as well as consistent changes in ST2 (2–6). Our group recently concluded the Applied Biomarkers of Late Effects of Childhood Cancer (ABLE)/Pediatric Blood and Marrow Transplant Consortium (PBMTC) 1,202 study, which included 302 pediatric transplant patients, 52 of whom had cGvHD (7, 8). This has allowed us to compare and contrast potential differences between children and adults in the immune cell and plasma cytokine profiles seen in our previous adults studies that correlate with the development of cGvHD.

Puberty is the result of activation of the hypothalamo-pituitary-gonadal axis and, as a consequence, of the increased production of androgens and estrogens usually starting between the age of 8 and 13 years in girls and 9 and 14 years in boys (9). Onset of puberty is associated with a decline in thymic function, and possibly splenic function as well (10, 11). Yet, the exact differences that age and puberty have on the development of immune reconstitution post-HSCT, GvHD, and the induction of immune tolerance are still incompletely studied. Comprehensive approaches to immune profiling, evaluation of thymic and splenic function, as well as the impact of sex hormones on the development of cGvHD are needed.

In this manuscript, we use our ability to perform broad immune cell (including T-, B-, and NK-cell populations) and plasma cytokine profiling to examine for differences in cGvHD markers between prepubertal children, pubertal adolescents, and adults. Based on these preliminary analyses, we develop hypotheses that could explain how cGvHD might be influenced by the recipient's age and pubertal status at the time of HSCT and provide insight into how cGvHD may be biologically different between children and adults.

METHODS

Study Populations

Adult Biomarker Studies

Samples from patients ≥18 years of age were collected as part of a Canadian Institutes of Health Research funded biomarker companion study based at the BCCH Research Institute, including 9 Canadian adult HSCT centers [all members of the Canadian Blood and Marrow Transplant Group (CBMTG)], 2 US centers, and 1 Saudi Arabian HSCT center. The companion study included patients enrolled on the CBMTG 0601 and 0801 trials as previously described (4, 12). Peripheral blood samples were collected on day 100 ± 14 days after transplantation, as well as at the time of cGvHD diagnosis, and evaluated for cellular and plasma markers. All samples were obtained after informed consent with institutional research ethics board approval.

Pediatric ABLE/PBMTC 1202 cGvHD Biomarker Study Design

Twenty-seven pediatric transplant centers (6 Canadian, 20 US, and 1 Austria) enrolled 302 patients <18 years of age between August 2013 to February 2017. All sites had institutional ethics board approval. The clinical study was described in detail (7). Centers assessed children for cGvHD according to the 2005 National Institutes of Health Consensus Criteria, with review by a central study adjudication committee composed of experts in cGvHD. Samples were evaluated by a broad immunophenotyping and plasma cytokine strategy (8).

Assessment of Pubertal Status in Children

Since our pediatric study did not include formal Tanner staging when designed, we evaluated the impact of the estimated pubertal status at the time of HSCT on the later development of cGvHD by approximating pubertal development, based upon the average signs of visible pubertal changes that reflect the secretion of gonadal hormones, corresponding to breast Tanner stage 2 in girls and genitalia (penis) Tanner stage 3 in boys (13). Based on these assumptions, we estimated a "puberty cut-off" (pre-pubertal versus pubertal) in girls as reflecting the 50^{th} percentile for Breast Tanner stage 2, occurring at 10.9 years (95% range; 8.9 to 12.9 years), and for boys as the 50^{th} percentile in penis Tanner stage 3 occurring at 12.4 years (95% range; 10.1 to 14.6 years) (13).

Shipping of Samples

Two different types of tubes were used for sample collection: heparinized tubes for plasma (BD Vacutainer) and Cyto-chex® BCT tubes, STRECK. INC. (Canada distributor: Inter Medico, Markham, ON, Canada) tubes for immunophenotyping. All peripheral blood samples were shipped to the Transplantation Applied Biomarkers laboratory at BC Children's Hospital Research Institute in Vancouver, BC, Canada *via* FedEx overnight priority shipping (delivered within 24 h after blood collection). Plasma isolation and storage: upon sample delivery, plasma was isolated from blood cellular component by primary centrifugation. Plasma aliquots were kept frozen at -80°C until usage. The tubes were shipped at room temperature overnight and phenotyping performed on the same day of sample delivery.

Phenotyping Procedure

Five panels were designed to look for different sub-populations in T, B, dendritic, and NK cells. All antibodies, corresponding conjugated dyes, clones, and vendors as previously described [(8), **Supplemental Table 3**]. One hundred microliter of blood was used for all panels except for the T_{reg} panel where 200 ul of blood was used. Samples were stained in the dark for 12 min at room temperature (RT) followed by treatment with fix/RBC lyze solution (eBiosceinces, Thermo Fisher Scientific, Waltham, US). For intracellular staining, cells were made permeable using BD

Perm II solution (BD Biosciences Mississauga, Canada). Flow cytometry data were acquired using BD LSR Fortessa X-20 Special Order four channel flow cytometer (BD Biosciences, San Jose, CA, US). A minimum of 300,000 events were acquired for all panels. Instrument settings was also standardized using SPHERO™ Rainbow Calibration particles 6 peaks (Sphereotech, Lake Forest, IL, US) to adjust laser power drifts over time. FCS files were analyzed using Kaluza software v2 (Beckman Coulter, INC. Mississauga, Canada). Flow cytometry accuracy, reproducibility was ensured by the detailed approaches as previously described (8).

Cytokine Measurement

Samples were collected and shipped as previously described (4, 8). Platelet depleted plasmas were isolated and frozen within 24 h of collection, as previously described (4). Batches of plasma samples were thawed and eleven cGvHD-associated markers were analyzed in both the adult and pediatric cohorts, including ST2, Osteopontin, sBAFF, sCD25, TIM-3, MMP3, ICAM-1, CXCL10, CXCL9, CXCL11, and soluble aminopeptidase N (sCD13). Reg3alpha was measured in the pediatric population only. CXCL9 and CXCL11 were measured using electrochemiluminescence dual-plex plate (Meso Scale Diagnostics LLC, Gaithersburg, US). sCD13 was measured using colorimetric assay based on enzymatic activity, as previously described (4). The remaining cytokines were measured by standard colorimetric ELISA (RnD Systems, Minneapolis, US). We found a high accuracy, reproducibility, and linearity for all assays measuring soluble biomarkers and a high stability of analytes upon 24 shipment as have been previously described in adults (4) and children (8).

Statistical Analysis of Results

Flow cytometry data was pre-processed by removing margin events, compensating the data, applying a logicle transform and using flowCut (14) to eliminate artifacts caused by poor flow. Files were then gated based on a designated gating strategy using flowDensity (15). After preprocessing the flow cytometry data, the flowType pipeline was used to identify cell populations as previously described (3). We looked at the 2-grouping cGvHD- versus cGvHD+. We conducted a statistical analysis of the cell frequencies as a percentage of their respective parent populations for all populations in pre-determined gating strategy. All three criteria were required to highlight biologically relevant markers including: a) $p \leq 0.05$, b) receiver operator curve (ROC) area under the curve (AUC) ≥ 0.60, and c) effect ratio of ≥ 1.3 or ≤ 0.75. The p-value of each marker was estimated based on the Wald test. ROC AUC was computed by estimating the true positive rate (proportion of cGvHD or late aGvHD correctly classified) against the false positive rate (proportion of controls falsely classified as cGvHD or late aGvHD) for different marker thresholds. The effect ratio was calculated as the average marker value of patients with cGvHD (or late aGvHD) divided by the average marker value of controls. For the T cell analysis, when using the flowType pipeline for the 2-grouping, we found X/Y-values <0.05. Of the immunophenotypes with significant p-values, we selected those with ROC AUC ≥ 0.6 and effect ratios of ≥ 1.3 or ≤ 0.75 for analysis with RchyOptimyx (16) in order to find the minimal and optimal set of immunophenotyping markers for the diagnosis of cGvHD.

Patients were assigned as having cGvHD or as a control in the analysis, as we previously have described (8). As our analyses were exploratory, no statistical adjustments were made for multiple testing. Given that there were numerous tests conducted, the probability of a Type I error likely exceeded 0.05, but this was moderated by the additional ROC AUC and effect ratio criteria. Because our data base did not include Tanner staging, we examined the impact of the puberty status at the time of HSCT on the later development of cGvHD by estimating the onset of puberty based where the average age of onset associated with an increase in the production of gonadal hormones that is sufficient to cause an increase in growth in height velocity: with breast Tanner stage 2 in girls and genitalia (penis) Tanner stage 3 in boys (13). Based on the puberty "cut-off" established above (13), each of the pediatric ABLE cohort (cGvHD, aGvHD, and controls) were divided <10.9 and 12.4 years and ≥10.9 and 12.4 years for boys and girls, respectively, and analyzed for each age group. The following clinical variables were modeled as confounding factors in the logistic regression model: a) prophylaxis or treatment with either alemtuzumab or ATG, b) prophylaxis or treatment with rituximab, c) recipient age, d) the use of a peripheral blood donor product or not, e) whether the donor was HLA-identical or not. In addition, all analyses were corrected for whether patients received TBI and the time of onset of cGvHD. All analyses were performed using MATLAB (MathWorks, Natwick, Mass, USA) and R (17).

RESULTS

Description of the Pediatric and Adult Population Utilized in the Comparison of Immune Profiling in cGvHD

For an initial comparison of immune profile differences between the adult and pediatric cohorts, we utilized the published results of 2 previously evaluated cohorts. The adult cohort (**Table 1**; N = 107) has been previously evaluated for B cell population profiles (18) and NK, T cell, and cytokine, populations at the onset of cGvHD (4). The pediatric cohort was from the later ABLE studies (**Table 1**; N = 302), and to date, only the analyses of the day 100 samples have been completed and described (8). The two cohorts are representative of the type of transplants performed in adults and children. While all HSCTs in the adult cohort were for malignant conditions, only 59% were in the pediatric cohort (**Table 1**). The pediatric cohort was characterized by greater use of cord blood as a donor source, compared to G-CSF mobilized peripheral blood progenitor cells (PBPC) being used more frequently in the adult cohort. Sibling donor transplants were more common in the pediatric cohort. The tissue distribution of cGvHD in the two cohorts was different, in that skin and lung cGvHD was more common in the adult population compared to the pediatric cohort. Atypical cGvHD presentations not associated with the diagnostic criteria

TABLE 1 | Baseline Characteristics of the pediatric (ABLE) and adult cohorts evaluated in these analyses[3].

Characteristic	Pediatric Cohort[1](Overall Percentages of Entire Evaluable Cohort n = 243)		Adult Cohort[2](Overall Percentages of Entire Evaluable Cohort n = 107)	
	No Late Acute GVHD or Chronic GVHD (n = 132)	Chronic GVHD (n = 51)	No cGvHD (n = 63)	cGvHD (n = 44)
Diagnoses				
Malignant	78 (59)	41 (80)	63 (100)	44 (100)
ALL	33 (25)	18 (36)	9 (14)	7 (16)
MDS/AML	37 (28)	15 (30)	25 (40)	11 (25)
			9 (14)	12 (27)
Mixed Lineage Acute Leukemia/Other	0 (0)	1 (2)	2 (3)	1 (2)
NHL	4 (3)	3 (6)	10 (16)	7 (16)
JMML	3 (2)	1 (2)	0 (0)	0 (0)
CML	1 (1)	3 (6)	4 (6)	1 (2)
CLL	0 (0)	0 (0)	2 (3)	2 (5)
MM	0 (0)	0 (0)	2 (3)	3 (7)
Non-Malignant	54 (41)	10 (20)	0 (0)	0 (0)
Sex				
Male (55.6%)	72 (55)	33 (65)	34 (54)	26 (59)
Female (44.4%)	60 (46)	18 (35)	29 (46)	18 (41)
Age at Transplant				
Median Age (Years, Range)	9.3 (0.2–17.9)	11.9 (2-18)		
<50 years			25 (40)	16 (36)
≥50 years			38 (60)	28 (64)
Donor and HLA Match				
HLA-Matched Family Donor (8/8)	54 (41)	8 (16)	17 (27)	19 (43)
Haploidentical Family Donor (with PTCy)	2 (2)	1 (2)	0 (0)	0 (0)
HLA-Matched Unrelated Donor (8/8)	48 (36)	22 (43)	17 (27)	14 (32)
HLA-Mismatched Unrelated Donor (≤7/8)	9 (7)	12 (24)	29 (46)	11 (25)
Cord Blood Matched and mismatched	19 (12)	8 (16)	0 (0)	0 (0)
Stem Cell Source				
Bone Marrow	94 (71)	25 (49)	4 (6)	3 (7)
PBSC	19 (14)	18 (35)	32 (51)	37 (84)
Cord Blood	18 (14)	7 (14)	27 (43)	4 (9)
Double Cord Blood	1 (1)	2 (3)	0 (0)	1 (2)
Conditioning Regimen				
Myeloablative	111 (84)	45 (88)	27 (43)	18 (41)
TBI 1200-1320 cGY +/- Other	33 (25)	19 (38)	19 (30)	10 (23)
Chemotherapy + 200-400 cGY TBI	7 (5)	1 (2)	0 (0)	0 (0)
Myeloablative w/o TBI	71 (5)	25 (50)	8 (13)	8 (18)
Reduced Intensity or Non-myeloablative	21 (16)	6 (12)	36 (57)	26 (59)
GVHD Prophylaxis				
CNI + MTX/MMF ± Sirolimus	115 (88)	50 (97)	48 (76)	38 (86)
CNI ± Sirolimus	0 (0)	0(0)	10 (16)	6 (14)
PTCy + CNI + MMF	5 (4)	1 (2)	0 (0)	0 (0)
CNI + Steroid	1 (1)	0 (0)	0 (0)	0 (0)
Other	11 (8)	0 (0)	5 (8)	0 (0)
History of Acute GVHD				
None	87 (66)	8 (16)	27 (43)	22 (50)
Yes	45 (34)	43 (84)	36 (57)	22 (50)
cGvHD Organ involvement in those affected by cGvHD				
Skin involvement		43.1%		61%
Oral involvement		62.7%		66%
GI involvement		39.2%		23%
Eye involvement		29.4%		45%
Joint involvement		5.9%		15%
Lung involvement		23.5%		47%
Liver involvement		27.5%		51%
Genital involvement		2%		16%
Other		21.7%		This data was not collected
• Pericardial effusion				
• Eosinophilia				
• ITP				
• Nephrotic syndrome				
• Cardiomyopathy				
• Neuropathy				

[1] Summarized data from the ABLE pediatric cohort (N = 183) as published (7, 8) [2] Summarized data from the adult cohort (N = 107) as published (3, 19); [3]These two cohorts were used for both the summary of the known published results presented in ***Table 2*** *as well as the prospective analyses presented in* ***Figures 1–3****.*

for National Institutes of Health cGvHD were relatively frequent (21.7%) in the pediatric cohort, but this data was not reliably collected in the adult cohort to allow a comparison.

B Cell, NK Cell, and Cytokine Differences Between Adult and Pediatric Populations in our Previously Published Studies

We have previously analyzed these two cohorts separately and published the results (4, 8, 18). While the pediatric analysis is only focused on immune profiles at day 100 post HSTC (8) and measured before the onset of cGvHD, and published adult results (4) were measured at the onset of cGvHD, we felt we could identify age-related patterns to guide additional comparisons of the two cohorts. We have previously performed comprehensive analysis of the B cell profiles in children (8) and adults (18) in the same laboratory and utilizing similar immune profiling strategies. For a cell population to be considered increased or decreased, it had to meet our definition of being a biologically relevant marker meeting all three of the following criteria including a) p value ≥0.05, b) ROC AUC ≥0.60; and c) effect ratio of ≥1.3 or ≤0.75. If it did not meet all three of these rigorous inclusion criteria we excluded it from the comparison. Comparison of B cell immune profiles identified population similarities and differences between the adult and pediatric cohorts (**Table 2**). We found that both adults and children had decreases in newly formed B cell populations (NF-B cells). Only the pediatric cohort, however, had decreases in the T2 and T3 transitional B cell populations. By contrast, $CD10^{hi}CD38^{hi}CD19^{+}$ B cells, another transitional B cell population commonly associated with the B_{reg} population, was depressed in adults at the time of cGvHD diagnosis, but not in children. Two B cell populations had opposite results between the pediatric and adult cohorts. $CD21^{low}$ B cells were increased in adult cGvHD, but in children with cGvHD, were significantly decreased (**Table 2**). By comparison, an increase in unswitched memory/Marginal zone-like B cells in pediatric cGvHD was observed versus a significant decrease in switched memory B cells in adult cGvHD (**Table 2**). Both the adult and pediatric cohorts exhibited decreases in mature naïve B cells.

In adults, we previously observed a similar decrease in NK_{reg} cells, both in the adult cGvHD cohort described in this paper (4) and in a separate adult donor cohort that had an evaluation of the infused donor product (3) (**Table 2**). In our pediatric cohort, we also saw a significant decrease in $CD56^{bright}$ noncytolytic NK cells (8), NK cells that are consistent with regulatory NK cells (NK_{reg}) (19, 20). Thus, unlike B cells, no age-related differences in the NK_{reg} population were observed, with decreases in NK_{reg} cells seen in both the adult and pediatric cGvHD cohorts.

Our group and others have observed plasma differences in a number of soluble factors between children and adults (4, 5). Our comparison of the ABLE pediatric cohort (8) found that ST2, in a similar manner to adults (5), is elevated in children (**Table 2**). Other cytokines such as CXCL10 and CXCL9 that were significantly elevated in adults with cGvHD (4, 6) could not be confirmed in the pediatric cohort. By comparison, aminopeptidase N (soluble CD13), originally found at cGvHD diagnosis by proteomic analysis in children (21), as well as ICAM-1, both appear increased in children with cGvHD.

TABLE 2 | Summary of Published Immune Profiling Studies Published by the BCCH group for B cells, NK cell and plasma marker populations associated with cGvHD in Separate Adult and Pediatric Cohorts.

Cell population		Pediatric (0–18 years; N = 241)[2] Day 100 in cGvHD	Adult (≥ 18 years;N = 107)[1] Onset of cGvHD
B cell populations			
T1 - Immature/Transitional B cell population consistent with Breg cells	$CD24^{hi}CD38^{hi}CD19^{+}$	NS	Decreased[4]
CD21 low B cells	$CD21^{lo}/CD19^{+}$	Decreased	Increased
T2 transitional	$CD38^{int}CD10^{int}$ of $CD19^{+}$	Decreased	NS
T3 transitional	$CD38^{dim}CD10^{lo}$ of $CD19^{+}$	Decreased	NS
Mature Naïve	$CD27^{-}$ $IgD^{+}CD19^{+}$	Decreased	Decreased
Unswitched memory/ Marginal-zone like	$\%CD27^{+}IgD^{+}$ of $CD19^{+}$	Increased	Decreased
Classical switched memory	$\%CD27^{+}IgD^{-}$ of $CD19^{+}$	NS	Increased
NK cell populations			
Regulatory $CD56^{bright}$ NK cells		Decreased Before onset – day 100	NA
		Decreased at onset	Decreased at Onset[1]
			Decreased in Donor cell infusion[3]
Plasma markers			
		Day 100	Onset of cGvHD
Aminopeptidase N (sCD13)		Increased	Increased but variable
ST2		Increased	Increased
CXCL10		NS	Increased
CXCL9		NS	Increased but variable
ICAM-1		Increased but variable	Increased but variable

[1]Rozmus (19) for adult data – N = 104; 44 with cGvHD onset (median of 207 days post-HCT; range 83–424 days) and 63 patients without cGvHD with sample collection a median of 194 days post-HCT (range 153–430 days); [2] Schultz KR (8) for pediatric data - N = 241 patients evaluated at day 100. [3]Adult data on donor product infused as part of the CBMTG0601 trial (N = 79) (3). [4] To be defined as increased or decreased, the marker had to meet our definition of being a biologically relevant marker which included all of the following 3 criteria a) p value ≤0.05; b) ROC AUC ≥0.60; and effect ratio of ≥1.3 or ≤0.75.

A Direct Comparison of T Cell Differences Between Adult and Pediatric Populations

We performed a direct comparison of the T cell populations evaluated at day 100 in both the adult and pediatric cohorts. Data was merged into a single data set with flow-cytometry reanalyzed to ensure identical gating between the two studies. An unsupervised comparison analyses (**Figure 1**) was then performed on the T cell populations at Day 100 after HSCT in the adult and pediatric ABLE cohort. Overall, T cell patterns appear to be more complex in the adult cGvHD patients compared to children at day 100 who would later go on to develop cGvHD (**Figure 2**; **Supplemental Table 1**). The only markers commonly affected in the same way between adults and children included an increase in overall $CD3^+$ T cells and Naïve Th cells ($CD31^-CD3^+CD4^+CD45RA^+$). $CD31^+$ (recent thymic emigrant or RTE) Naïve Th cells were also affected in both the pediatric and adult cohorts, but in opposite directions, being increased in adults compared to decreased in children (**Figure 2**). Additional changes in adults included an increase in the $PD1^-$ Naïve Th cell population. While we could demonstrate no differences in T_{reg} cells in the overall pediatric cohort, we did see an increase in both $PD1^-$ and $CD31^+$ T_{reg} populations in the adult cohort. Memory Th cells were also significantly different in adults, with an increase in $PD1^-$ memory Th cells and a decreased in $PD1^+$ memory Th cells (**Figure 2**).

Impact of Puberty Status Pre HSCT on Immune Profiles in cGvHD in the Pediatric Population

The pediatric ABLE cohort included patients 0–18 years of age. We hypothesized that the differences we had identified between adults and children potentially would begin to change at the onset of puberty, with adolescent cGvHD markers becoming more adult-like after puberty. Based on standardized data for the onset of puberty in North American children (9), we estimated the average age of onset of Tanner stage 2 breast development in girls (10.9 years) and Tanner stage 3 penis development in boys (12.4 years) as reflecting secretion of gonadal hormones the point between being prepubertal and pubertal. We divided the <18 year old pediatric cohort from the ABLE study into a prepubertal (< 10.9 years for girls and < 12.4 years for boys) and pubertal age group based upon these age cut-offs, (≥ 10.9 years in girls and ≥ 12.4 years in boys) grouping and evaluated the impact of puberty at the time of HSCT on cGvHD immune profiles. All analyses were adjusted for a number of clinical factors including the impact of TBI and the time of onset of cGvHD (**Figure 3**). We evaluated the identical T cell populations identified in **Figure 2**. We found that naïve Th cells were significantly increased in both the prepubertal and pubertal cohort (**Figure 3**; **Supplemental Table 2**). PD1 expression on naïve Th cells continued to have no significance on the pediatric subpopulation analysis. Interestingly, the significant decrease in $CD31^+$ (RTE) naïve T cells in the overall pediatric group was due primarily to a decrease in the prepubertal group (**Figure 3**; **Supplemental Table 2**), with an effect ratio of 0.5 in the prepubertal children (**Figure 3**), increasing to an effect ratio of 1.2 in the pubertal group, which approaches that of the adults (effect ratio of 2.1). While we identified no difference in T_{reg} populations in the overall pediatric cohort, our sub analysis identified that $PD1^-$ memory T_{reg} cells were increased in the prepubertal group (**Figure 3**; **Supplemental Table 2**), and $PD1^+$ memory T_{reg} cells were significantly increased in the pubertal group. A second T_{reg} population was altered in the prepubertal group, with a decrease in RTE ($CD31^+$) naïve T_{reg} cells, whereas these cells were not significantly different in the pubertal group.

We evaluated the impact of puberty at the time of HSCT on B cell populations at day 100 in the ABLE cohort. We selected the six B cell populations that were significantly different between the adult and pediatric cohorts (excluding mature naïve B cells, as they were decreased in both adults and children). We found that the T2 and T3 transitional B cell populations were only decreased in prepubertal children, but were not significantly different in the pubertal population. In prepubertal populations, the effect ratios of 0.44 and 0.44 for the T2 and T3 transitional B cell populations, respectively, were significant; however, neither were significant for the pubertal population. (**Figure 3**; **Supplemental Table 2**). Evaluation of the unswitched memory/marginal zone-like B cells demonstrated that these B cells were only significantly increased in prepubertal children (effect ratio of 3.7) and had a non-significantly lower effect ratio (1.4) in the pubertal population. By contrast, we saw a significantly decreased effect ratio in of $CD21^{low}$ B cells in prepubertal with a non-significant decrease in this population pubertal children to 0.53. Switched memory B cells continued to be unchanged in cGvHD in the pediatric cohort.

NK_{reg} cells were decreased with cGvHD (**Figure 3**) in both the prepubertal group with an effect ratio of 0.67 ($p = 0.006$; ROC AUC = 0.68) and for pubertal children with an effect ratio of 0.68 ($p = 0.05$; ROC AUC = 0.69). The impact of puberty pre HCT on cytokines expression in plasma was also evaluated (**Figure 3**; **Supplemental Table 2**). ST2 was significantly elevated in both prepubertal children with an effect ratio of 1.5 ($p = 0.04$; ROC AUC = 0.68) and pubertal children with an effect ratio of 1.5 ($p = 0.04$ ROC AUC = 0.68). By contrast, Aminopeptidase N or sCD13 was only significantly increased in prepubertal children with an effect ratio of 1.6 ($p = 0.004$; ROC AUC 0.67) and non-significantly increased in pubertal children with an effect ratio of 1.3 ($p = 0.11$; ROC AUC = 0.69). Similar to sCD13, ICAM1 was only significantly elevated in prepubertal children with cGvHD with an effect ratio of 1.4 ($p = 0.01$; ROC AUC = 0.63). Neither CXCL10 or CXCL9 were increased in either group.

DISCUSSION

Although there appear to be consistent cGvHD immune profile patterns in NK_{reg} cells, naïve Th cells, and ST2, regardless of age (**Table 3**), the comparisons we have performed support that significant differences exist in a number of T and B cell subpopulations and cytokine profile patterns between pediatric and adult HSCT patients with cGvHD. For cellular populations that were different between the pediatric and adult cohorts in our

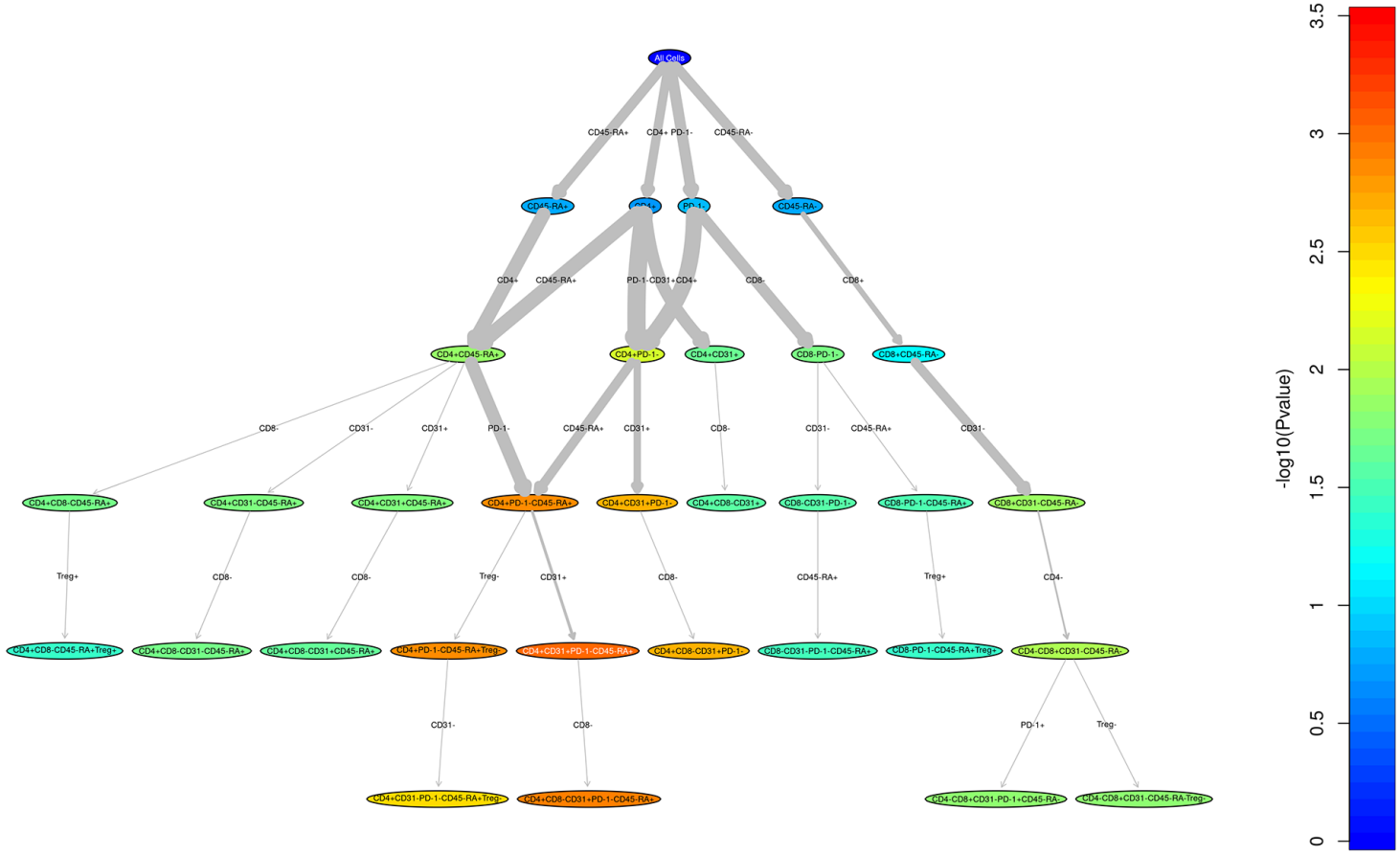

FIGURE 1 | Unstructured analysis of adult and pediatric T cell populations in cGvHD. This RChyOptimyx plot depicts the results of the unstructured statistical analysis conducted to find combinations of markers which best predict cGvHD. Colors correspond to the p-values and the width of the arrows corresponds to the change in p-value after including an additional marker.

studies, pubertal patients often had immune profile values in-between adults and prepubertal children (**Table 3**), suggesting a transition in cGvHD mechanisms associated with the onset of puberty.

Age Related T Cell Differences

T cell similarities and differences identified appear to involve primarily Naïve Th cells (**Table 3**). Thymic function is highest before puberty, with intermediate function at the onset of puberty and further decline towards adulthood (10). Both children and adults in our cohorts had significant expansions of naïve Th cell associated with cGvHD, although the pattern of naïve Th cell expansion was different. In adults, we observed that PD1 expression in naïve Th cells may play a role in the development of cGvHD, with an expansion of $PD1^-$ naïve Th cells (**Figure 2**). By contrast, PD1 expression on naïve Th cells was not important in the cGvHD pediatric cohort. One other difference in the T cell pattern we observed between pediatric and adult cohorts was that in adults (but not children) $PD1^-$ memory Th cells were increased, whereas $PD1^+$ memory Th cells were decreased. This would be consistent with the dependence on PD1 as a regulator of peripheral tolerance, the primary mechanism of T cell tolerance in adult and post pubertal recipients (22). PD1 independent and thymic-dependent mechanisms may therefore play a greater role in the prepubertal population in regulating cGvHD development.

Age Related B Cell Differences

Our group was the first to identify the critical role of B cells in the development of cGvHD, in mice (23), followed by identification of significant B cell abnormalities in children (24). Interestingly, in the current analysis, the B cell compartment is where we identified no common B cell profiles and some of the greatest differences between the pediatric and adult cohort (**Table 3**). The primary impact of cGvHD, both in adults and children, appears to be in what has been recently designated as newly formed B cells (NF-B), including all transitional and immature B cells (25). In the pediatric cohort, we saw a broad depression of the NF-B cell populations associated with cGvHD including the T1-$CD21^{lo}$, T2, T3, and mature naive B cell populations, with these differences primarily seen in the prepubertal subgroup. The only population that was increased in cGvHD in the pediatric group was the unswitched memory/marginal zone-like B cell population. In contrast to the pediatric group, adults had a significant increase in the transitional T1- $CD21^{lo}$ B cell population (associated with autoimmunity) and a significant decrease in transitional T1-$CD24^{hi}CD38^{hi}$ B cell populations (associated with a B_{reg} population). This suggests that B_{regs} may play a much greater role in controlling cGvHD in adults compared to children.

The different B cell patterns seen in NF-B cells between children and adults suggests there are significant differences in the role that B cell regulation may play in pediatric versus adult cGvHD. There is evidence that patients with autoimmune disease

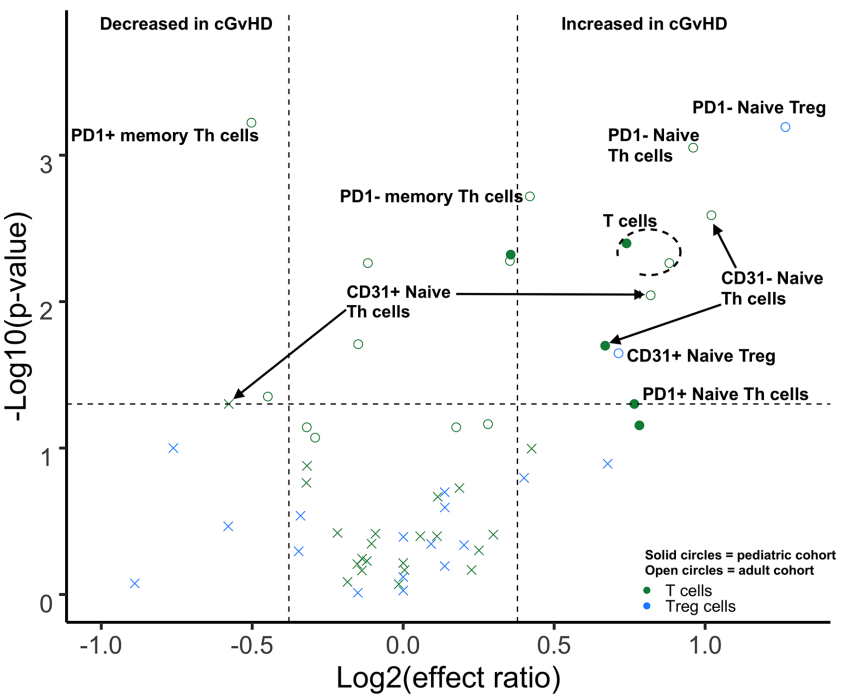

FIGURE 2 | Differences of Day 100 adult and pediatric T cell populations in cGvHD. Volcano plots that met our definition of a biologically relevant markers for cGvHD were required to meet all 3 criteria of a i) p-value ≤0.05 (y-axis), ii) receiver operator curve (ROC) area under the curve (AUC) of ≥0.60 (circle: ≥0.60 and cross: <0.6), and iii) effect ratio ≥1.3 or ≤0.75 (x-axis). A circle that is on either the upper right quadrant (higher in cGvHD) or upper left quadrant (lower in cGvHD) was considered a significant markers whereas a cross in these same quadrants, while meeting the criteria for effect ratio and p value, did not have an ROC AUC ≥0.60. Cell populations are identified by color with green = T cells and light blue = T_{reg} cells. Solid circles = the pediatric cohort and open circles = the adult cohort.

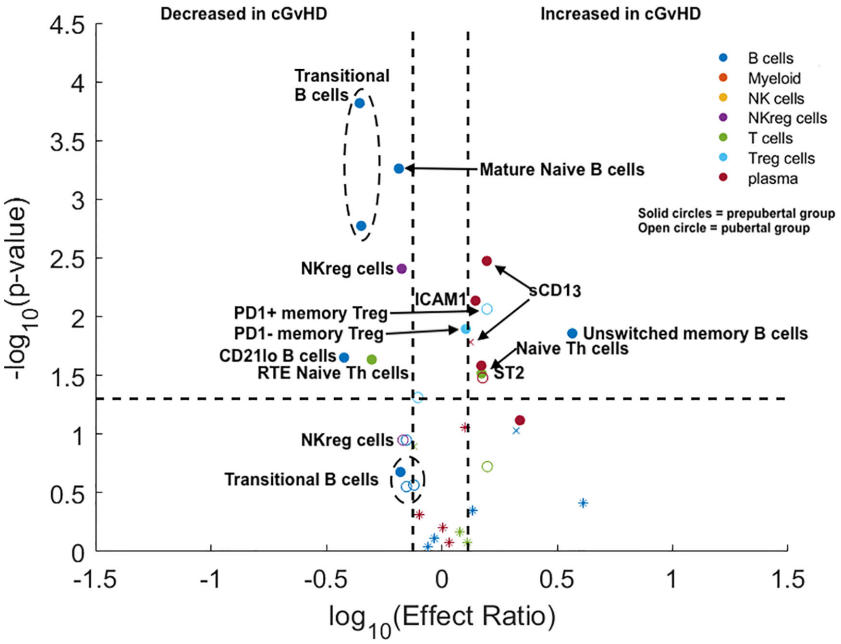

FIGURE 3 | Evaluation of the impact of estimated pubertal status pre HSCT on cGvHD Immune Profile. Volcano plots that met our definition of a biologically relevant marker for cGvHD were required to meet all 3 criteria of a i) p-value ≤0.05 (y-axis), ii) receiver operator curve (ROC) area under the curve (AUC) of ≥0.60 (circle: ≥0.60 and cross: <0.6), and iii) effect ratio ≥1.3 or ≤0.75 (x-axis). A circle that is on either the upper right quadrant (higher in cGvHD) or upper left quadrant (lower in cGvHD) was considered a significant markers whereas a cross in these same quadrants, while meeting the criteria for effect ratio and p value, did not have an ROC AUC ≥0.60. Cell populations are identified by color with dark blue = B cells, orange = myeloid populations, yellow = NK cells, purple = NK_{reg} cells, green = T cells, light blue = T_{reg} cells, and dark red = plasma cytokines. Solid circles = the prepubertal group and open circles = the pubertal group. We note the following clinical variables were modeled as confounding factors in the logistic regression model: **(A)** prophylaxis or treatment with either alemtuzumab or ATG, **(B)** prophylaxis or treatment with rituximab, **(C)** recipient age, **(D)** the use of a peripheral blood donor product or not, **(E)** whether the donor was HLA-identical or not. The onset of puberty was estimated as 10.9 years in boys and 12.4 years in girls. The results are corrected for both the time of onset after HSCT and for the use of TBI.

suffer from defects in early B-cell tolerance checkpoints in the T1 transitional NF-B cell populations, resulting in selection of autoreactive NF-B cells (26, 27) that potentially present self-antigen to T cells, similar to early murine models of cGvHD (23). In the area of autoimmunity, increased circulating NF–B cells are found in SLE, type 1 diabetes, and juvenile dermatomyositis (28, 29) possibly as an important source of pathogenic autoantibodies. It is suggested that autoreactive NF-B cells contains clones that may develop into $CD27^{-}$ $CD21^{-/lo}$ B cells after the acquisition of somatic hypermutations that improve affinity for self-antigens (27). In adults, we observed an inverse relationship between increased $CD21^{lo}$ B cells and decreased T1 $B_{regs,}$ suggesting a possible inhibitory impact of B_{reg} cells on $CD21^{lo}$ B cells at the early B cell checkpoint.

In children, we saw a very different pattern that also appeared to be a result of an early B cell checkpoint abnormalities that resulted in a broad suppression of a number of NF-B cell populations including $CD21^{lo}$ B cells. These two different patterns of B cell abnormalities resulted in increased classic switched memory and decreased unswitched B cell in adults and increased unswitched memory B cell in children. NF–B cell activation appears to be driven by TLR7 and TLR9 activation by recognition of RNA and DNA motifs (30–32). Our previous observation that a TLR-9 responsive B cell population was associated with the onset of cGvHD in children (24) suggests an aberrant NF-B population in children. In children, there is one other possible mechanisms by which B cell may impact on development of post HSCT tolerance whereby intrathymic B cells may support development of T_{regs} through cognate help and can shape the T_{reg} repertoire (33).

Regulatory Cells in cGvHD

Of all of the regulatory populations, NK_{reg} cells (19) appear to have a consistent age-independent role in suppression of cGvHD. $CD56^{bright}$ NK cells represent 10% of peripheral NK cells and are similar to decidual NK cells, with regulatory function, that inhibit placental rejection (34). The NK_{reg} populations is characterized by expression of granzyme K rather than expression of either perforin or granzyme B (35) and many times are considered as non-cytolytic. With large patient populations, we have identified in both children and adults, a non-cytolytic $CD56^{bright}$ NK population (NK_{reg}) closely correlating with a lack (or inhibition) of cGvHD. In adults, we have seen increased $CD56^{bright}$ $CD335^{+}$ $CXCR3^{+}$ NK_{reg} cells associated with decreased cGvHD (4); increased $CD56^{bright}$ NK_{reg} cells in adult donor product that correlated with suppression of cGvHD (3); and increased NK_{reg} cell numbers induced by ATG-treatment day at 100 post HSCT in 38 adults on

TABLE 3 | Summary of Recipient Age on cGvHD markers.

	Pre pubertal	Pubertal[1]	Adult
Naïve T cells			
• Naïve Th cells	Increased	Increased(NS)	Increased
• RTE Naïve Th cells	Decreased	NS	Increased[2]
• PD1- or PD1+ Naïve Th cells	NS	NS	Increased
Memory T cells			
• $PD1^+$ memory Th cells	NS	NS	Decreased
• PD1- Naïve Th cells	NS	NS	Increased
Newly formed B cells			
• $CD21^{lo}$ B cells	Decreased	NS	Increased
• T2 transitional	Decreased	NS	NS
• T3 transitional	Decreased	NS	NS
Peripheral B cells			
• Mature Naïve	Decreased	NS	Decreased
• Unswitched memory/Marginal-zone like	Increased	Increased(NS)[3]	Decreased
• Classical switched memory	NS	Increased(NS)	Increased
Regulatory populations			
Regulatory T cells			
• $PD1^-$ memory T_{reg} cells	Increased	Decreased(NS)	NS
• PD1+ memory T_{reg} cells	NS.	Increased	NS
• RTE memory T_{reg}	Decreased	NS	NS
• PD1- Naïve Treg	NS	NS	Increased
• RTE Naïve Treg	NS	Increased(NS)	Increased
Regulatory NK cells	Decreased	Decreased	Decreased
T1 - Transitional population consistent with Breg cells	NS	NS	Decreased
Cytokines and Chemokines			
• ST2	Increased	Increased	Increased
• CXCL10	NS	NS	Increased
• CXCL9	NS	NS	Variable
• Aminopeptidase N (sCD13)	Increased	Increased (NS)	Variable
• ICAM-1	Increased	NS	NS

[1]Prepubertal was defined as a girl aged <10.9 years or boy <12.4 years at time of HSCT and pubertal was defined as a girl ≥10.9 years or boy ≥12.4 years at time of HSCT. [2]CD31 expression was not important in adults with both $CD31^+$ and $CD31^-$ Th cell populations elevated. [3]the effect ratio met criteria of either ≤0.75 or ≥1.3 but was not statistically significant due to smaller number of patients.

the CBMTG 0801 trial where ATG significantly decreased cGvHD (12). Similarly, in the pediatric ABLE studies, we found increased noncytolytic $CD56^{bright}$ NK_{reg} cells in pediatric recipients at 3 months post HSCT in those who did not later develop cGvHD (8).

It has been postulated that T_{reg} cells play a role in cGvHD, but there are many conflicting studies regarding their role (36–38). Part of differing findings may be a result of age-related differences in the role of T_{reg} cell subpopulations in cGvHD (**Table 3**). We found that differences in memory T_{reg} cells were more predominant in prepubertal and pubertal groups whereas naïve T_{reg} cells appeared to be more important in adults (**Table 3**). In the prepubertal group we observed both an increase in $PD1^-$ memory T_{reg} cells numbers and a decrease in $CD31^+$, RTE memory T_{reg} cells (**Table 3**). Interestingly, a previous analysis of our group of the pediatric cohort at day 100 in those patient that had already developed cGvHD found an increase in $PD1^-$ memory T_{reg} cells and a concomitant decrease in $PD1^+$ memory T_{reg} cells (8). By contrast adults had no observable differences in memory T_{reg} cells but did have an increase in naïve T_{reg} cells either expressing CD31 (RTE) or lacking PD1 expression (**Figure 2**, **Table 3**).

The role of PD1 in regulation of memory and naive T_{reg} cells appears to be inadequate where PD1 blockade increases the proliferation of highly suppressive $PD1^+$ memory T_{reg} cells and inhibition of antitumor immunity (25). The absence of PD1 along with partial FoxP3 insufficiency, however, can result in T_{reg} cells with proinflammatory properties and expansion of effector/memory T cells that contributed to the autoimmunity (39). Others have established that PD1 is critical in modulating T_{reg} homeostasis during low does IL-2 therapy for cGvHD (41). They observed that $PD1^-$ memory T_{regs} showed rapid Stat5 phosphorylation and proliferation with IL-2 initiation followed by higher Fas and lower Bcl-2 expression decreased the effectiveness of IL-2 on memory T_{regs} (40). The importance of PD1 expression on either memory or naïve T_{reg} cells is further supported by a study that demonstrated that PD1 upregulated on T_{reg} cells and its interaction with PD1 ligand on effector T cells resulted in potent T cell suppression (41).

The other regulatory population that may be more prominent in the adult population is the T1- $CD24^{hi}CD38^{hi}$ B cell population, many times associated with a B_{reg} function. While we acknowledge that the only definitive way to evaluate B_{reg} cells is by a functional assay and thus cannot be sure this T1 B cell population is in fact a B_{reg} cell population, we could identify only a decrease in the B_{reg} phenotype in adults and not in children.

Age Related Cytokine Differences

Soluble ST2 was the only cytokine consistently increased in all age groups. Elevation in ST2 associated with cGvHD has been described in multiple adult studies and in our previous ABLE pediatric study (4, 5, 8, 22). The ST2-related chemokines, CXCL9

and CXCL10 were not elevated in the younger population suggesting a greater role in adults. By contrast, two cytokines may play a greater role in the pediatric population, aminopeptidase N (sCD13) and ICAM-1 (**Figure 3**), neither which is associated with ST2 functionally. We had initially found elevation of sCD13 in a pediatric study, COG ASCT0031, through proteomic discovery and validation (21). Subsequently, we and others have seen an elevation of sCD13 in adults. Our current analysis of the prepubertal versus the pubertal group showed that the association was strongest in prepubertal children. The source of sCD13 in the HSCT cGvHD environment is still not known. One group did find an apparently distinct $CD13^{+}CD33^{+}$ population of leukemic cells contributing to a proinflammatory microenvironment that was detrimental to long-term normal hematopoiesis (42) suggesting an inflammatory role of sCD13 in the hematopoietic microenvironment. It is possible that the increased sCD13 may impact early B cell lymphopoiesis in cGvHD. Support for this hypothesis is provided by data demonstrating the impact of Bestatin, a sCD13 inhibitor, on B cell lymphopoiesis. In mice, treatment with Bestatin increased the total number of thymocytes, splenocytes, and lymphocytes of mesenteric lymph nodes. Inhibition of sCD13 by bestatin decreased peripheral Th and Tc cells and augmented B cells in the peripheral lymphatic sites (43). In humans, Bestatin had a similar effect, augmenting immune reconstitution following HSCT with significant increases in NK cells and B cells (44). Thus, it is possible that the suppression of NF-B lymphopoiesis and thymopoiesis seen in children with cGvHD may be impacted by elevations of sCD13.

Impact of Sex Hormones on Immune Function in HSCT and cGvHD

It is well established that the onset of puberty appears to initiate the involution of the thymus and a decrease in thymic function (10). The thymus involutes after estradiol treatment or during pregnancy, in both mice and humans (45, 46). Since testosterone partially is converted to estradiol, testosterone treatment probably also has an identical impact. Moreover, decreasing recipient thymic function is hypothesized to be one of the major reasons for the increase in cGvHD seen in adult compared to children. Some have proposed that using steroid ablation will result in thymic regeneration in adults and potentially both help in both immune reconstitution after HSCT, but also may impact on the development of cGvHD (47). Our studies are limited in that we evaluated the pubertal status at the time of HSCT and could not evaluate the impact of hormonal levels on immune reconstitution after HSCT. We know that all myeloablative preparative regimens, whether including total body irradiation (TBI). However, steroid-secreting cells (Leydig cells in boys and granulosa cells in girls) are more resistant to TBI and high-dose cytotoxic drugs. Whereas germ cell producing Sertoli cells, in boys, and oocytes, in girls, are much more sensitive (48). It is also possible that alteration in sex hormone levels post HSCT may impact on immune reconstitution and possible the onset of cGvHD. We attempted to accommodate for some of these variables in our analysis of the prepubertal and pubertal sub analysis by considering a number of clinical covariates including both TBI and time of cGvHD onset as covariates and none of these factors had a major impact on the final results.

Proposed Model for Age Related Differences in cGvHD Immune Profiles

While many questions remain, we conclude that recipient age at the time of HSCT impacts on the immune profile of cGvHD cell populations and cytokines and needs to be taken into consideration when evaluating the biomarkers and immunology of cGvHD. We summarize the age related differences we have been able to identify and have attempted to develop a working model for the recipient impose differences in cGvHD development (**Figure 4**). In general, there appears to be some major differences in the immune profiles of pediatric versus adult cGvHD. In the T cell compartment, all age groups appear to have an increase in naïve Th cells. Interestingly, the increase in adults seem to be primarily due to an increase in an RTE $PD1^{-}$ naïve Th cell population. We saw more striking differences in B cells (**Figure 4**). Children had a broad suppression of NF-B cells where as many of the difference in adults appeared to be at the T1 transitional stage with an increase in $CD21^{lo}$ B cells that probably are due to an aberrant early B cell check point inhibition that may have prevented the development of this population. We hypothesize that there may be age related differences in how B cell abnormalities develop in NF-B cells at the early checkpoint associated with T1-trasnional B cells. In adults, there appears to be a major divergence to increased $CD21^{low}$ B cells whereas children develop a broad suppression of almost all NF-B cell lymphopoiesis.

Three regulatory populations appear to be important in preventing the development of cGvHD. All age groups appeared to depend on NK_{reg} cell suppression of cGvHD, but only adults appeared to have an association with cGvHD suppression by a T1 transitional B cell population that phenotypically is similar to B_{reg} cells (49). Children seem to develop abnormalities primarily in memory T_{reg} cells whereas adults had abnormalities in naïve T_{reg} cells. These findings suggest that the dynamics of the regulatory losses may be different before and after puberty. While ST2 is consistently elevated regardless of age, aminopeptidase N (sCD13), which is not known to be affected by ST2, was more prominently elevated in the prepubertal group and we hypothesize may be impacting on both dysfunctional B cell lymphopoiesis and thymopoiesis.

One factor that may have influenced the comparison of adult to pediatric cGvHD biology are the differences in HSCT approaches between the two age cohorts including the greater use of myeloablative regimens, higher number of non-malignant indications, and low use of PBPC as a donor source in the pediatric population compared to adults. In our multivariate analyses, we corrected for these co-variates as much as possible and still observed significant differences leading to our conclusion that there are recipient age-related differences. Moreover, our comparison of the prepubertal to the pubertal subgroups essentially compared groups receiving he identical transplant approaches as they were limited to pediatric HSCT centers.

In summary, these data support that the impact of pre HSCT age and pubertal development may both explain why children have a lower cGvHD, possible different organ distribution, and most

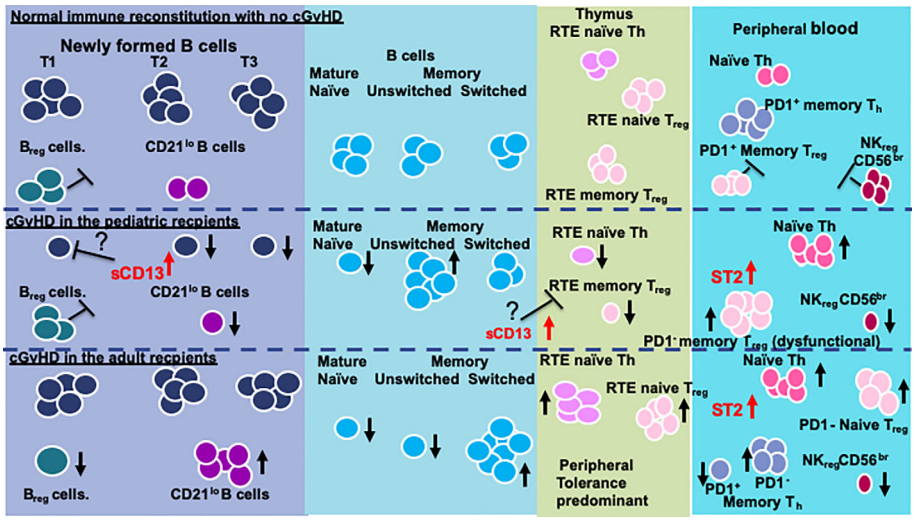

FIGURE 4 | Model of the differences in between children and adults.

importantly have distinct biological differences in some of the pathways that result in the development of cGvHD. One thing to emphasize in that we many times found cell population patterns in the pubertal group somewhere between that in prepubertal children and adults suggesting that the onset of puberty begins to affect cGvHD patterns somewhere between children and adults. Moreover, the impact of post HSCT sex hormone production on the development if immune tolerance and cGvHD is also probably impacted by the pre HSCT pubertal status. We have tried to develop a model that only partially explains the observed differences and more information is required.

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 University of British Columbia. Written informed consent to participate in this study was provided by the participant or the participant's legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

GC: clinical lead of the pediatric ABLE studies with study performance, interpretation of data, and writing. AL: interpretation of T cell analysis and writing of the manuscript. SD: interpretation of T cell analysis and writing of the manuscript. AK: performance of all analyses, interpretation of results, and writing of the manuscript. SA: performance of all analyses, interpretation of results, and writing of the manuscript. JR: performance of B cell studies, interpretation of the results, and writing. BN: performance of all pediatric cohort statistical analyses and writing. SM: oversight of all statistical analyses and writing. RB: oversight of all T cell analyses, interpretation, and writing. KS: overall analysis, study design, performance, interpretation, and writing. All authors contributed to the article and approved the submitted version. J-PC is a pediatric endocrinologist. His contributions to the present paper include: Substantial contributions to the interpretation of data for the work (puberty); Revising the work critically for important intellectual content in the endocrine area (puberty, hormones); Final approval of the version to be published; Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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MINI REVIEW
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Considerations in Preparative Regimen Selection to Minimize Rejection in Pediatric Hematopoietic Transplantation in Non-Malignant Diseases

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The variables that influence the selection of a preparative regimen for a pediatric hematopoietic stem cell transplant procedure encompasses many issues. When one considers this procedure for non-malignant diseases, components in a preparative regimen that were historically developed to reduce malignant tumor burden may be unnecessary. The primary goal of the procedure in this instance becomes engraftment with the establishment of normal hematopoiesis and a normal immune system. Overcoming rejection becomes the primary priority, but pursuit of this goal cannot neglect organ toxicity, or post-transplant morbidity such as graft-versus-host disease or life threatening infections. With the improvements in supportive care, newborn screening techniques for early disease detection, and the expansion of viable donor sources, we have reached a stage where hematopoietic stem cell transplantation can be considered for virtually any patient with a hematopoietic based disease. Advancing preparative regiments that minimize rejection and transplant related toxicity will thus dictate to what extent this medical technology is fully utilized. This mini-review will provide an overview of the origins of conditioning regimens for transplantation and how agents and techniques have evolved to make hematopoietic stem cell transplantation a viable option for children with non-malignant diseases of the hematopoietic system. We will summarize the current state of this facet of the transplant procedure and describe the considerations that come into play in selecting a particular preparative regimen. Decisions within this realm must tailor the treatment to the primary disease condition to ideally achieve an optimal outcome. Finally, we will project forward where advances are needed to overcome the persistent engraftment obstacles that currently limit the utilization of transplantation for haematopoietically based diseases in children.

Keywords: transplantation, preparative, childhood and adolescence, hematopoeific stem cells, engrafted survival outcomes

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INTRODUCTION

Since its first attempts in the 1950s, allogeneic hematopoietic stem cell transplantation (HSCT) has rapidly evolved over time (1). Initially used for the most desperate of situations, it has now become a standard of care for many disease conditions. This transformation is a product of many advancements including: (1) Improving our understanding of hematopoiesis and immune reconstitution. (2) Improvements in supportive care, (3) Improvements in the prevention of graft-versus-host disease (GVHD), (4) Expansion of donor pools, (5) Refinements in preparative regimen selection and design. These advancements have produced a steady decline in transplant related mortality rates which now approach 10% in some instances. Thus, HSCT is now viewed as a viable option for virtually any disease that originates from the hematopoietic system. Continued improvements must now take into account not only mortality, but also minimizing the long-term toxicities that a surviving patient must confront after achieving cure of their primary disease.

Long term toxicities can be a consequence of several variables. 1.) Organ damage from the preparative regimen, 2.) Sequelae from the transplant course such as mucositis, infection, or excessive bleeding, 3.) Chronic GVHD, 4.) Toxicity from other medications administered (calcineurin inhibitors, steroids, etc.) (2). Although some of these complications may be unpredictable, the choice of the preparative regimen can have a significant impact. For non-malignant conditions, the primary goal of the transplant procedure is to achieve stable engraftment that is sufficient to rectify the underlying disease yet minimize long term toxicity (3). In its simplest view, the primary obstacle of HSCT is rejection of the graft. Thus, the choice of preparative regimen should focus on its immunosuppressive properties, optimizing engraftment yet avoiding an excessive immunocompromised state leading to life threatening infections (4). This "balance" can be difficult to achieve, and the optimal regimen, which varies with the primary disease, has not been established for any condition.

This mini-review will summarize both the history and current state of the repertoire of preparative regimens that have been utilized for HSCT for non-malignant conditions. We will discuss the variables which should be considered in choosing the appropriate preparative regimen and how different conditions may warrant different approaches. Finally, we will discuss future directions where advances in preparative regimen design may improve the outcome for these patients.

INDIVIDUAL AGENTS UTILIZED FOR PREPARATIVE REGIMEN DESIGN

Established preparative regimens have historically been developed utilizing standard phase I designs which advance dose intensity until a dose limiting toxicity was encountered. Hematologic toxicity was disregarded due to its reversal with the infusion of hematopoietic stem cells of the graft. Thus, doses and schedules of individual agents were limited by toxicities outside the hematopoietic system.

Modern day regimens are typically classified into three categories (3, 5, 6). *Myeloablative regimens* typically requires a stem cell graft infusion to reconstitute hematopoiesis. *Non-myeloablative regimens*, as the name implies, are less intensive and, even in the absence of a stem cell infusion, spontaneous hematopoietic recovery is expected. *Reduced intensity regimens*, whose definition has not been rigorously defined, falls somewhere in-between the two extremes, and is an acknowledgement that non-myeloablative regimens are associated, by their nature, with an increased risk of rejection. Reduced intensity regimens thus, fall short of full myeloablative dosing, but may achieve engraftment with less toxicity. Regardless of the type of preparative regimen, below are the components which constitute most modern day therapies.

Total Body Irradiation (TBI)

One of the first modalities developed, TBI was the primary modality utilized in early transplant studies in animals because of its known immunosuppressive and myeloablative properties (7, 8). Clinical experience in humans quickly raised awareness of TBI's effects on the lungs and strategies that fractionated doses and shielded the lung fields led to improvements in survival (9). TBI's toxicity unfortunately does not spare any tissue, often leading to irreversible damage to exposed organs making it less attractive for non-malignant diseases. Subsequent investigations have strived to reduce the dose and presumably the toxicity to exposed organ systems because of its usefulness in overcoming rejection particularly in mismatched donors. Long term studies have failed to identify doses that are free of significant rates of infertility, thyroid disease, and growth hormone deficiency making the use of this modality problematic.

Cyclophosphamide

A well-established alkylating agent, cyclophosphamide has maintained its role in HSCT due to its highly immunosuppressive properties and the relative resistance of hematopoietic stem cells to this agent even the highest doses (8, 10, 11). Recent studies have utilized cyclophosphamide post graft infusion to improve the outcomes of haploidentical transplant procedures (12–14). The success of this strategy has probably entrenched this agent as a major element of transplant therapy. Acute toxicities including hemorrhagic cystitis, and cardiac toxicity have been reduced with improved supportive care, with persistent long term toxicities that include sterility and secondary malignancies.

Busulfan

One of the first agents to be utilized in non-TBI containing preparative regimens, the establishment of pharmacokinetic modeling to project optimal dosing for this drug has reduced rejections and hepatotoxicity (8, 10, 11, 15). Seizures, a common complication of this agent has been minimized with prophylactic anti-epileptic drugs. Sinusoidal obstruction syndrome, (SOS) continues to be a clinical problem, but pharmacokinetic dose adjustments have reduced its risk.

Treosulfan

A structural analog of busulfan, its use is increasing with its potent immunosuppressive properties and favorable toxicity profile (16–19). Future trials will determine whether it supplants busulfan as a primary agent for preparative regimens.

Thiotepa

An alkylating agent, thiotepa has gained increasing popularity due to its immunosuppressive effects and its ability to lower rejection rates in reduced intensity preparative regimens (8, 20, 21). Its toxicity profile is comparable to other alkylating agents although it does have unique properties that lead to significant cutaneous toxicity which is typically managed with supportive care.

Melphalan

Another popular alkylating agent, its use has increased over the years as its toxicity is limited outside of the hematopoietic system particularly at doses used in modern reduced intensity regimens (22).

Etoposide

A phase specific, topoisomerase II inhibitor, etoposide has continued to be a common component of modern day preparative regimens due to its predictable toxicity profile and its ability to be combined with alkylating agents without adding excessive side effects (8). Most short term toxicities outside of myelosuppression has been restricted to gastrointestinal and dermatologic which can be typically managed, and severe liver toxicity is observed only with high doses (23). Etoposide's association with an increased risk of secondary leukemia limits its use and makes it a somewhat less attractive agent for transplantation in non-malignant conditions.

Fludarabine

A purine analog, fludarabine's popularity in its incorporation into more modern day preparative regimens is due to its relatively potent immunosuppressive properties without significant organ toxicity (10, 11, 24). Early use of this agent was associated with neurologic toxicity which has been overcome with dosing adjustments. Its successful incorporation into several reduced intensity preparative regimens for non-malignant diseases would indicate that it will a remain central element in HSCT for the foreseeable future.

Antibody Agents

Antibodies directed at the lymphoid compartment have an inherent attractiveness due to their lack of toxicities on other organ systems (3). Such agents can help overcome rejection. In addition, their typical long half-life allows for its persistence in the recipient where it can potentially impact GVHD, depleting T cells from the infused donor product. Appropriate premedication can overcome most infusion reactions. The greatest challenge is to tailor the dosing and schedule of administration to minimize rejection yet avoid sustained suppression of the T cell compartment that would lead to excessive opportunistic infections. Although many agents have been utilized over the years, only a few have maintained a stable presence in this field.

Anti-Thymocyte Globulin (ATG)

Two sources of anti-thymocyte globulin encompass most of its use: 1) ATGAM (horse polysera) 2. Thymoglobulin (rabbit polysera). ATGAM has been utilized for many more years than the rabbit formulation (25), but the latter is a more potent agent (26, 27). Studies with ATGAM have demonstrated that its use reduces the duration of other immunosuppressive agents (28). Both have been shown to improve engraftment rates when added to conventional preparative regimens and given their retained presence in the host, their use has reduced rates of both acute and chronic GVHD to varying degrees (29–32).

Anti-T Lymphocytes Globulin (ATLG)

Anti-T lymphocytes globulin, derived from rabbit polysera from immunization with a Jurkat T cell leukemia line, is also gaining in popularity (27, 33, 34). Most trials comparing the efficacy between ATG and ATLG have been performed in patients with malignant disease where more effective lymphodepletion and subsequent reductions in GVHD have been offset by increased rates of relapse of the primary cancer (35). More robust trials in non-malignant diseases are needed.

Alemtuzumab

A humanized monoclonal antibody against CD52, alemtuzumab has been shown to target T and B cells, NK cells, and antigen-presenting cells. It has been incorporated into several reduced intensity preparative regimens and has been used successfully for immunodeficiencies, hemophagocytic lymphohistiocytosis, lysosomal storages disease, thalassemia and sickle cell disease. Like other anti-lymphocyte products, it is associated with an increased risk for infections (36). However, since it is a monoclonal product, the clinical responses may be less variable from patient to patient in comparison to the polyclonal products listed above.

Co-Stimulation Blockade

Recent investigations have begun to examine T cell co-stimulation blockade as an additional means of immunosuppression to both reduce the risk of rejection and GVHD. Abatacept, a CTLA4-Ig agents can block the CD28-CD80/86 interactions needed for T cell activation has been incorporated into newer preparative regimens (37). Preliminary studies have demonstrated low rates of GVHD with an acceptable toxicity profile. Further trials are needed to further define its role.

Agents Less Commonly Used in Preparative Regimens for Non-Malignant Disease

Other chemotherapy agents which were initially advanced into preparative regimens have not sustained their presence in modern day treatments for non-malignant diseases due to their inherent toxicities and the lack of a need for their anti-neoplastic activity. Platinum agents, other alkylating agents, anthracyclines, are examples of agents that have not sustained their presence in modern day regimens (8).

Strategies in Preparative Regimen Selection for Non-Malignant Diseases

Lacking the necessity of eradicating malignant cells, the transplant physician contemplating HSCT for a patient with a non-malignant disease must take several considerations into account which may or may not be specific to the patient's disease state. These include: 1) What are the specific vulnerabilities of a particular disease population that lead to transplant related complications from the preparative regimen selection? 2) How has the patient's primary disease and the corresponding treatment to treat that disease impacted the patient's vital organs? 3) What are the barriers to achieve engraftment which would guide minimizing the intensity of the preparative regimen? 4.) What are other immunological features beyond rejection that influence transplant outcome? Thoughtful consideration for each of these variables will optimize the course of the patient.

Specific Vulnerabilities of a Particular Disease Population

The different diseases which are considered for HSCT have different clinical phenotypes which are linked to problems, some which are severe. Although a successful HSCT procedure may ultimately alleviate the condition, specific elements of a particular preparative regimen may exacerbate a patient's clinical condition to serious levels. An appreciation of the specific vulnerabilities for a particular disease will provide insight for thoughtful decision making to select a preparative regimen (**Table 1**). Given the diversity of clinical difficulties that each disease possesses and given the expected patient to patient variability in clinical courses, having a transplant team with sufficient experience for a particular disease will ensure optimal management of the unique complications that a patient may experience.

How Has the Patient's Primary Disease and the Corresponding Treatment to Treat That Disease Impacted the Patient's Vital Organs?

The natural history of a particular disease may lead to organ compromise that may make the patient less tolerant to preparative regimens with specific toxicities. For instance, patients with leukodystrophies with substantial demyelination of the CNS may not tolerate TBI or high doses of neurotoxic chemotherapy such as busulfan (66, 67). A patient with sickle cell disease who has acquired substantial renal injury may handle agents cleared by the kidney poorly leading to heightened toxicity (81, 82). Alternatively, a patient with an immune compromised state such as chronic granulomatous disease may have incomplete clearance of infections which may worsen and progress once the full immunosuppressive effects of the preparative regimen have taken hold (50, 51). Thus, not only must the clinician be sufficiently familiar with the inherent vulnerabilities of the patient's disease state, but an evaluation that sufficiently characterizes an individual's susceptibilities to the procedure is a critical facet of the process. Preparative regimen selection and agent dosing may need to be individualized for a patient to minimize the toxicities while still striving toward a successful procedure. A sensitivity to these issues will minimize the transplant related morbidity and mortality for the patient, who could otherwise survive for a substantial number of years in the absence of the transplant procedure.

What Are the Barriers to Achieve Engraftment Which Would Guide Minimizing the Intensity of the Preparative Regimen?

The barriers to engraftment are primarily immunologic, with its magnitude dictated by the patient's underlying disease and past treatment history (54, 57, 71). Certainly immunodeficiencies are presumed to be less capable of rejecting infused grafts, but there is wide variability in the immune competence between primary diagnoses and even for patients with the same disease. This may not necessarily be reflective in obvious differences in phenotype, but it will manifest itself in rejection (43–45). There is a tendency to provide as minimal intensity as possible for patients with immunodeficiencies to try and reduce toxicities, particularly if the patient presents with a preexisting infection. However, rejections from an inadequate preparative regimen will invariably lead to a need to repeated procedures of increasing preparative regimen intensity to avoid another rejection. Such escalation will invariably result in the accumulation of toxicities potentially leading to an unsatisfactory result.

Other disease states that are amenable to HSCT may in fact have intact immune systems. In contrast to patients with malignancies in which prior chemotherapy exposure may reduce the likelihood for rejection, non-malignant diseases, such as lysosomal storage diseases, leukodytrophies, and hemoglobinopathies may require preparative regimens with substantial immunosuppressive properties, perhaps even requiring fully myeloablative regimens (20, 66, 71, 72, 82). Such transplant procedures will lead to more severe long term toxicities.

Conditions of bone marrow failure further illustrate the complexities of choosing the right preparative regimen. Aplastic anemia, typically a disease of T cell mediated destruction of the hematopoietic system, is a condition where prior blood product exposure may sensitize the donor to an even greater risk of rejection (55). Alternatively, other conditions such as Fanconi's Anemia or Dyskeratosis Congenita, possess difficulties in DNA repair with intolerance to the even most modest doses of radiation or alkylating agents (57–60, 64). Thus, even conditions of poor marrow function present with a wide array of clinical challenges.

What Are Other Immunological Features Beyond Rejection That Influence Transplant Outcome?

Beyond rejection, the immune system plays a central role in the clinical course of the transplanted patient. The expansion of alloreactive T cells will ultimately result in varying degrees of GVHD, and will have a substantial impact on both long term toxicity and treatment related mortality. Simultaneously, the newly reconstituting immune system is striving to achieve a protective state against infections, building new B and T cell repertoires while priming to new antigens (38, 86–90). Further complicating this process is the impact specific preparative regimen agents may have on the newly emerging lymphocyte population. Antibodies with specificity to different lymphocyte

TABLE 1 | Disease-specific vulnerabilities and the influence of preparative regimens on HSCT course.

Disease	Specific vulnerabilities	Impact of preparative regimen toxicities	Agents to be used with caution	Agents with less associated toxicity
SCID (22, 38–42)	Pre-existing infection	Disruption of mucosal barriers Prolonged myelosuppression/ immunosuppression Pulmonary toxicity/Pneumonitis	TBI, High-dose busulfan,	Fludarabine Dose adjusted busulfan Cyclophosphamide Melphalan Lymphocyte depleting antibodies (ATG, ALG, Alemtuzimab)
Other immunodeficiencies (22, 43–49)	Pre-existing infection Autoimmune disease Higher rates of rejection	Disruption of mucosal barriers Prolonged Myelosuppression /immunosuppression Autoimmune cytopenias Pulmonary toxicity/Pneumonitis	TBI, High-dose busulfan,	Fludarabine Dose adjusted busulfan Treosulfan Cyclophosphamide Melphalan Lymphocyte depleting antibodies (ATG, ALG, Alemtuzimab)
Chronic granulomatous disease (21, 50–52)	Chronic aspergillus pneumonitis Granulomatous lung disease Inflammatory bowel disease Anti-Kell alloimmunization	Pulmonary toxicity/Pneumonitis Fungal sepsis. Bowel injury	TBI High-dose busulfan	Fludarabine Dose adjusted busulfan Treosulfan Cyclophosphamide Melphalan Lymphocyte depleting antibodies (ATG
Aplastic Anemia (53–56)	Blood product sensitization Iron overload Chronic neutropenia/infection	Mucositis SOS Hemorrhagic cystitis	TBI	Cyclophosphamide Lymphocyte depleting antibodies (ATG, ALG, Alemtuzimab)
Fanconi's Anemia (57–63)	Poor DNA repair Endocrine deficiencies MDS/AML	Mucositis SOS Pulmonary toxicity/Pneumonitis Renal insufficiency Hemorrhagic cystitis	Radiation, Alkylating agents	Dose adjusted busulfan Cyclophosphamide Fludarabine ATG
Inherited Bone Marrow Failure Syndromes, other than Fanconi's anemia (19, 64, 65)	DNA repair defects (DKC) Endocrinopathies Chronic neutropenia/infection	Severe mucosal injury Pulmonary toxicity SOS Infection Hemorrhage	TBI, high dose Alkylating agents	Fludarabine Cyclophosphamide Melphalan Lymphocyte depleting antibodies (ATG, ALG, Alemtuzimab)
Leukodystrophies (66–70)	Leukoencephalopathy, Adrenal insufficiency	Seizures, decline in neurologic and cognitive function, Adrenal insufficiency (ALD) Swallowing difficulties, Impaired ambulation	Radiation High dose busulfan	Dose adjusted busulfan Cyclophosphamide Fludarabine Lymphocyte depleting antibodies (ATG, ALG, Alemtuzimab)
Hurler's Disease (66, 71–73)	Upper airway patency, Heart failure	Mucositis, Airway obstruction	Radiation	Dose adjusted busulfan Cyclophosphamide Fludarabine Lymphocyte depleting antibodies (ATG, ALG, Alemtuzimab)
Thalassemia (74–80)	Iron overload	Mucositis SOS Pulmonary toxicity/Pneumonitis Hemorrhage	Radiation High-dose busulfan	Dose adjusted busulfan Cyclophosphamide Fludarabine Treosulfan Lymphocyte depleting antibodies (ATG
Sickle cell anemia (81–85)	History of stroke/vasculopathy Recurrent Chest Syndrome/Pulmonary compromise	Mucositis Seizures PRES Renal injury	Radiation High-dose busulfan	Dose adjusted busulfan Cyclophosphamide Fludarabine Lymphocyte depleting antibodies (ATG, ALG, Alemtuzimab)

(Continued)

TABLE 1 | Continued

Disease	Specific vulnerabilities	Impact of preparative regimen toxicities	Agents to be used with caution	Agents with less associated toxicity
	Renal insufficiency Red cell alloimmunization			

ATG, anti thymocyte globulin; ALG, anti-lymphocyte globulin; PRES, posterior reversible leukoencephalopathy syndrome; SOS, inusoidal obstruction syndrome; TBI, total body irradiation.

populations (ATG, ATLG, alemtuzumab etc.) will linger in the body many days after their infusion and impact not only the infused lymphocyte populations of the graft but also the newly emerging populations. The amount of antibody present as the engrafting lymphocytes develop varies with the agent, dose administered, and between patients. Thus, the transplant physician must use information from past clinical trials in selecting the appropriate regimen for an individual patient in contrast to making empiric decisions. A reduced effect on the emerging immune system may lead to extensive GVHD, while an excessive one may lead to life threatening infections (91). The inability to "fine tune" this effect is a limiting feature of the use of antibody agents.

Thoughtful Use of Preparative Regimens in HSCT in Non-Malignant Diseases

It is apparent from this review that many challenges confront the clinician when choosing a preparative regimen for a transplant candidate. Over the past several decades, investigators have reported their successes and challenges exploring different strategies (**Table 2**). It is apparent that virtually every element of the transplant course from rejection risk to overall survival vary tremendously from report to report. Furthermore, variables such as donor source, age of the patient, and disease status prior to the transplant procedure can influence the transplant outcome further obscuring the impact of the preparative regimen. This variability is in part due to differences in the condition of the patient population transplanted, the agents used to formulate the preparative regimen, the graft selection, (matched sibling, matched or mismatched unrelated donor, cord blood, peripheral blood verses bone marrow), and graft manipulation (T cell depletion) which will result in varying outcomes. Furthermore, many reports merge outcomes of several different preparative regimens or combine multiple diseases together, sometimes making it impossible to link specific outcomes from a preparative regimen to a specific disease. Thus, comparisons between reports can be difficult. Programs and groups that commit to a specific preparative regimen "backbone," and then refine elements from this backbone in well-defined cohorts will provide the most useful information on how to select a preparative regimen for a patient.

TABLE 2 | Variation of HSCT outcomes.

Disease	Successful Preparative Regimens (#patients)	Graft Failure/ Rejection Rate	aGVHD	cGVHD	TRM	EFS	OS
SCID (22, 38–42)	Range of Reported Outcomes[0,51,55-58]	0–82% 42%*	0–65% 38%	0–39% 0%	0–24% 0%	60–95% 95%	67–84% 95%
	None (21) (92)	11%	22%	22%	33%	67%	67%
	Bu/Cy (9) (93)	0%	60%	33.3%	20%	80%	80%
	Flu/Mel (5) (94)	0%	50%	-	33%	67%	67%
	Bu/Cy/ATG (6) (95) Bu/Flu/ATG Treo/Flu Treo/Cy						
Other immunodeficiencies	Range of Reported Outcomes [22,47,48,67-70]	0–66.7% 0%	17.4– 87.5%	0–20% 14.2%	0–44% 14.2%	33– 100%	62.5– 94%
	Bu/Cy (7) (93)	0%	57%	0%	0%	86%	86%
	Alem/Treo/Flu (13) (48)	12.5%	62%	0%	12.5%	100%	100%
	Treo/Flu/Thio/RTX/ATG (8) (48)	66.7%	87.5%	-	25%	87.5%	87.5%
	Alem/Flu/Mel (12) (46)	20%	-	20%	20%	33%	62.5%
	Flu/Mel/ALG (5) (22) Bu/Cy/PTN Bu/Cy/ATG Bu/Flu/ATG Treo/Flu/		50%			80%	80%
Chronic granulomatous disease (21, 50–52)	Range of Reported Outcomes[9,34,35,63]	0–20% 0%	4–60% 33%	0–20% 4.8%	0–40% 4.8%	80–91% 97.2%	60–100 97.2%

(Continued)

TABLE 2 | Continued

Disease	Successful Preparative Regimens (#patients)	Graft Failure/ Rejection Rate	aGVHD	cGVHD	TRM	EFS	OS
	Bu/Flu/ATG (96)	9%	39%-	9%	6%	91%	91%
	Bu/Flu/Alem (96)	0%	-	-	0%	100%	100%
	Bu/Alem(5) (52)	12%	-	-	3%	76%	85%
	Bu/Alem/LD TBI (33) (52)	0%	60%	20%	40%	60%	60%%
	Treo/Flu (5) (21)	13%	40%	14%	4.5%	90%	95%
	Treo/Flu/Alem (22) (21)	20%	40%	0%	0%	80%	100%
	Treo/Flu/Thio/ATG(5) (21)	25%	50%	25%	0%	75%	75%
	Alem/Flu/Mel (4) (97)						
	Bu/Cy						
	Bu/Cy/ATG						
	Bu/Flu/Cy/ATG						
Aplastic Anemia (53–56)	Range of Reported Outcomes (7, 40, 41, 51)	0–6% 6%	8–37.5% -	6– 37.5%	5.7– 32.1%	64.3– 93.1%	67.9– 96.6
	Cy/ATG (33) (98)	3.6%	35.7%	35.7%	12%	81%	89%
	Flu/Cy/ATG (28) (55)	3.4%	37.5%	37.5%	32.1%	64.3%	67.9%
	Flu/Cy/ATG# (29) (55)	0%	29%	35%	3.5%	93.1%	96.6%
	Alem/Flu/Mel (17) (99)				12%	88%	88%
	Bu/Cy						
	Bu/Cy/ATG						
	Cy						
	Cy/TBI						
Fanconi's Anemia	Range of Reported Outcomes[46,52,54,55,74,76]	0–11% 4%	6.7–23% 11%	4–36% 5%	5.7– 44%	70.5– 94%	53.6– 94%
	Cy (109) (62)	5.7%	23%	12%	12%	88%	88%
	Cy/TAI/ATG (35) (58)	2.2%	6.7%	6.7%	5.7%	89%	89%
	Bu/Flu/Cy/ATG (45) (100)	0%	27%	4%	17.8%	77.8%	80%
	Flu/Cy/ATG (44) (60)				29.5%	70.5%	70.5%
Inherited Bone Marrow Failure Syndromes, other than Fanconi's anemia	Range of Reported Outcomes [19,53,77]	0–17% 10%	9–70% 70%	10– 31%	7–33% 20%	62–93% 70%	63.3– 93%
	Bu/Cy/ATG (101)	0	43%	10%	7%	93%	80%
	Treo/Flu/ATG (14) (19)	0%	33%	14%	33%	67%	93%
	Alem/Flu/Mel (6) (102)	9%	9%	16%	18%	82%	67%
	Alem/Flu/Mel (11) (103)			27%			82%
	Bu/Flu/Mel						
	Mel/Flu/Cy						
	Flu/Cy						
	TBI/Mel/Cy						
Leukodystrophies	Range of Reported Outcomes [38,39,78-80]	0–12% 14.2%	31–44% 71.4%	10– 25.9%	0–44% 14.2%	48– 100%	52– 100%
	Alem/Flu/Mel (7) (104)	9%	40%	0%	25%	85.7%	85.7%
	Bu/Cy/ATG (12) (105)	11.1%	40.7%	10%	25.9%	66.7%	66.7%%
	Bu/Cy/ATG (27) (106)	0%	75%	25.9%	0%	66.7%	74.1%
	Bu/Flu/Cy/ATG (4) (107)			-		100%	100%
	Bu/Cy						
	Bu/Flu/ATG						
Hurler's Disease (71–73)	Range of Reported Outcomes [6,42,73]	0–37.4% 12.5%	12.2– 16%	0– 14.8%	0– 45.8%-	41.2– 100%	60.8– 100%
	Bu/Cy (8) (108)	0%	12.5%	0%	12.5%	75%	87.5%
	Bu/Cy/ATG (7) (109)	15%	28.6%	0%	0%	100%	100%
	Bu/Cy/ATG (20) (21)	0%	25%	10%	15%	85%	85%
	Bu/Flu/Mel/ATG (8) (110)	14.2%	25%	0%	0%	100%	100%
	Alem/Flu/Mel (7) (104)		71.4%	-	14.2%	85.7%	85.7%
Thalassemia (74–80)	Range of Reported Outcomes [4-80]	0–16.7% 16.7%	14–75% 16.7%	2–40% 16.7%	0– 37.5%	62.5– 100%	62.5– 100%
	Bu/Cy/ATG(12) (76)	0%	8.3%	8.3	0%	83%	100%
	Bu/Flu/Cy/ATG (48) (75)	9%	14%	2%	0%	100%	100%
	Thio/Treo/Flu/ATG (60) (77)	7.1%	14.3%	10%	7%	84%	93%
	Thio/Treo/Flu (28) (76)	0%	75%	25%	21.4%	71.4%	78.5%
	Bu/Flu/Thio (8)	0%	16.7%	25%	37.5%	62.5%	62.5%
	Bu/Flu/Thio/Abet (24) (111)	0%	-	-	0%	100%	100
	Alem/Flu/Mel (9) (112)	3%	33%	21%	0%	100%	100%
	Alem/Flu//Thio/Mel (33) (79)				18%	64%	82%

(Continued)

TABLE 2 | Continued

Disease	Successful Preparative Regimens (#patients)	Graft Failure/ Rejection Rate	aGVHD	cGVHD	TRM	EFS	OS
Sickle cell anemia	Range of Reported Outcomes [40,41 89-91]	0–18% 18%%	0–33.3% 9%	0–62% 4.5%	0–28 9%	69–100%	79–100%
	Bu/Cy (22)	0%	13%	0%	0%	73%	91%
	Bu/Cy/ATG (16)	14%	14%	14%	0%	100%	100 (115)
	Bu/Flu/Cy/ATG (14) (113)	4.5%	18%	27%	9%	100%	%
	Bu/Flu/ATG (22) (84)	0%	10%	10%	10%	86%	100%
	Alem/Flu/Mel (29) (83)	0%	0%	0%	0%	80%	91%
	Alem/TBI (30) (85)	10%	20%	10%	10%	97%	90%
	Flu//Thio/Mel/ATG (10) (114)					80%	97% 90%

Range of Reported Outcomes summarized in the first line of each category. Number of patients specifically cited marked by () following listed preparative regimen. Preparative regimens used in respective disease where the specific preparative regimen could not be directly attributed to a specific disease specific population are left blank. Outcome measures missing or not extractable from the report are designated with a "-" symbol aGVHD, acute graft-versus-host disease; cGVHD, chronic graft versus host disease; TRM, transplant-related mortality; EFS, event-free survival; OS, overall survival; Alem, alemtuzumab; ATG, anti thymocyte globulin; Bu, busulfan; Cy, cyclophosphamide; Flu, fludarabine; Mel, melphalan; Treo, treosulfan; Thio, thiotepa; TAI, thoraco-abdominal irradiation; TBI, Total Body Irradiation; LD TBI, Low Dose TBI.

**Includes patients who lost B cell but retained T cell function.*

#Reduced dose Cyclophosphamide, increased Fludarabine.

Considerations of the vulnerabilities of the primary disease, the clinical status of the individualized patient, the essential needs of overcoming rejection yet temporizing GVHD and life threatening infections must all be weighed in making the appropriate decision for the patient. Unfortunately, despite over three decades of experience, there is no "formula" that can be utilized to assemble a combination of agents that will give a predictable outcome fulfilling the needs of both the clinician and the patient. Large scale studies with detailed reports of outcomes and toxicities provide our only resource to guide the clinician to make thoughtful decisions for their patient. Further research with well-designed clinical trials with full characterization of outcomes are needed to enhance our understanding of this topic.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Mesenchymal Stromal Cells in Pediatric Hematopoietic Cell Transplantation a Review and a Pilot Study in Children Treated With Decidua Stromal Cells for Acute Graft-versus-Host Disease

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Mesenchymal stromal cells (MSCs) are rare precursors in all organs of the body. MSCs have profound anti-inflammatory effects and reduce alloreactivity *in vitro* and *in vivo*. In pediatric allogeneic hematopoietic cell transplantation (HCT), MSCs have mainly been used to treat acute graft-versus-host disease (GVHD). MSCs are commercially available for this indication in Canada, Japan, and New Zeeland. More rare indications for MSCs in pediatric patients include graft failure and chronic GVHD. MSCs from bone marrow, adipose tissue, umbilical cord, Wharton's jelly, placenta tissue, and decidua have been used, but the optimal clinical stromal cell source has not been compared in clinical trials. More experimental clinical indications using MSCs, such as sepsis, acute respiratory distress syndrome, hemorrhages, pneumo-mediastinum, and neuroinflammation have primarily been explored in animal models or adult HCT patients. MSCs have almost no if any side-effects. In this pilot study we report the outcome of six children treated with decidua stromal cells (DSCs) for steroid refractory acute GVHD. At 6 months, complete response was seen in four patients and partial response in two patients. One child with high-risk ALL died from relapse and a boy with sickle cell disease died from a cerebral hemorrhage. Five-year survival was 67% and all survivors showed a Lansky score of 100%. To conclude, MSCs from various organs are well-tolerated and have shown an encouraging outcome for acute GVHD in pediatric patients.

Keywords: graft-versus-host disease (GVHD), mesenchymal stromal cell (MSC), pediatric haematopoietic stem cell transplantation, cell theraphy, decidua stromal cells (DSCs)

INTRODUCTION

Hematopoietic cell transplantation (HCT) is an established treatment for children with both malignant and non-malignant hematopoietic diseases and inborn errors of metabolism (1–4). The main obstacles to success are relapse of the disease, infections, graft failure, toxicity of various organs, hemorrhagic cystitis, and graft-versus-host disease (GVHD). To prevent GVHD, patients

are treated with immunosuppressive drugs, most commonly, calcineurin inhibitor combined with Methotrexate (5). Despite this, a majority of the patients developed acute GVHD, with a considerable mortality, even if this was significantly lower in children compared to adults (6). To confirm the gastrointestinal GVHD histopathological biopsies is recommended, since e.g., viruses could cause gastrointestinal symptoms (7–9). Cortisone is first-line therapy for acute GVHD (10) and almost all immunosuppressive therapies are used as a secondary treatment with varying degrees of success (11). Friedenstein et al. were the first to describe MSCs (12). We introduced mesenchymal stromal cells (MSCs) as a new therapy for acute GVHD (13, 14). MSCs are rare in all tissues in the body and can differentiate into several cells of mesenchymal cell lineages, such as bone, cartilage, tendon, cardiomyocytes, muscles, and fat (15, 16). There is no specific CD marker for MSCs. However, they stain positive for CD29, CD73, CD90, CD105, and CD166. They are negative for hematopoietic markers, CD34, CD45, and CD14. They are not true stem cells because they cannot regenerate and maintain a whole tissue compartment. MSCs express HLA class I molecules and contain intracellular HLA class II that is expressed on the cell surface after interferon-γ stimulation (17). After injection, MSCs do not appear to be long lived and have been demonstrated in the circulation only shortly after infusion into patients who underwent autologous HCT for breast cancer (18).

IMMUNOSUPPRESSION

MSCs have potent immunomodulatory effects and inhibit phytohemagglutinin induced T cell proliferation and alloreactivity in mixed lymphocyte cultures (MLC) (17, 19, 20). MSCs' inhibition of alloreactivity *in vitro* is independent of the major histocompatibility system (21). Furthermore, after differentiation into osteocytes, chondrocytes and adipocytes, immunosuppression was still induced (17). MSCs also prolonged skin allograft survival in baboons (19). Several factors and mechanisms are involved in MSC-mediated immune modulation.

Bone marrow MSCs (BM-MSCs) are susceptible to complement activation after contact with human blood (22). This results in cell dysfunction or cell death (23). When in contact with blood, BM-MSCs also elicit activation of clotting factors (24).

MSC immunosuppression has been studied extensively (25–28). Stromal cells from various organs such as BM, Wharton's jelly, placenta tissues and cord blood have varying immunosuppressive effects in the MLC (17, 19–21, 29, 30). The MLC is also inhibited by skin fibroblasts (31). Immunosuppressive factors produced by MSCs include prostaglandin E2 (32), HLA-G5 (33), and galectins (34). MSCs also produce indoleamine-2,3, dioxygenase (IDO), which inhibits T cells by converting of tryptophan to kynurenine [(35), **Figure 1**]. IDO is involved in the induction of regulatory T cells and the inhibition of Th17 differentiation (36). IDO produced by MSCs also promotes differentiation of macrophages toward M2 phenotypes (37). MSCs also induce contact-dependent immunosuppression. Among these are activation of the PD-1 pathway (38), by activation of VCAM-1 and ICAM-1 (39), purification of CD39 and increased adenosine production (40), and Fas-mediated T-cell apoptosis (41). There are differences in various species and, in mice, several models failed to reduce alloreactivity and GVHD (42). To inhibit GVHD in mice, MSCs need to be licensed by IFN-γ, nitric oxide, or transduced with IL10 to prevent GVHD. In a colitis model in mice, it was shown that prevention of colitis by MSCs requires CD11b+ macrophages (43). In a murine model of GVHD, it was demonstrated that MSCs are actively induced to undergo perforin-dependent apoptosis by recipient cytotoxic T-cells, and that this process is essential to initiate MSC-induced immunosuppression (44). After IV infusion, recipient phagocytes engulf apoptotic MSCs and produce IDO, which is necessary for immune suppression. MSCs produce exosomes and microparticles, some of which are small complexed entities that contain both immunomodulatory proteins, micro RNA and mediators for homing abilities (45). Exosomes were also used to reverse acute GVHD (46).

MESENCHYMAL STROMAL CELLS FOR TREATMENT OF ACUTE GVHD

We introduced MSCs, as a therapy for acute GVHD, by treating a 9-year-old boy with life-threatening grade IV acute GVHD, as well as a phase-I study in GVHD patients whom were resistant to several immunosuppressive therapies (13, 14). We also performed a multi-center phase II study, including 55 patients with severe steroid resistant GVHD (47). Complete responders had lower transplantation-related mortality 1 year after infusion than patients with partial or no response (11 [37%] of 30 vs. 18 [72%] of 25; $p = 0.002$). Patients with complete response to MSCs had a 2-year survival of 53% as opposed to 16% in partial and non-responders. Children had a trend for better response (64%) as opposed to adults (47%). Subsequently, several single-center studies were performed with varying results using various sources of stromal cells, for instance, adipose tissue (48). Lucchini et al. gave platelet lysate expanded MSCs to children with severe steroid refractory acute or chronic GVHD with varying responses (49). Commercial MSCs (prochymal) were given to 12 children with therapy-resistant grade III and IV acute GVHD (50). A complete response was seen in seven children (58%), a partial response in two (17%), and mixed responses were recorded in three (25%) of the children. The 100-day survival was 58%. Osiris performed a double-blind placebo controlled phase 2/3 study using prochymal for severe acute GVHD (51). The children were given 8×10^6 MSCs/kg twice a week or placebo. Among 260 patients, including children and adults, who were randomized in this trial, a complete response at 28 days was 74% in the MSCs group and 30% in the placebo group (52). However, the 180-day durable response of liver GVHD was 29% in the MSC group compared to 5% in the placebo group ($p = 0.047\%$). Among patients with acute GVHD grades III–IV, Remestemcell-L demonstrated significantly higher overall response, 65%, as opposed to 23% in the placebo arm ($p =$

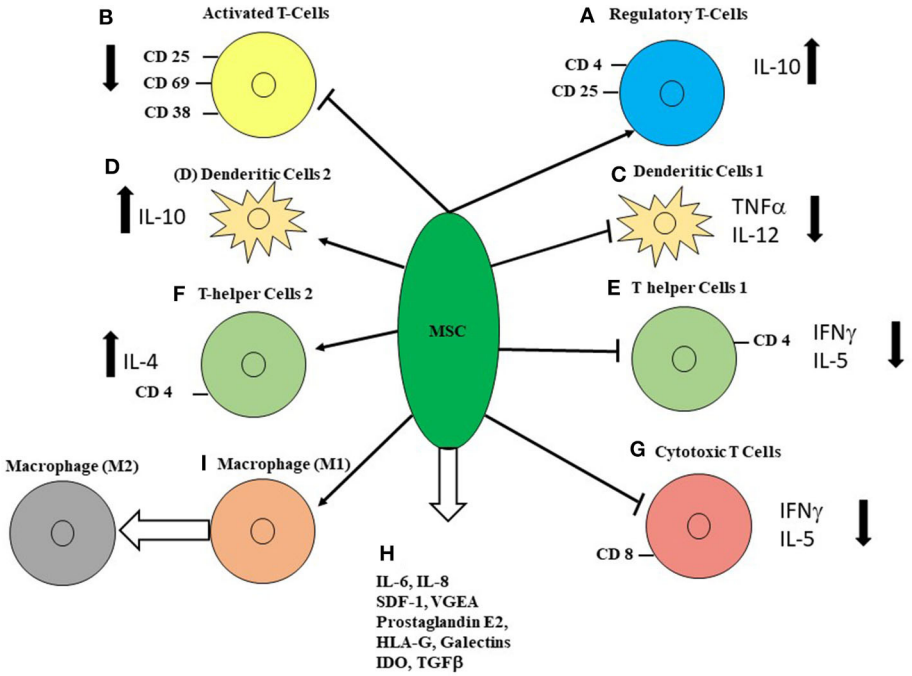

FIGURE 1 | The multiple effects of MSCs on immune cells. **(A)** MSCs increase the proportion of CD4+CD25+ cells and IL-10 production. **(B)** MSCs decrease markers for activated T cells, CD25, CD69, and CD38. MSCs delayed maturation of APC and decreased expression of HLA-DR. **(C)** Dendritic cell type 1 when stimulated had decreased TNF-α and IL-12, when co-cultured with MSCs. **(D)** MSCs increased IL-10 secretion by LPS-stimulated dendritic cells type 2, CD4+ cell had decreased IL5-secretion. **(E)** T-helper cell type 1 IFN-γ production was significantly decreased by MSCs. **(F)** T-helper cell type 2 increased IL-4 secretion in the presence of MSCs. **(G)** MSCs inhibit mixed lymphocyte cultures and subsequent development of cytotoxic T cells by a soluble factor. **(H)** Several soluble factors are produced by MSCs, amongst them are IL-6, IL-8, stem-cell derived factor 1 (SDF1), vascular endothelial growth factor (VEGF). Soluble factors that have been suggested to inhibit T-cell activation are prostaglandin E2, which induces regulatory T-cells, indoleamine 2,3-dioxygenase (IDO), which is induced by IFN-γ which catalyzes the conversion from tryptophan to kynurenine and inhibits T-cell responses. Other soluble factors that have been suggested to inhibit T-cell responses are TGFβ1, hepatocyte growth factor and IL-2. **(I)** MSC induce macrophage differentiation from M1 to M2. (References are mentioned in the text).

0.05). Children had a better outcome of treatment with MSCs for acute GVHD as compared to adults. These pediatric patients were also reported separately (53). Ball and coworkers reported on 37 children treated with MSCs for steroid-refractory grade III–IV acute GVHD (54). A response was observed in 65% of the children. The 3-year survival was 37%. Kurtzberg et al. reported on 241 children with steroid refractory acute GVHD who were treated for 4 weeks with infusion of 2×10^6 MSCs/kg (Remestemcel-L) twice weekly (55). The overall response rate at day +28 was 65%. Survival at 100 days was 82% among the responders and 39% among the non-responders ($p \leq 0.001$). In a Brazilian multicenter study, involving 16 children and 30 adults with steroid refractory GVHD, half of the patients responded and 1-year survival was 20% (56). A study using platelet-lysate-expanded MSC for steroid refractory acute GVHD included eight children and 22 adults. The overall response rate at day +28 was 50% in the adults and 88% in the children ($p = 0.099$). The survival was 88% in the children as opposed to 25% in the adults ($p = 0.003$) (57).

A study used BM-MSCs pooled from multiple third-party donors (58). The study included 92 adult and pediatric patients with steroid refractory acute GVHD. The patients received a median of three doses of pooled MSCs without toxicity. The overall response was 82% and 6-month survival was 64%. In a previous separate analysis of children, the overall response at day 28 was 77% and the 2-year survival was 77% (59). At our unit, long-term follow up of patients treated with BM-MSCs with steroid refractory GVHD included nine children and 22 adults (60). Two-year survival was only 26%. Patients receiving MSCs from passage 1–2 had significantly better survival than those receiving MSCs from passage 3–4 ($p < 0.01$). A meta-analysis reported that children had a better response to MSCs therapy for steroid refractory acute GVHD, with an overall response rate of 82%, as opposed to 70% in adults ($p = 0.04$) (61). A more recent meta-analysis included children and adults given MSCs for prophylaxis ($n = 651$) and for treatment of acute GVHD ($n = 149$) and chronic GVHD ($n = 76$) (62).

MESENCHYMAL STROMAL CELLS FOR TREATMENT OF CHRONIC GRAFT-VERSUS-HOST DISEASE

Chronic GVHD is a great burden for many patients after HCT (63, 64). It seems logical to use MSCs to treat chronic GVHD, which resembles auto-immune disorders. MSCs were reported to be successful in many models of autoimmune diseases (65, 66). There are only a few reports on MSCs for chronic GVHD and

most are about adults (14, 67, 68). Lucchini et al. used platelets-lysate expanded MSCs in four children with chronic GVHD (49). Transient benefits were noted. One child had a complete response that subsequently re-flared.

DSCs appear to have a stronger immunosuppressive effect than MSCs from bone marrow (30, 69). Thus, we used DSCs to treat chronic GVHD in three pediatric patients with severe grade 3 chronic GVHD (Based on National Institute of Health, NIH) (70). The three pediatric patients were affected in several organs such as the skin, mouth, eyes, gastrointestinal tract, liver, lungs and joints, fascia. Two patients received two doses of DSC and one patient received one dose. Two patients had a partial response in the liver, normalization of elevated liver enzymes and, in one patient, esophageal varices disappeared. However, the overall grading of chronic GVHD remained very severe (3) according to NIH grading (71). A meta-analysis of 76 children and adults with chronic GVHD suggested improved survival using MSCs (62).

PREVENTION OF GVHD AND GRAFT FAILURE

In mice, MSCs were shown to prevent the development of lethal GVHD (72). Lazarus et al. performed co-transplantation of HLA-identical sibling bone marrow and donor MSCs in 46 patients (73). No patient had graft failure and grades III–IV acute GVHD were seen in 15% of the patients. We performed co-transplantation of HCT and MSCs to enhance engraftment (74). All patients had engraftment and full donor chimerism. A prospective randomized study of HCT and with co-infusion of MSCs or placebo reported decreased risk of acute GVHD and increased likelihood of relapse (75). Engraftment of neutrophils and platelets was similar in the two groups. Most studies of co-transplantation of HCT and MSCs are performed in adult patients or in a combination of pediatric and adult patients (76, 77). In a pediatric study, parental haplo-identical MSCs were used to promote engraftment in unrelated donor umbilical cord blood transplantation (78). In another pediatric study, MSCs were given to recipients of haplo-identical grafts (79). No patient had graft failure as opposed to 10% of the retrospective controls. A meta-analysis, which included 651 children and adults, showed improved survival in patients treated with MSCs as prophylaxis (62). MSCs may also be used to treat graft failure (80, 81).

MSCs FOR METABOLIC DISORDERS

Hurler's disease is deficiency of the enzyme alfa-L-iduronidase. HCT may partially prevent disease progression if performed before the patient is 2 years of age (82, 83). HCT patients with Hurler's disease and metachromatic leukodystrophy were given MSCs to enhance enzyme production after HCT (84). The rationale for using MSCs was because these cells express high levels of alpha-L-iduromidase and arylsulphatase-A. Four out of five patients with metachromatic leukodystrophy had improved nerve conduction velocity. Five patients with osteogenesis imperfecta who underwent HCT had donor osteoblast engraftment, new dense bone, increased total bone mineral content and improved growth velocity (85). The frequency of bone fractures decreased. Gene-marked MSCs were given to six HCT patients with MSC engraftment in bone and accelerated growth velocity. In a fetus with bilateral femur fractures due to severe osteogenesis imperfecta, *in utero* transplantation of MSCs showed 7% engraftment and the patient had fewer fractures than expected after birth (86).

MSCs FOR HEMORRHAGES AND SIDE-EFFECTS

We used MSCs for hemorrhagic cystitis, colon perforation, and pneumomediastinum after HCT (87). Adult patients are more vulnerable and had more toxicity after HCT as opposed to pediatric patients. However, toxicity also occurs in children with advanced hematological malignancies treated with multiple rounds of chemotherapy prior to transplantation. Stromal cells induce clotting and may stop or prevent bleeding. This effect appears to be stronger for DSCs than BM-MSCs (88). Yim et al. reported on two patients with pneumomediastinum/pneumothorax with resolution after MSCs treatment (89).

MATERIALS AND METHODS

Patients

Six children diagnosed with grade II–IV acute gastrointestinal GVHD, with or without skin involvement, were treated with DSCs (**Table 1**). The patients comprised five boys and one girl aged from 10 months to l6 years. Informed consent was obtained from the legal guardians of the patients. Diagnoses were pre-B-ALL in two children, Langerhans cell histiocytosis (LCH), sickle cell anemia, osteopetrosis, and severe combined immunodeficiency (SCID). The conditioning therapy was total body irradiation and etoposide in the two patients with leukemia. The four children with other disorders were given fludarabine together with treosulfan in three patients and with the addition of thiotepa in one patient with sickle cell anemia. A boy with osteopetrosis was given a low dose of busulfan, in addition to fludarabine. Donors were matched unrelated in three patients, cord blood in two children, and bone marrow from an HLA-identical sibling donor in one patient. Post-transplant immunosuppression consisted of tacrolimus together with sirolimus in four patients (**Table 1**). Three patients were given antithymocyte globulin (90).

Acute GVHD was graded according to Seattle criteria (91). The diagnosis of gastrointestinal GVHD was based on biopsies from endoscopies (7–9). Skin biopsies were not performed. Donor recipient chimerism was followed by PCR and patients with acute GVHD were full-donor chimeras (9, 92). Cytomegalovirus (CMV) was followed weekly by PCR and reactivation was treated with ganciclovir (93). Epstein-Barr virus (EBV) PCR was only regularly followed in patients with an EBV-mismatched donor (94). Adenovirus was

TABLE 1 | Characteristics of pediatric patients treated with DSCs for acute GVHD.

No	UPN	Sex/age	Diagnosis	Conditioning	Donor	Graft	Immuno-suppression	Acute GVHD grade	Organs involved	Day of acute GVHD	Day of DSC
1	1555	M/1	Langerhans cell, histiocytosis	Flu treo	UD	CB	Prograf rapamune	III SR	GI skin	+ 20	+23, +43
2	1625	F/16	High risk pre B-ALL	TBI+VP16	MUD	BM	Prograf rapamune ATG	II SR	GI skin	+ 18	+30
3	1687	M/9	Intermediate risk pre B-ALL	TBI+VP16	HLA id sister	BM	Prograf rapamune	III SR	GI skin	+ 9	+31, +38, +45, +55, +284, +298
4	1692	M/14	Sickle cell anemia	Flu treo TT	MUD	BM	Prograf rapamune ATG	II SR	GI	+ 182	+200
5	1707	M/1	Osteopetrosis	Flu Bu2	MUD	PB	Prograf methotrexate ATG	II SR	GI	+ 17	+ 21, +70, +78, + 86, +93
6	UAH	M/1	SCID	Flu treo	UD	CB	Cyclosporine MMF	IV SR	GI skin	+ 33	+ 57, +64, +71, +78, +92, +113

M, male; F, female; Flu, Fludarabine; Treo, Treosulphan; UD, unrelated donor; CB, cord blood; GI, gastro-intestinal tract; TBI, total body irradiation; VP16, vepesid; BM, bone marrow; ATG, anti-thymocyte globulin; TT, Thiotepa; Bu, short course busulphan; SR, steroid refractory; MUD, matched unrelated donor; HLA id, human leukocyte antigen identical; UAH, Uppsala Academic Hospital; SCID, severe combined immunodeficiency; MMF, murophenolate mofetil.

not monitored routinely (95), and only when an infection was suspected.

Ethics

We received ethical approval from the regional ethic committee to harvest DSCs from Caesarian section placentas and use them for treatment of GVHD and toxicity after HCT (2009/418-31-34 and 2010/2061-32, 2010/452-31/4, and 2014-2132-32). The procedure for using DSCs was also later approved by the Central Ethical Review Board in Sweden (Dnr 011-2016). The method for clinical culture of DSCs was also approved by the Swedish Product Agency (Dnr 6.1.3-42994/2013).

Decidua Stromal Cell Culture

The method to culture and expand DSCs was previously published in detail (96). DSCs express CD166, CD105, CD73, CD44, and CD29. They did not express hematopoietic markers CD34, CD14, and CD45. DSCs were negative for bacteria, mycoplasma, and fungi before infusion. The DSCs were cultured and expanded in a good manufacturing process laboratory. DSCs were stored in liquid nitrogen, thawed, and resuspended in CliniMACS PBS/EDTA buffer, supplemented with 10% AB plasma or 5% albumin (69). The cells were washed three times and resuspended in NaCl and 10% AB serum or 5% albumin. The infusion solution was filtered through a 70 μM cell strainer (BD Bioscience, Franklin Lakes, NI) before being transferred to a heparinized syringe (Leo Pharma, Ballerup, Denmark) at 2×10^6 cells/ml. The DSC was infused intravenously using a central venous line. The central venous line was flushed with 2–5 mL of NaCl with 25 IE heparin/ml in children weighing over 15 kg and 12.5 IE heparin/ml in children weighing under 15 kg.

RESULTS

Patient 1 (UPN, unique patient number, 1555). A male baby boy was presented with disseminated LCH disease including bone marrow involvement and was pretreated with steroids and chemotherapy, followed by a HCT. The boy received an unrelated cord blood transplant. We previously reported that LCH can be cured by HCT (97, 98). Due to poor engraftment he was treated with granulocytes colony-stimulating factor (G-CSF) from day +20 after HCT. He reached absolute neutrophil counts (ANC) $>0.5 \times 10^9$/L on day +27. On day +20 after HCT, he started vomiting and had watery diarrhea 10 times/day. His diarrhea deteriorated and he developed a skin rash on the back of his body. He was given high- dose prednisolone (2 mg/kg). Due to unresponsiveness he was treated with DSCs 3 days later and one additional dose was administered 3 weeks after the steroids had been introduced (**Table 1**). DSC doses were above 2×10^6/kg and viability was 78 and 95% in the two infusions, respectively (**Table 2**). At day 28 after DSC infusion, he had a partial response (PR). At day 56 and at the 6-month follow-up he showed no signs of acute GVHD (**Table 2**). He was diagnosed with a CMV reactivation on day +61 treated with ganciclovir. He is currently alive and well more than 8 years after HCT and from last follow up he showed Lansky score of 100%.

TABLE 2 | Decidua stromal cells (DSCs) for therapy of acute GVHD characteristics, cell dose and outcome.

Patient	UPN	DSC dose no	DSC viability %	Cell dose $\times 10^6$/kg	Passage	Day 28 response	Day 56 response	6 months response	Chronic GVHD grade	Outcome
1	1555	1	78	2.7	2	PR	CR	CR	0	Alive and well, Lansky score 100%
		2	95	2.4	2					
2	1625	1	91	1.7	3	CR	CR	CR	0	Died from leukemic relapse 2 years post HCT
3	1687	1	97	1.2	4	CR	PR	PR	2	Moderate chronic GVHD obstructive bronchiolitis. Presently Lansky score 100%
		2	69	1.1	3					
		3	96	1.1	4					
		4	96	1.1	4					
		5	96	1.2	4					
		6	94	1.5	3					
4	1692	1	96	0.9	4	PR	PR	PR	2	Died from cerebral hemorrhage
5	1707	1	89	1.9	4	CR	PR	CR	0	Alive and well. Lansky score 100%
		2	100	1.7	4					
		3	91	1.5	4					
		4	95	1.6	4					
		5	82	1.5	3					
6	UAH	1–6	69–100	1.0	3–4	PR	CR	CR	0	Alive and well, Lansky score 100%

PR, partial response; CR, complete response; UAH, Uppsala Akademic Hospital.

Patient 2 (UPN 1625). A 16-year-old female with high risk B-ALL in 2nd complete remission (CR) received bone marrow from an unrelated donor. The patient was treated pre-HCT according to the NOPHO (Nordic Pediatric Hematology Oncology) ALL protocol 2008 and was in complete remission pre HCT, including MRD <0.01% (99). She experienced CMV reactivation on day +19, treated with ganciclovir. ANC reached $>0.5 \times 10^9$/L on day +23. Eighteen days post-transplant, she developed steroid refractory grade II acute GVHD of the gastro-intestinal tract and a skin rash. She was treated with a high dose of steroids from day +20, but did not respond. Due to steroid resistance, she was treated with one dose of DSCs 30 days after HCT (**Table 1**). The DSC dose was 1.7×10^6/kg with 91% viability (**Table 2**). Her symptoms of acute GVHD disappeared and she was considered to be in a complete response at day 28 and remained so. However, the patient died from leukemic relapse 2 years after HCT.

Patient 3 (UPN 1687). A 9-year-old boy with an intermediate risk of B-ALL in CR2 received a bone marrow graft from his HLA-identical sister. He was previously treated according to the NOPHO ALL protocol 2008 (99). Both donor and recipient were CMV seropositive. He had no CMV reactivation. On day +10 he had hemorrhagic cystitis grade II that resolved. Already on day 9 after HCT he developed acute GVHD of the gastrointestinal tract and erythema of the skin. He did not respond to high doses of steroids and was considered steroid refractory. On day +30 he also developed a varicella-zoster reactivation. One month after HCT he was given 1.2×10^6 DSC/ $\times 10^6$/kg with a viability of 97% (**Table 2**). At day 28 after DSC treatment was initiated, he had complete resolution of all signs of acute GVHD but received another three additional weekly doses (**Tables 1**, **2**). However, at 6 months, it was evident that he had developed signs of chronic GVHD as sicca and lichenoid changes of the skin, treated with extracorporeal psoralene and ultraviolet light (PUVA). After another 2 months he developed signs of a more generalized GVHD, with symptoms from both the skin, the liver, and the gastrointestinal tract. The biopsy from the GI-tract revealed GVHD, grade II (8) and he was given two more doses of DSC (**Tables 1**, **2**). The symptoms of acute GI-GVHD disappeared but one and a half year after transplant he was still having symptoms of moderate chronic GVHD, mainly symptoms of bronchiolitis obliterans. 6.5 years after HCT he is suffering from NIH grade 2 chronic GVHD and is now treated with a JAK2 inhibitor, but from his last follow up he scored Lansky 100%.

Patient 4 (UPN 1692). A 14-year-old boy arrived in Sweden, from an African country with an untreated severe sickle cell disease. He had a history of multiple sickle cell crises, as severe pain, osteonecrosis, cerebral infarctions, and bleedings and was therefore planned for a HCT. Before HCT he was treated with Hydrea capsules, but the treatment showed very moderate effect. He was finally transplanted and received bone marrow (0.25×10^6 CD34+ cells/kg) from an unrelated donor (12/12 match). He reached ANC $>0.5 \times 10^9$/kg on day +19. On day +28 he was treated with acyclovir for a herpes simplex virus infection. Immunosuppression was tacrolimus combined with sirolimus. During discontinuation of immunosuppression on day 182 after HCT he developed diarrhea diagnosed as gastrointestinal GVHD. Steroids were administered, but the diarrhea continued. One

week later he was given 0.9 × 10^6 DSC/ × 10^6/kg (**Table 2**). He had a partial response at 28 days and at follow-up at 6 months. Seven months after HCT he had CMV reactivation treated with ganciclovir. One year after the transplant he developed chronic GVHD, NIH overall score 2 (**Table 2**). However, the patient died from severe cerebral hemorrhage 1 year and 9 months after HCT, where previous cerebral damage pre HCT probably contributed to cerebral hemorrhage post HCT.

Patient 5 (UPN 1707). A 1-year-old boy with osteopetrosis rejected the first graft and was re-transplanted 2 months later. He received a peripheral blood graft from an unrelated donor (**Table 1**). HLA-match was 10/12 with one antigen-HLA-C and –DP-mismatches. CD34+ cell dose was 34 × 10^6/kg. He had CMV reactivation on day +11, treated with ganciclovir. On day 17 after HCT he developed diarrhea grade II that did not respond to steroids. He was subsequently given five doses of DSC in doses ranging from 1.5 to 1.9 × 10^6/kg per kg (**Table 2**). The viability of the cells ranged from 82 to 100%. At day 28 after initiation of DSC therapy, he had a complete response. At day 56 he had some abdominal pain and a loose stool. At the 6-month follow-up the stool was normal. He did not develop any chronic GVHD and is currently alive and well 6 years after transplantation, with a Lanskys score of 100%.

Patient 6 The boy, born at term, non-consanguineous parents, was admitted to the hospital at the age of 9 months, with symptoms of severe respiratory infections, failure to thrive, and low lymphocytes. He was investigated for suspected severe combined immunodeficiency (SCID). Genetic analysis revealed a JAK-3 gene mutation (two heterozygous variants, leading to a frame shift and premature stop codon; p.Ser 449LysfsX71). At 12 months of age the boy was transplanted, with cord blood as a stem cell source. Pre-HCT the boy was colonized with rhinovirus, which also was observed after transplantation. On day 29, PCR-chimerism analysis revealed 60% donor cells. Subsequently, during immunosuppressive tapering, he developed a skin rash and, a few days later, also massive diarrhea due to gastrointestinal GVHD. This was diagnosed on a colon biopsy showing crypt destruction with several apoptotic bodies and regenerated features of grade IV gastrointestinal GVHD (8). He did not respond to steroids or mycophenolate mofetil therapy (**Tables 1**, **2**). From day 57 after HCT, he was treated with weekly doses of DSCs. He had a partial response at day 28 and continued to need albumin transfusions. He received a total of six doses of DSCs before the resolution of gastrointestinal GVHD. At day 56 and 6 months after transplant he had a complete response and was doing well. Apart from rhinovirus, no viral, or fungal infections were diagnosed post-HCT. He is currently alive and well, 5 years after transplantation. He doesn't need any medications goes to school and shows Lanskys score of 100%.

Overall Follow Up

The outcome among these six children treated for severe gastrointestinal and sometimes also acute skin GVHD at the 28-day follow-up was a complete response in three patients and a partial response in three patients (**Table 2**). At 6 months, a complete response was seen in four patients and a partial response in two patients. Two patients developed moderate chronic GVHD. One patient with high risk pre-B-ALL died of leukemic relapse 2 years after transplantation. A boy with sickle cell anemia died of cerebral hemorrhage 1 year and 1 month after HCT, although he had a history of multiple severe sickle cell crises before HCT. Three patients are alive and well and one patient is suffering from moderate chronic GVHD with obstructive bronchiolitis but responded to Jak-2 inhibition. Now he scores Lansky 100%. Overall, there is a 5-year survival of 67%.

DISCUSSION

Although this is only a small series of pediatric patients treated for acute GVHD, it still holds some promise. None of the children died from GVHD and 6-year survival was four out of six (67%). This is similar to what was achieved with DCSs with 21 patients, most of them older adults with a 4-year survival of 57% (100). The two deaths were due to relapse in the patient with high-risk ALL and cerebral hemorrhage in the patient with sickle cell disease. These are unfortunate yet expected complications after HCT. Patients who survived acute GVHD have an reduced risk of leukemic relapse (101). The graft-versus-leukemia (GVL) effect did not prevent relapse in this girl with high risk B-ALL. She did not develop chronic GVHD. The study from the International Registry suggested that acute GVHD had a profound GVL effect in ALL patients (102). A European study in ALL patients found that chronic GVHD was more important to decrease relapse probability (103). There were only two patients who developed moderate chronic GVHD. Children have a relatively low risk of chronic GVHD (104, 105). However, there is an increased risk of chronic GVHD in patients who survive acute GVHD (101). Children have a better outcome than adults after HCT and this is striking in patients with severe acute GVHD (6). In a prospective randomized study performed by Osiris, it was reported that children treated for severe acute GVHD, as opposed to adults, had a better outcome (51). The first multicenter study using MSCs for acute GVHD also showed a better outcome in children than adults (47). However, this was not supported by a meta-analysis, which showed that survival following MSC therapy for acute GVHD did not differ in children and adults (106).

An advantage of using MSCs as opposed to other drugs to treat acute GVHD is safety, with few, if any side-effects (107, 108). There were no side-effects caused by the stromal cells in any of the six children treated with DSCs.

The ideal source of stromal cell for treatment of acute GVHD, MSC from bone marrow, adipose tissue, Wharton's jelly, umbilical cord, placenta tissue or DSCs, may be discussed. In a humanized mouse model, it was shown that MSCs from BM, umbilical cord, and adipose tissue had different properties (109). In **Table 3** is listed the different properties of MSCs from bone marrow compared to DSCs. Bone marrow aspiration is quite a painful procedure. Thus, alternative sources such as adipose tissue, cord, placenta tissue, or fetal membrane, stromal cells are more easily accessible. We found that DSCs had a stronger immunosuppression of alloreactivity *in vitro* in mixed lymphocyte cultures compared to MSCs from other

TABLE 3 | Differences between bone marrow-derived mesenchymal stromal cells and placenta-derived decidual stromal cells.

Characteristic	MSC	DSC
Expansion potential	++	++++
Differentiation to fat and cartilage	+++	+/–
Size, volume	4600 fl	2400 fl
Express PDL-1, PDL-2	+	++
Express CD49d, homing to inflammatory tissue (integrin)	+	++
Vascular cell adhesion molecule 1 (VCAM-1) expression	+	–
Express HLA class II after IFNγ stimulation	+	–
Pro-coagulant tissue factor	6%	39%
CD55 complement regulatory activity	62%	98%
Reduction in clotting time	55%	70%
Prevent alloreactivity *in vitro* (MLC)	++	+++
Needs direct contact for immunosuppression	–	+
Overall response in steroid refractory acute GVHD	75%	100%

MLC, mixed lymphocyte culture.

sources. We therefore selected DSCs for further investigation (30). DSCs also appeared to be more effective for treating acute GVHD compared to BM-MSCs (69). However, it is unlikely that different sources of stromal cells will be compared in prospective randomized studies for the treatment of acute GVHD. Currently, there are several promising drugs for treating acute GVHD, such as Ruxolitinib, Vedolizumab and Etanercept (110–112). However, it seems that an advantage of using MSCs is the toxicity profile.

The first child (UPN1555) was treated with G-CSF for poor engraftment. G-CSF was reported to be associated with severe acute GVHD because it can trigger alloreactive T-cells (113, 114). G-CSF may have potentiated acute GVHD in this child.

Several large studies have been using MSCs, as shown from a single report on children from Kurtzberg et al. who recently reported on 241 children with grade II–IV steroid refractory acute GVHD (115). The 28-day overall response rate was 65% with a 14% complete response. The 100-day survival was 67%. These results were achieved with the commercially available MSCs Remestemcel-L. The randomized study by Osiris, which did not show an overall improvement in the placebo-controlled trial, showed that pediatric patients had a significantly better outcome using MSCs compared to the placebo group (53). Bonig et al. used MSCs pooled from multiple donors to treat acute GVHD (58). They reported an overall response rate of 82% following a median of three doses of pooled MSCs. Overall, 6-month survival was 64%.

MSCs have mainly been used for treatment of acute GVHD in pediatric patients. They have not been used much for chronic GVHD. This is because stromal cells have a strong anti-inflammatory effect, which may be more effective for acute inflammatory processes such as acute GVHD and less effective in chronic fibrotic processes (116). Another indication for MSCs, mainly used in adults, is hemorrhagic cystitis (117, 118). MSCs have also been used for the treatment of acute respiratory distress syndrome (ARDS). There is a wealth of experimental data suggesting the potential of MSCs for sepsis and ARDS (119–121). We treated a young boy who developed ARDS after HCT with MSCs (122). He died from massive Aspergillus infection. DSCs were shown to dramatically reverse ARDS in a male patient early after HCT (123). There is limited clinical experience (124). The lack of data on pediatric patients for these more novel indications could be because they are under development. If effective in the adult studies, MSCs will also be used for hemorrhagic cystitis, ARDS, and other indications that are more experimental today.

In addition to acute GVHD, MSCs have also been used to prevent and reverse graft failure, enhance engraftment, or as prophylaxis to reduce GVHD (74, 79–81). These studies include pediatric patients and adults.

As discussed above, the immunosuppressive effects of MSCs are induced by direct contact, as well as via several soluble factors. Exosomes and microvesicles derived from MSCs were shown to protect from acute kidney injury (125), myocardial ischemia (126), and pulmonary hypotension (127) in animal models. Exosomes for MSCs were also demonstrated to reverse severe acute GVHD (46). Since exosomes will only transfer soluble effect by MSCs and not a direct immunosuppressive effect, it is less likely that exosomes will replace MSCs in the near future.

To conclude, MSCs from various sources are mainly used in pediatric patients to treat severe acute GVHD and have shown encouraging response rates and survival efficacy. Thus, commercially available MSCs are registered as a drug in Canada, Japan and New Zeeland (128). Furthermore, MSCs also have the potential to cure other acute inflammatory and toxic disorders seen in pediatric patients, such as hemorrhages, ARDS, poor engraftment, and possibly also neuroinflammatory disorders (129).

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because There is no restriction for the authors of this article to use this datasets. However it is not publicly available. The reviewers are of course welcome to request any data they feel are of importance for evaluating the study properly. Requests to access the datasets should be directed to Olle.ringden@ki.se; Britt.Gustafsson@ki.se.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical committee of Karolinska Institutet, Stockholm, Sweden. Approval to collect placentas and isolate decidual stromal cells (DSC), Dnr 20019/413-31/4 and 2010/2061-32. To use DSCs for treatment of acute GVHD and toxicity after hematopoietic cell transplantation (Dnr 2010/452-31/4 and 2014/2132-32). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

OR, BG, and BS: concept and design, collection and assembly of data, manuscript writing, and final approval of manuscript. OR and BG: financial support. OR and BS: administrative

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

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REVIEW
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Monogenic Immune Diseases Provide Insights Into the Mechanisms and Treatment of Chronic Graft-Versus-Host Disease

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Chronic graft-versus-host disease (GvHD) has become a leading cause of morbidity and mortality following allogeneic hematopoietic stem cell transplantation (HSCT) and can burden patients with devastating and lifelong health effects. Our understanding of the pathogenic mechanisms underlying chronic GvHD remains incomplete and this lack of understanding is reflected by lack of clear therapeutic approaches to steroid refractory disease. Observations predominantly from mouse models and human correlative studies currently support a three phase model for the initiation and development of chronic GvHD: 1) early inflammation and tissue damage triggers the innate immune system. This leads to inflammatory cytokine/chemokine patterns that recruit effector immune cell populations; 2) chronic inflammation causes the loss of central and peripheral tolerance mechanisms leading to emergence of pathogenic B and T cell populations that promote autoimmune and alloimmune reactions; 3) the dysregulated immunity causes altered macrophage polarization, aberrant tissue repair leading to scarring and end organ fibrosis. This model has led to the evaluation of many new therapies aimed at limiting inflammation, targeting dysregulated signaling pathways and restoring tolerance mechanisms. However, chronic GvHD is a multisystem disease with complex clinical phenotypes and it remains unclear as to which cluster of patients will respond best to specific therapeutic strategies. However, it is possible to gain novel insights from immune-related monogenic diseases. These diseases either share common clinical manifestations, replicate steps from the three phase chronic GvHD model or serve as surrogates for perfectly targeted drugs being investigated in chronic GvHD therapy. In this review, we will summarize the evidence from these monogenic immune related diseases that provide insight into pathogenic pathways in chronic GvHD, rationales for current therapies and novel directions for future drug discovery.

Keywords: primary immunodeficiency diseases, chronic graft-versus-host disease, hematopoietic stem cell transplantation, inflammation, T cell, B cell

CHRONIC GRAFT-VERSUS-HOST DISEASE

Chronic graft-versus-host disease (cGvHD) is now the leading cause of morbidity and mortality post-hematopoietic stem cell transplantation (1, 2). cGvHD is a pleomorphic syndrome that resembles autoimmune and other immunologic disorders that occurs between 3 and 15 months after HCT. Chronic GvHD can affect almost any organ including skin, liver, eyes, mouth, lungs, gastrointestinal tract, neuromuscular system, or genitourinary tract. The spectrum of disease manifestations and diagnostic criteria were updated in 2014 after the second National Institutes of Health (NIH) Consensus Conference on cGvHD (3). The rates of cGvHD depend on several variables and can range from as low as 6% in matched sibling cord blood transplants to as high as 65% in matched unrelated donor (MUD) peripheral blood stem cell (PBSC) transplants (4).

Our understanding of the pathophysiology of cGvHD has improved over the last decade to the point where there is now a well-accepted three phase model of cGvHD development supported by mouse models, correlative clinical studies and clinical trials (5). The three phases are: 1) acute inflammation and tissue injury trigger inflammatory cytokine/chemokine patterns, mediated through the innate immune system, that recruit effector immune cell populations; 2) chronic inflammation causes a loss of tolerance that disrupts the homeostasis of the adaptive immune system leading to the emergence of pathogenic B and T cell populations; 3) the dysregulated immune response causes altered macrophage polarization causing an aberrant tissue repair mechanism leading to excessive end organ fibrosis and scarring.

Despite these insights, clinicians continue to struggle to identify the optimal therapy for patients with cGvHD who do not respond to front-line corticosteroids or patients who cannot be successfully weaned off corticosteroids.

WHY STUDY RARE DISEASES?

Rare inherited monogenic diseases affecting innate and adaptive immunity provide a unique opportunity to understand the role of specific genes, molecules, pathways and cell types in our immune system (6). Unfortunately, in the past these rare diseases were often considered medical outliers and neglected compared to more polygenic, multifactorial common disorders. However, they all operate under the same biological principles and these rare diseases are actually much simpler pathologically then common diseases. These human models demonstrate the function of a particular gene in an otherwise controlled experiment of nature, in which everything else is identical except for the one single factor which is the root cause of the resulting disease phenotype. Better understanding rare diseases not only directly benefits those patients afflicted but the recognition of a molecular defect can lead to potential therapies. Mutations that alter the level of activity of gene products can be thought of as surrogates for perfectly targeted drugs (7).

Their study provides the means to better understand complex acquired diseases in a number of ways:

1. An acquired disease may have a specific phenotype that is specifically missing in an inherited disease due to the absence of a key molecule. By pharmacologically inhibiting this factor you may eliminate the phenotype, therefore the inherited disease informs a potential new target.
2. An acquired disease may have a specific phenotype that mimics that seen in an monogenic disease associated with altered function (gain or loss of function) of a key molecule or cell type; inherited disease again provides potential new target or supports the addition of a key factor into treatment, such as adding an agonist or cell type. The emergence of cellular therapies has given clinicians the ability to treat disease with a wide variety of manipulated cell types in addition to the well-established therapy of hematopoietic stem cell transplant.
3. A new targeted therapy may be trialed based on mouse models or human correlative clinical studies of a specific disease and there is a corresponding monogenic disease involving that factor; the rare disease may provide insights into unintended consequences of targeting that factor in other biological pathways.

With these principles in mind, the purpose of this review is to use our evolving understanding of monogenic immune disorders to provide a rationale for previous and ongoing therapies in cGvHD and potentially provide new avenues for intervention based on the pathophysiology of cGvHD (**Table 1**).

SIMILARITIES BETWEEN CHRONIC GVHD AND PRIMARY IMMUNE DISORDERS

Chronic GvHD is fundamentally a disorder of immune regulation. A successful HCT requires: 1) reconstitution of normal innate and adaptive cellular immune responses to infectious pathogens and the 2) induction of immune tolerance to non-self antigens and in the case of malignant disease, while preserving the graft-versus-tumor effect.

The persistent alloreactivity in cGvHD is driven step-wise by increased expression of host-derived molecules that result from tissue damage. This leads to the expansion of pathogenic T and B cell populations that escape tolerance and are allowed to persist due to the failure of suppressive regulatory mechanisms. This promotes chronic inflammation that triggers aberrant repair mechanisms. Therefore, this review will focus on primary immunodeficiencies associated with defects in intrinsic or innate immunity, autoimmunity and dysregulation of lymphocyte homeostasis.

DEFECTS INVOLVING THE INNATE IMMUNE SYSTEM

The intestinal epithelium, an integral component of innate immunity, is altered in a number of ways during the HCT

TABLE 1 | Potential therapies targeting the pathophysiology of each phase of chronic graft-versus-host disease.

cGvHD Phase	Phase 1	Phase 2	Phase 3
Pathophysiology	Acute inflammation and tissue injury activates the innate immune system including the complement system leading to the recruitment of pathogenic cell populations	Loss of tolerance mechanisms disrupts the homeostasis of the adaptive immune system	Aberrant tissue repair mechanism leading to excessive end organ fibrosis and scarring
Relevant monogenic diseases	*Primary immune deficiencies (PID) with inflammatory bowel disease (IBD)-like pathology* *MyD88 and IRAK-4 deficiencies* *Complement deficiencies*	*Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED)* *Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome* *LPS-responsive beige-like anchor protein (LRBA) deficiency* *Cytotoxic T lymphocyte antigen-4 (CTLA-4) haploinsufficiency* *Autosomal dominant hyper-IgE syndrome (AD-HIES)* *IL-12/23 receptor beta 1 (IL-12/23Rβ1) and IL-12/23 cytokine p40 subunit deficiency* *Glut1 deficiency* *Leptin deficiency* *Wiskott-Aldrich syndrome (WAS)* *B cell activating factor (BAFF) receptor deficiency* *Gain-of-function mutations in PIK3CD* *X-linked agammaglobulinemia (XLA)*	*Matrix metalloproteinase-2 (MMP-2) deficiency* *Stiff skin syndrome (SSS)*
Potential therapies	***Treatment of gut dysbiosis:*** selective use of antibiotics, pre- and probiotics, fecal microbiota transplantation (FMT) ***TLR inhibition:*** MyD88 and IRAK-4 inhibitors Statins Hydroxychloroquine ***Complement inhibitors:*** Eculizumab [anti-C5 monoclonal antibody (mAb)], narsoplimab (IgG-4 mAb against MASP-2), and coversin (C5 inhibitor)	***Thymic transplantation:*** Medullary thymic epithelial cells (mTECs) ***Reduce donor T cell migration:*** Sphingosine 1-phosphate receptor (S1PR) agonists ***Increase number and function of regulatory T cells (Tregs):*** Rapamycin (mTOR inhibitor), abatacept (CTLA4-Ig), hydroxychloroquine, low dose IL-2, extracorporeal photopheresis, and infusion of donor-specific or third party Tregs. ***Targeting the Th17 subset:*** ROCK2 inhibitor KD025, tocilizumab (mAb against IL-6 receptor), ustekinumab (IL-12 and IL-23 antagonist), pirfenidone ***Targeting T cell metabolic reprogramming:*** Inhibition of glycolysis, leptin, glutamate-oxaloacetate transaminase (GOT1), and glutaminase (GLS) ***Targeting B cell mediated autoimmunity:*** Rituximab (anti-CD20 mAb), belimumab (anti-BAFF mAb), Syk inhibitors, PI3K inhibitors, ibrutinib (BTK inhibitor)	***Target activation of collagen-producing fibroblasts and myofibroblasts:*** Imatinib mesylate (tyrosine kinase inhibitor), sonidegib (sonic hedgehog pathway inhibitor) ***Increase levels of dermal MMP-2:*** Narrowband ultraviolet-B light therapy ***Integrin inhibition:*** Natalizumab (mAb against α4-integrin) and vedolizumab (α4β7 inhibitor)

The bolded text in italics describes the mechanism/class of medications that addresses the pathophysiology of that phase of cGvHD.

process; complications of the primary disease, gastrointestinal infections, conditioning chemotherapy and radiation cause direct intestinal damage and the use of broad-spectrum antibiotics and varied diets disrupt gut microbiota. Affected cell types include: 1) intestinal stem cells, impairing epithelial regeneration, 2) intestinal epithelial cells, which comprises barrier function, 3) Paneth cells, leading to decreased secretion of antimicrobial peptides, and 4) goblet cells, which depletes the mucus barrier. The cumulative effect is a dysbiosis associated with decreased commensal bacterial function and diversity, increased gut permeability and bacterial translocation leading to increased local inflammation that disrupts immune homeostasis (8).

Many primary immune deficiencies are associated with microbial dysbiosis, which manifests clinically as inflammatory bowel disease (IBD)-like pathology (9) and may alter the clinical phenotype of a common genetic susceptibility. Underlying pathogenic mechanisms include the absence of secretory IgA which normally promotes the clearance of antigens and pathogenic bacteria from the gut microbiota (10) and increased translocation of lipopolysaccharide (LPS) (11). Therapies such as the selective use of antibiotics, prebiotics, probiotics and fecal microbiota transplantation, aimed at restoring the gut microbiota may prove beneficial in cGvHD and are actively being investigated (12).

Early inflammation in patients post-HCT is triggered by the activation of innate pattern-recognition receptors (PRRs) such as Toll-like receptors (TLRs) and nucleotide oligomerization domain (NOD)-like receptors (NLRs) on host antigen presenting cells (APCs) by viral and bacterial components and endogenous dangerous molecules termed danger-associated molecular patterns (DAMPs). These signals are released due to endothelial and epithelial cell damage in the GI tract caused by underlying disease, infection and transplant conditioning. TLR signaling in APCs such as dendritic cells enhance antigen

endocytosis and autophagy and augments the assembly of key antigen transport and processing systems (13). In turn, activated host and donor APCs stimulate donor T cells either directly through donor T-cell receptors that recognize minor histocompatibility antigens, foreign MHC molecules and allogeneic peptides or indirectly through the release of pro-inflammatory cytokines and chemokines such as IL-1β, IL-6, IL-8, IL-10, IL-12, IL-21, IL-23, TGFβ and TNFα. Compared with non-GVHD patients after HSCT and healthy donor controls, TLR4-mediated NF-κB signaling-related genes including TLR4, NF-κB, IL-6 and intercellular adhesion molecules 1 (ICAM-1) were significantly increased in patients with cutaneous cGVHD (14).

In innate immune cells such as dendritic cells, MyD88 is the critical adaptor molecule that bridges TLRs to the IRAK family of kinases, which in turn stimulate a signaling cascade that results in NF-κβ activation (15, 16). Germline MyD88 and IRAK-4 deficiencies predispose patients to recurrent life-threatening bacterial diseases, such as invasive pneumococcal disease in particular, with weak signs of inflammation (17).

There is evidence that TLR signaling contributes to the early activation of APCs and priming of donor T cells. TLR inhibition can be achieved either by blocking the binding of agonists to corresponding TLRs or inhibiting the intracellular signaling of the TLR pathways.

The use of a novel MyD88 inhibitor, TJ-M2010-5 in a fully MHC-mismatched murine model inhibited the LPS-stimulated activation of dendritic cells and the priming of donor allogeneic T cell proliferation (18). Administration of the inhibitor ameliorated the inflammatory environment, increased tissue repair in GvHD target organs and suppressed lethal GvHD. Administration of an IRAK-4 inhibitor also ameliorated GvHD in a mouse model of allo-SCT (19). In this model, MyD88 in donor T cells was not essential for graft-versus-leukemia (GvL) effects. There are other well-established medications that have been repurposed in cGvHD because of their effects on TLR signaling including statins, which decrease TLR4 expression and downstream signaling (20, 21) and hydroxychloroquine, an inhibitor of TLR9 signaling (22). Any strategy to block TLR signaling pathways incur significant risk, particularly during post-transplant immune reconstitution as TLR-mediated inflammation functions to protect the host against infection.

Another major part of the innate immune response, the complement system, is also implicated in cGvHD. The complement system is composed of a number of diverse signaling pathways that causes specific plasma proteins to react with one another to generate: 1) activated complement proteins that bind pathogens triggering opsonization by phagocytes, 2) fragments of some complement proteins that serve as chemoattractants and 3) membrane attack complexes that damage bacteria by creating pores in the outer bacterial membrane. All the pathways merge at the proteolytic cleavage of C3 to generate a larger fragment, C3b, that marks a target for opsonization, and a small one, C3a, which serves as an anaphylatoxin which triggers the release of inflammatory mediators from nearby cells. Subsequent cleavage of another complement protein, C5, results in C5a, which is also an anaphylatoxin and chemotactic factor, and C5b which initiates formation of the membrane attack complex (23). The anaphylatoxins C3a and C5a exert their biological function by binding to their cognate G protein-coupled receptors C3aR and C5aR on cells of the innate and adaptive immune system.

Human C3 deficiency is associated with impairments in dendritic cell maturation suggesting complement activation could play a role in the dendritic cell regulation of GvHD in first acute phase of inflammation (24). The generation of C3 and C5 complement proteins during complement activation has previously been implicated in the pathogenesis of GvHD. Expression of C3aR and C5aR on donor T cells is essential for GVHD development after HCT (25). Reduced GvHD in C3-deficient mice is associated with decreased donor Th1/Th17 differentiation (26). C3aR/C5aR-mediated signaling directly induces secretion of IFN-γ and IL-2 from T cells driving Th1/Th17 differentiation and suppressing Treg generation (27, 28). C3aR/C5aR signaling suppresses lethal mitophagy in dendritic cells after HCT. Blockade of C3aR/C5aR activation significantly enhanced mitophagy in recipient dendritic cells which correlated with improved GvHD outcomes and the studies also showed that treatment with C3aR/C5aR antagonists effectively separated GvHD and GvL responses making it a promising therapeutic approach for GvHD treatment especially in malignant diseases dependent on the GVL effect (29, 30).

In the post-allo-HCT setting, complement inhibitors such as eculizumab, (anti-C5 monoclonal antibody), narsoplimab (IgG-4 monoclonal antibody that inhibits the effector enzyme MASP-2 of the lectin complement pathway), and coversin (C5 inhibitor) have already been used in the treatment of transplant-associated thrombotic microangiopathy (TA-TMA) (31–33). It is possible that these therapies could be used as prophylaxis for cGvHD.

Targeting these innate pathways appears promising as they would ideally interrupt the early inflammatory cascade underlying cGvHD development while preserving the GvL effect.

A potential issue would be the timing of these interventions as they target the early stages of cGvHD which are difficult to appreciate clinically and maybe better served as prophylactic agents against GvHD or used in specific patients early post-HCT with predictive biomarkers (34).

T CELL IMMUNE DYSREGULATION

Ongoing damage to epithelial and connective tissue releases DAMPs that activate cells of the innate immune system such as dendritic cells triggering the release of IFNα, IL-1β, TNFα and IL-6. This inflammatory cytokine profile induces Th1/Th17 differentiation and subsequent recruitment to the injured tissue. They are activated by APCs and continue the cycle of tissue damage. Dysfunctional thymic negative selection frees alloreactive T cells targeting host antigens that continually feed this vicious cycle leading to chronic tissue inflammation. This part of the review will focus on two key elements of this pathological process: 1) loss of thymic negative selection and 2) skewing of T cell

repertoire toward Th1/Th17 lineages at the expense of regulatory T cells. Both of these biological processes have parallels with monogenic immune disorders that provide insights into pathology and the basis for existing and potential new therapies.

Loss of T Cell Thymic Selection

Early after HCT, mature donor alloreactive T cells transferred with the allograft are activated by host APCs and mediate direct tissue destruction. In particular, thymic epithelial cells are damaged leading to release of self-reactive T cells. Severe histopathological damage to the thymus is a feature of aGvHD and plays a prominent role in the second phase of cGvHD. Using murine models of allogeneic HCT it has previously been shown that donor T cells can damage primary lymphoid tissue including the thymus. Thymic aGvHD impaired the compartment of medullary thymic epithelial cells (mTEC) that express the autoimmune regulator (AIRE) (35, 36). Loss of AIRE + mTEC led to a failure to clonally delete self-reactive T cells. This is likely caused by the decreased heterogeneity of tissue specific auto-antigens from cGVHD target organs presented by thymic mTEC cells in order to select functional but tolerant T cells. Accordingly, donor-derived T cells possessing cGvHD antigen reactivity escape deletion and expand. This loss of thymic negative selection is further exacerbated by the physiologic process of age-related thymic atrophy/involution (37). This pool of self-reactive T cells is under constant homeostatic pressure to expand due to overall lymphopenia in GvHD caused by the dysfunction of the peripheral niches essential for the survival of naïve T cells (38).

The AIRE gene is mutated in autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), a rare monogenic recessive disorder characterized by a variety of autoimmune diseases that target endocrine organs, liver, intestine and skin (39). This is caused by immune reactions against an assortment of autoantigens (40). Murine studies suggest that AIRE promotes ectopic transcription of self-antigens in mTECs and therefore important for negative selection of autoreactive T cells or in the case post-HCT, alloreactive T cells (41). All patients with APECED also have neutralizing antibodies against type I interferons and they are present before the development of autoimmune conditions (42). Antibodies to IFNα have also been recognized as an autoantibody that develops after allogeneic BMT in association with cGvHD (43, 44). Lastly, APECED patients also have a decrease in the regulatory T cell population (45) similar to patients with cGvHD.

It is unclear whether thymic transplantation, which has been used successfully in the treatment of differentiative thymic disorder related to FOXN1 mutations (46), would alter the process of negative selection by the thymus. The transplantation of recipient-type thymus at 4 weeks post-BMT in an established chronic GvHD model prevented the development of cGvHD and increased survival (47). Perhaps in the future it will be possible to generate mTECs from recipients, for example through the use of induced pluripotent stem cells, prior to HCT that can be used as prophylactic treatment post-HCT to support normal T cell development (48).

Another potential therapeutic target is preventing the trafficking of alloreactive T cells to the thymus in the early stages post-HCT thereby limiting damage to the thymus thus preserving tolerance mechanisms. This same principle could be applied to the trafficking of pathogenic T cells to target organs and ideally preserving trafficking of regulatory T cells. Sphingosine 1-phosphate (S1P) is a sphingosine containing lipid intermediate obtained from ceramide that plays a key role in lymphocyte migration through concentration gradients and binding and activation of G-protein-coupled receptors known as S1P receptors (S1PR1) (49). It has been shown that prophylactic, not therapeutic, administration of a S1PR agonist reduced donor T cell migration to the host thymus, thus significantly attenuating thymic aGvHD in murine model of unconditioned recipients of haploidentical donor T cells (50). This approach was successfully used in a patient with severe CNS GVHD (51).

Skewing of T-Cell Repertoire During Chronic GvHD Development

The acute inflammation of the first phase of cGvHD creates an environment that favors excessive pro-inflammatory Th17 cells over regulatory T cells that suppress inflammation. The development of cGvHD has been shown to be associated with a dynamic imbalance that favors the production, expansion, and persistence of effector T cells, in particular Th17 cells driven by BCL2 expression over CD4 regulatory T cells (52). Patients with active cGvHD had a significantly lower frequency of circulating T follicular helper cells (cTFH) compared with patients without cGvHD. This was associated with higher CXCL13 plasma levels suggesting increased homing of TFH to secondary lymphoid organs. The cTFH phenotype was skewed toward a highly activated profile with predominance of Th2/Th17 subsets and demonstrated increased functional ability to promote B cell immunoglobulin secretion and maturation (53). Again their survival was preferentially promoted by BCL-2.

The creation of this immune imbalance in patients with active cGvHD lends itself to potential therapies either previously used in PIDs associated with T cell disorders or provides information about which gene products should be targeted to create an effect that mimics the PID phenotype; if cGvHD is associated with elevated Th17 cells then we should target affected proteins/pathways in monogenic diseases associated with loss of Th17 cells.

The potential impact of current therapies and new avenues of treatment are discussed in the context of known PIDs with abnormal T cell homeostasis.

Strategies to Increase the Number of Regulatory T Cells

The prototypical genetic autoimmune disease involving Tregs is Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome which is caused by mutations in the FOXP3 gene and characterized by markedly decreased or absent FOXP3+ Tregs (54). Many other primary immunodeficiencies with prevailing lymphoproliferation, such as LPS-responsive beige-like anchor protein (LRBA) deficiency and cytotoxic T

lymphocyte antigen-4 (CTLA-4) haploinsufficiency, are also associated with decreased or dysregulation of Tregs (55, 56).

Rapamycin is a small molecule inhibitor of mechanistic target of rapamycin (mTOR) that selectively inhibits effector T cell proliferation while sparing rapamycin-resistant Treg cells thereby supporting the relevant expansion and function of Treg cells (57). Rapamycin has significant clinical benefits in patients with IPEX syndrome (58) and has previously shown efficacy as a cGvHD therapy (59, 60).

Abatacept (CTLA4-Ig) is a fusion protein consisting of an IgG1 Fc domain fused to the CTLA-4 extracellular domain that has successfully been used to control autoimmune inflammation and interstitial lung disease in patients with CTLA-4 haploinsufficiency and LRBA deficiency (61, 62). In a phase I clinical trial, abatacept resulted in a clinical response in 44% of patients with steroid-refractory cGvHD with both decreased prednisone use and T cell PD-1 expression in responders (63).

Inhibition of lysosomal degradation *via* chloroquine/hydroxychloroquine rescued CTLA4 expression in LRBA deficient cells *in vitro* and improved lymphoproliferative lung pathology in a patient with LRBA mutation *in vivo* (64) and long term outcome of patients with LRBA deficiency (65). A phase II trial of hydroxychloroquine in patients with steroid-resistant or steroid-dependent cGvHD resulted in a 53% response rate and all responders tolerated a >50% reduction in their steroid dose while receiving hydroxychloroquine (66).

IL-2 is a critical cytokine for the maintenance and function of FOXP3+ Treg cells. High CD25 expression confers to Treg cells the ability to respond to low doses of IL-2, whereas effector T cells require higher IL-2 concentrations to support their proliferation. Patients with Wiskott-Aldrich syndrome who received low dose IL-2 therapy had statistically significant increase in platelet counts, a trend toward higher T, B, and NK cell numbers and higher T regulatory cell percentages (67). Low dose IL-2 has been shown to provide durable clinical improvement in active cGvHD and extended therapy is well-tolerated (68).

It still remains controversial as to whether extracorporeal photopheresis (ECP) has a clinically significant effect on the number and function of Tregs in cGvHD (69–71).

Multiple studies have demonstrated that developing mixed chimerism post-HCT in non-malignant disease is associated with a lower incidence of aGvHD and cGvHD and among patients with mixed chimerism, cGvHD is associated with a more frequent evolution toward complete chimerism (72). The proportion of Treg cells is increased in patients with mixed chimerism after SCT and acts to suppress the alloreactive immune response (73). In non-malignant diseases, especially those undergoing reduced intensity conditioning resulting in dynamic chimera states, interventions to increase Tregs may stabilize mixed chimerism and lead to lower rates of cGvHD.

Lastly, the Infusion of donor-specific or third-party regulatory T cells have been tested in patients with steroid-refractory or dependent cGvHD. A phase I trial utilizing donor derived Tregs enriched by CD25+ immunomagnetic selection from a non-mobilized peripheral blood apheresis product and purified by high speed flow cytometry demonstrated feasibility, safety and tolerability with encouraging preliminary clinical responses with a single infusion of cells (74). Patients have also been treated with umbilical cord blood derived regulatory T cells (75).

Targeting the Th17 Subset

Autosomal dominant hyper-IgE syndrome (AD-HIES), formerly known as Job syndrome, caused by loss of function mutations in STAT3, is associated with impaired Th17 development (76). Th17 cell development is directed by multiple cytokines, including IL-1β, IL-6, TGF-β, IL-21 and IL-23 which leads to activation of the transcription factors STAT3 and interferon regulatory factor 4 and subsequent expression of retinoic acid-related orphan receptor (ROR)yt. It has been shown that oral administration of the selective ROCK2 inhibitor KD025 to healthy subjects or rheumatoid arthritis patients attenuates the ability of T cells to secrete IL-17 in response to stimulation ex vivo *via* a STAT3-dependent mechanism. ROCK2 inhibition significantly diminished STAT3 phosphorylation and binding to IL-17 and IL-21 promoters and reduced interferon regulatory factor 4 and nuclear hormone RORyt protein levels in T cells derived from healthy subjects or rheumatoid arthritis patients. Simultaneously, KD025 also promoted the suppressive function of regulatory T cells through up-regulation of STAT5 phosphorylation (77). KD025 has been shown to ameliorate cGvHD in multiple murine models and inhibit the secretion of IL-21, IL-17 and interferon y along with decreasing phosphorylated STAT3 and reduced protein expression of interferon regulatory factor 4 and B-cell lymphoma (BCL6) in human peripheral blood mononuclear cells purified from active cGvHD patients (78).

IL-6 is a proinflammatory cytokine that activates the STAT3 signaling cascade and promotes Th17 differentiation. Tocilizumab, the monoclonal antibody against the IL-6 receptor, has been used to treat STAT3 gain of function disease (79). Tocilizumab appears to be a promising treatment option in advanced cGvHD but further evaluation within a phase II trial is required (80).

Inherited IL-12/23 receptor beta 1 (IL-12/23Rβ1) and IL-12/23 cytokine p40 subunit deficiency are rare primary immunodeficiencies associated with impaired generation of IL-17 producing cells (81). Anti-p40 treatment attenuated the severity of sclerodermatous cGvHD in a murine model (82). Ustekinumab, a human IL-12 and IL-23 antagonist, delivered by subcutaneous injection on day -1 and day +20 after peripheral blood mobilized hematopoietic stem transplantation from HLA-matched sibling or unrelated donors significantly improved overall survival and National Institute of Health (NIH) moderate/severe cGvHD-free, relapse-free survival (83). It has not yet been tested in patients with existing cGvHD.

Pirfenidone has been shown to inhibit IL-17A facilitated macrophage infiltration in a mouse model of cGvHD lung disease. In addition, pirfenidone significantly reduced the percentage of IL-17a-producing CD4+ T cells but did not affect the percentage of Tregs (84).

TARGETING METABOLIC REPROGRAMMING AS A POTENTIAL THERAPEUTIC STRATEGY

A number of dysregulated metabolic pathways have previously been identified in PIDs and in turn congenital defects in metabolism are often associated with immune defects. Targeting these pathways in cGvHD offers new avenues of potential therapy.

It has been shown that glycolysis is required for optimal function of alloantigen-activated T cells and induction of GVHD. T cells switch from fatty acid β-oxidation and pyruvate oxidation *via* the tricarboxylic (TCA) cycle to aerobic glycolysis. Inhibition of glycolysis through specifically targeting mTORC1 or PFKFB3 ameliorated GVHD in a preclinical BMT model (85).

Glut1 deficiency selectively impairs metabolism and function of thymocytes and effector CD4 T cells while sparing Treg cells (86). Allo-reactive Glut1-deficient T cells have dramatically decreased ability to induce lethal GvHD due to reduced IL-17 production.

Congenital deficiency of the adipocyte hormone leptin is associated with reduced numbers of circulating CD4+ T cells and impaired T cell proliferation and cytokine release (87). In contrast, increased serum leptin concentrations may contribute to T cell activation during development of cGvHD (88).

There is literature that shows that by simply inhibiting transamination in differentiating T cells, Th17 cell fate can be epigenetically redirected toward the Treg lineage. A recent study identified a compound, (aminooxy)acetic acid (AOA), that is able to reprogram differentiating Th17 cells into Foxp3-expressing iTreg cells by inhibiting the activity of glutamate-oxaloacetate transaminase (GOT1) (89). Another group were able to show that transiently inhibiting glutamine metabolism by targeting glutaminase activity lead to impaired differentiation of Th17 cells and increased Th1 and CTL effector cell function (90).

Selectively targeting metabolic pathways in order to alter the balance of TH17/Treg cells may represent a novel strategy to treat chronic GvHD.

THE ROLE OF B CELL MEDIATED AUTOIMMUNITY

Chronic GvHD has many clinical, histological and serological manifestations that resemble the autoimmunity and dysgammaglobulinemia associated with primary B-cell related immunodeficiencies. Multiple lines of evidence point to an important role for B cells in the pathogenesis of cGvHD. Antibodies to both alloantigens and nonpolymorphic autoantigens are frequently associated with cGvHD (91, 92). Stimulatory antibodies to the platelet-derived growth factor (PDGF) receptor (PDGFR) are selectively found in patients with extensive cGvHD and activate the generation of reactive oxygen species which stimulates type 1 collagen gene expression suggesting a role in the development of fibrosis (93). Allogeneic HY antibodies detected at 3 months after female to male HCT predict cGvHD in humans (94). B cells facilitate autoimmunity not just by secreting host-reactive antibodies, but also by secreting proinflammatory cytokines and by presenting autoantigens to T cells (95). Conversely, an impaired ability of B cells to produce IL-10 was found in patients with active cGvHD (96). Perhaps the best evidence for B cell involvement is the success of rituximab, a chimeric anti-CD20 monoclonal antibody, in corticosteroid-free primary treatment of cGvHD (97–99).

The emergence and persistence of host-reactive B cells in cGvHD results from acquired failures in tolerance mechanisms. Central B cell tolerance is compromised in cGvHD due to altered B cell signalling that affects the negative and positive selection of B cells during development, skewing the emerging B cell repertoire towards a host or self-reactivity. Autoantibody production may also occur due to disturbed T cell- B cell interaction and regulation (100). The highest rate of autoimmune cytopenias following HCT are reported in children undergoing HCT for non-malignant indications with anti-thymocyte globulin (ATG) or alemtuzumab-containing conditioning regimens (101).

The most important driver of immature bone marrow B cell tolerance is B-cell receptor (BCR) signaling after encountering self-antigens. Developmental fate is based on strength and location of BCR engagement, the form of self-antigen and synergy with other co-receptor signals (102). It is clear there are intrinsic and extrinsic factors that can skew this process and overcome other processes that would normally remove autoreactive B cells. Two of the most critical signaling pathways that integrate with BCR signaling in B cell survival and tolerance are Toll-like receptor (TLR) and B cell-activating factor receptor (BAFFR) signaling.

TLR activation appears to contribute to both the negative and positive selection of autoreactive B cells depending on the developmental stage based on observations in PID patients. Patients who lack MyD88 or IRAK-4 exhibit defects in central and peripheral B cell tolerance, implicating TLR-dependent innate signaling pathways in negative selection of immature autoreactive B cell clones (103, 104). In contrast, there is evidence for TLR signaling promoting transitional B cell positive selection in patients with Wiskott-Aldrich syndrome (WAS). There is enhanced signaling downstream of both the BCR and TLRs in B cells from WAS patients that promotes the positive selection of autoreactive transitional B cells (105, 106).

B cell activating factor (BAFF) plays a fundamental role in the survival and differentiation of B cells (107). Its' principal cognate receptor in early B cell development is the BAFF receptor (BAFF-R). Without BAFF-R, B-cell development is arrested at the stage of transitional B cells and the numbers of all subsequent B cell stages are severely reduced (108). Increased BAFF levels rescue low-affinity self-reactive transitional B cells by co-opting BCR signaling through phosphorylation of proximal BCR signaling components such as spleen tyrosine kinase (Syk) (109). BAFF also enhances TLR7/9 expression on B cells and TLR-mediated production of autoantibodies (110). In turn, TLR signaling

promotes BAFF receptor expression creating a positive feedback loop (111). Murine models of B cell autoimmunity suggest that excess BAFF and a reduced pool of naïve B cells are both necessary to promote the survival of autoreactive B cells (112, 113). BAFF has also been shown to selectively enhance the survival of plasmablasts which would promote the subsequent production of host-reactive antibodies (114).

Chronic GvHD is associated with reduced transitional and naïve B cell counts (115), elevated levels of sBAFF (116) and Syk hyperresponsiveness in B cells.

Belimumab, a fully human monoclonal IgG1λ anti-BAFF antibody, is currently being tested as prophylaxis against chronic GvHD in a phase 1 trial (NCT03207958). Inhibition of Syk with fostamatinib in mice with established cGvHD with bronchiolitis obliterans was able to reverse disease. It also decreased the frequency of GCs and expression of the activation costimulatory molecules CD80 and CD86 in CD11c + cells *in vivo*. Most importantly, human cGvHD B cells had increased death when treated with fostamatinib (117). Inhibiting Syk kinase activity abrogates the BCR-driven ex vivo proliferative and survival advantage of human cGvHD B cells (118).

Another example of a PID with a B cell specific break in self-tolerance are patients with gain-of-function mutations in PIK3CD, encoding the p110δ catalytic subunit of phosphoinositide 3-kinase (PI3K), who present with production of germline autoreactive IgM antibodies (119). PI3K expression has been shown to be increased in cGvHD patients (120). The effective treatment of mice with active cGvHD with PI3K-specific inhibitors support future clinical trials of approved PI3K inhibitors for cGvHD therapy in humans (121).

In humans, central B cell tolerance checkpoints are also abrogated in the absence of Bruton's tyrosine kinase (BTK), an essential BCR signaling component (122). Patients suffering from X-linked agammaglobulinemia, caused by loss of function mutations in the BTK gene, have a severe decrease of peripheral B cells and serum immunoglobulin. B cell differentiation is severely affected at the pro- to pre-B transition but the few B cells that do develop are paradoxically enriched in autoreactive clones. The use of antileukemic drugs that inhibit Btk signaling to promote apoptosis of malignant B cells, especially in chronic lymphocytic leukemia, theoretically may also affect B cell selection by interfering with normal BCR signaling leading to the release of autoreactive B cells. Autoimmune cytopenias have been observed in patients with chronic lymphocytic leukemia treated with ibrutinib (123, 124).

Treatment of patients with active cGvHD with inadequate response to corticosteroid-containing therapies with ibrutinib, a BTK inhibitor, in a phase II clinical trial resulted in clinically meaningful responses with acceptable safety leading it to become the only FDA-approved second-line therapy for steroid-resistant cGvHD (125, 126).

Targeting Btk in cGvHD patients with ibrutinib also highlights the potential for phenotypic differences between germline presentations and the effects of an imperfect inhibitor. In addition to its critical role in B cell development, BTK is important for collagen signaling *via* the collagen receptor glycoprotein VI (GPVI) in platelets (127). Ibrutinib has been reported to increase rates of major hemorrhage through selective inhibition of platelet signaling and functions downstream of the collagen receptor GPVI and strongly affects firm platelet adhesion on von Willebrand factor (VWF) under arterial flow (128). In contrast to ibrutinib-treated subjects, patients with XLA do not bleed excessively. The risk of bleeding is attributed to off-target effects of ibrutinib on several other intracellular molecules important for platelet signaling including Tec, another kinase of the Tec family of protein-tyrosine kinases that includes Btk (129). Ibrutinib can also affect T cells due to the off-target inhibition of IL-2 inducible T cell kinase (ITK) with shares significant homology with BTK. Ibrutinib treatment in chronic lymphocytic leukemia (CLL) patients markedly increases CD4+ and CD8+ T cell numbers, decreases the Treg/CD4+ T cell ratio and reduced PD-1 and CTLA-4 expression in T cells (130). It remains unclear if the efficacy of ibrutinib in targeting B cells in cGvHD will be offset by changes in T cell populations, significant risk of bleeding and potential flares of autoimmunity.

FIBROTIC END STAGES OF CGVHD

Fibrosis represents the end stage of the chronic inflammation that occurs in cGvHD and once fixed is poorly amenable to any known therapies. It is thought to result from an aberrant wound-healing process driven by M2-polarized macrophages that in turn produce transforming growth factor-β (TGF-β) and platelet-derived growth factor-α (PDGF-α) leading to the activation of collagen-producing fibroblasts and myofibroblasts partly through sonic hedgehog signaling (131). The prominent role of TGF-β, PDGF-α and sonic hedgehog (SHH) in stimulating fibroblasts has led to the use of tyrosine kinase inhibitors such as imatinib mesylate and the Hedgehog pathway inhibitor sonidegib in the treatment of sclerotic cGvHD (132–134).

Even though there is a lack of a strong association between primary immune disorders and fibrosis, sclerotic or scleroderma-like changes, there are other rare monogenic diseases that have already or may provide new therapeutic avenues. Mutations in MMP2, an antifibrotic metalloproteinase, may result in scleroderma-like skin thickening (135). Patients post-HCT with low levels of plasma MMP-2 were more likely to develop sclerotic cGvHD (136). Narrowband ultraviolet-B light therapy, which is known to increase the level of dermal MMP-2 (137), has successfully been used to treat sclerotic cGvHD (138).

Mutations in fibrillin-1 cause stiff skin syndrome (SSS), an autosomal dominant congenital form of scleroderma (139). These mutations all localize to the domain in fibrillin-1 that harbours a motif needed to mediate cell-matric interactions by binding cell-surface integrins. Aggressive skin fibrosis in mouse lines harbouring analogous mutations was prevented by integrin-modulating therapies and reversed by antagonism of TGF-β (140). Perhaps there is an role for integrin inhibition in the prophylaxis or treatment of cGvHD analogous to the use of

natalizumab (monoclonal antibody against α4-integrin) and vedolizumab (α4β7 inhibitor) in the treatment of steroid refractory aGvHD of the gut (141, 142).

CONCLUDING REMARKS

Immune disorders due to single gene defects offer invaluable insights into understanding the immune dysregulation that occurs during all three phases of cGvHD development. One of the issues that clinicians continue to struggle with is the timing of interventions either as prophylaxis or treatment and the ideal therapy or combination of therapies depending on the specific clinical cGvHD phenotype. It is clear that by the time many of the clinical manifestations of cGvHD, in particular fibrotic and sclerotic changes, are evident many of the therapies targeting earlier phases of inflammation may be ineffective. This only reinforces the urgent need to develop predictive and prognostic biomarkers that properly identify earlier stages of the disease where interventions may be more effective.

It is clear the cGvHD is a heterogeneous disease with multiple pathogenic pathways operating simultaneously and superior treatments will only emerge from an improved understanding of disease mechanisms.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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