IMMUNE PROFILE AFTER AUTOLOGOUS HEMATOPOIETIC STEM CELL TRANSPLANTATION FOR AUTOIMMUNE DISEASES: WHERE DO WE STAND?

EDITED BY: Kelen Cristina Ribeiro Malmegrim, Maria Carolina Oliveira, Antoine Toubert and Dominique Farge-Bancel

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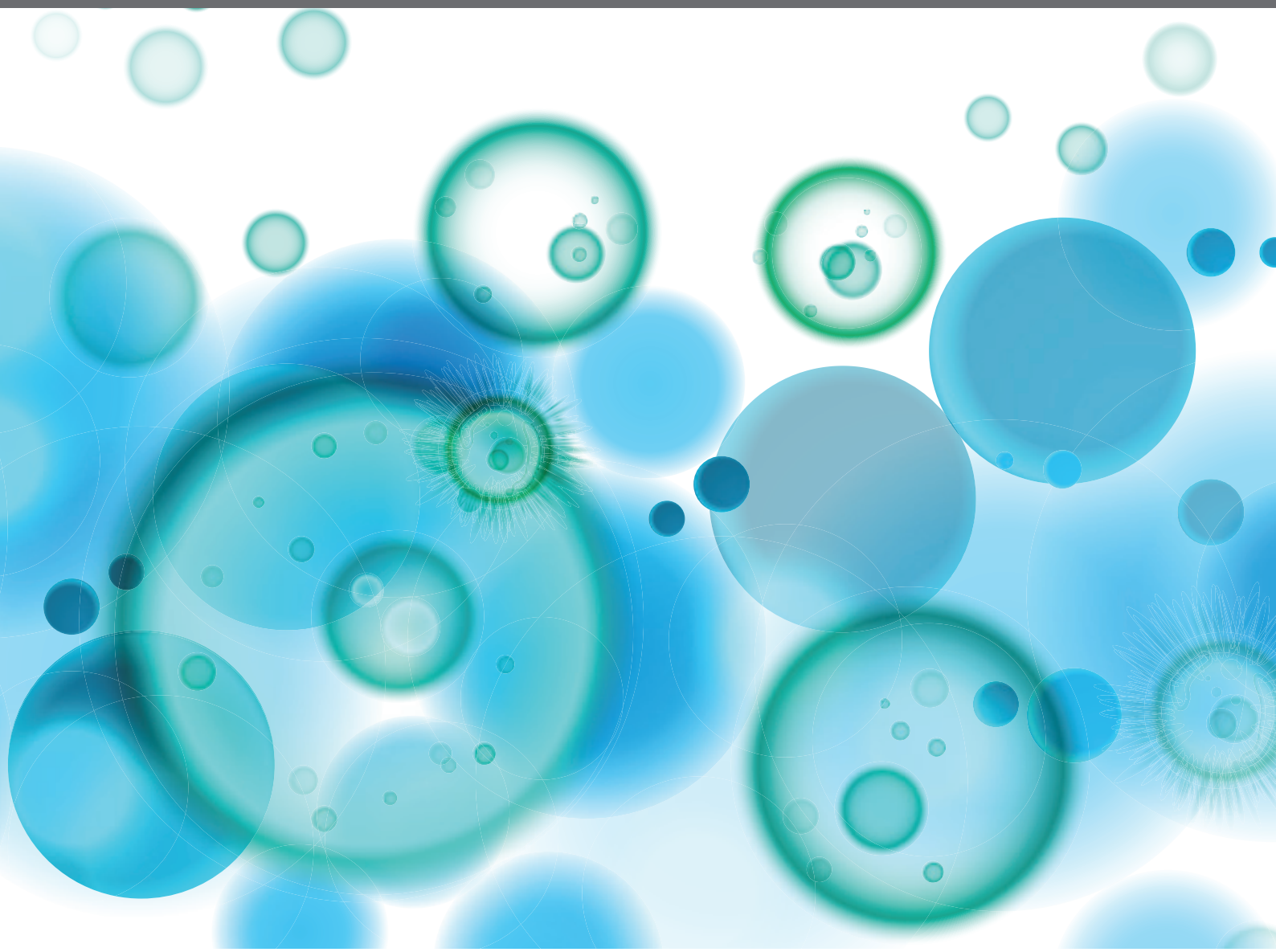

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IMMUNE PROFILE AFTER AUTOLOGOUS HEMATOPOIETIC STEM CELL TRANSPLANTATION FOR AUTOIMMUNE DISEASES: WHERE DO WE STAND?

Topic Editors:
Kelen Cristina Ribeiro Malmegrim, University of São Paulo, Brazil
Maria Carolina Oliveira, University of São Paulo, Brazil
Antoine Toubert, Paris Diderot University, France
Dominique Farge-Bancel, Assistance Publique Hopitaux De Paris (AP-HP), France

Autologous hematopoietic stem cell transplantation (AHSCT) was proposed 20 years ago as an alternative therapeutic approach for patients with severe and refractory autoimmune diseases. Since then, this innovative therapy has been successfully used to treat several autoimmune diseases, such as neurological disorders (Multiple Sclerosis), connective tissue diseases (Systemic Sclerosis and Systemic Lupus Erythematous), gastrointestinal inflammatory diseases (Crohn's Disease) and others (Juvenile Arthritis, Type 1 Diabetes, Vasculitis).

Experience in phase I-II and III clinical trials over the years has led to increased safety and efficacy of this procedure. Recent studies have demonstrated superior therapeutic efficacy of AHSCT versus conventional therapies, showing that transplantation can induce long-term disease remission in the absence of any further immunosuppressive treatment. Immune monitoring studies have showed that AHSCT is able to reduce the inflammatory milieu, to reset the immune balance and to promote the generation of a new auto-tolerant immune repertoire. However, some patients fail to remain in remission, and disease is re-activated after some time post-transplantation. This scenario indicates that further immunological interventions may be still required to improve AHSCT efficacy. Clear understanding of the operating immune mechanisms that contribute to specific clinical outcomes is vital to enable improvement of AHSCT protocols for autoimmune diseases.

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EDITORIAL
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Editorial: Immune Profile After Autologous Hematopoietic Stem Cell Transplantation for Autoimmune Diseases: Where Do We Stand?

Kelen Cristina Ribeiro Malmegrim[1,2], *Antoine Toubert*[3,4,5], *Dominique Farge*[6,7,8] *and Maria Carolina Oliveira*[2,9]*

[1] Department of Clinical Analysis, Toxicology and Food Science, School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Ribeirão Preto, Brazil, [2] Center for Cell-Based Therapy, Regional Hemotherapy Center of Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil, [3] Université de Paris, Institut de Recherche Saint Louis, EMiLy, Paris, France, [4] INSERM UMRS 1160, Microenvironment, Lymphocyte Development and Homing, Paris, France, [5] Laboratoire d'Immunologie et d'Histocompatibilité, AP-HP, Hopital Saint-Louis, Paris, France, [6] Unité de Médecine Interne, Maladies Auto-immunes et Pathologie Vasculaire (UF 04), Hôpital St-Louis, AP-HP, Paris, France, [7] Université de Paris, IRSL, Recherche Clinique Appliquée à l'Hématologie, Paris, France, [8] Department of Medicine, McGill University, Montreal, QC, Canada, [9] Division of Rheumatology, Allergy, Immunology and Immunotherapy, Department of Internal Medicine, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil

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Edited by:
Aurore Saudemont, GlaxoSmithKline, United Kingdom

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Philippe Saas, INSERM U1098 Interactions Hôte-Greffon-Tumeur and Ingénierie Cellulaire et Génique, France
Luca Castagna, Humanitas Research Hospital, Italy

***Correspondence:**
Maria Carolina Oliveira
mcarolor@usp.br

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Keywords: hematopoietic stem cell transplantation, autoimmune diseases, immune reconstitution, immune monitoring, cell therapy

Editorial on the Research Topic

Immune Profile After Autologous Hematopoietic Stem Cell Transplantation for Autoimmune Diseases: Where Do We Stand?

Autologous hematopoietic stem cell transplantation (AHSCT) induces long-term remission in autoimmune diseases (AD) without further use of immunosuppression (1, 2). Recently, randomized trials have proven greater efficacy of AHSCT when compared to conventional therapies for multiple and systemic sclerosis (3–6). However, despite the overall positive outcomes, subgroups of patients reactivate the AD after AHSCT due to reasons not yet completely understood, indicating that additional specific immunological interventions may still be required to improve or sustain therapeutic efficacy of AHSCT.

This Frontiers Research Topic, combines reviews, opinions, and original research from the most active researchers in the field of AHSCT for AD. Here, clinical outcomes and immune mechanisms of AHSCT, as well as insights for future studies are presented in the setting of different AD.

Different concepts, from basic knowledge to translational medicine, are integrated in two Perspective articles. Malmegrim et al. critically review the current knowledge about the operating immune mechanisms of AHSCT for AD, and propose strategies for future immune monitoring studies and biobanking. Harris et al. present data from a trial on AHSCT for poor prognosis multiple sclerosis to illustrate post-transplantation immune reconstitution and discuss experimental challenges and strategies to identify biomarkers of clinical response to AHSCT.

Two review articles address mechanistic effects of AHSCT. Massey et al. describe the pathogenesis of multiple sclerosis (MS) and how AHSCT restores immunological balance and, therefore, tolerance. The authors revise most of the available clinical trials on HSCT for MS and discuss aspects such as the lymphopenia-induced proliferation (LIP) that takes place early after transplantation and how it correlates with the later thymic rebound and T-cell diversification. Pockley et al. also describe the dynamics of immune reconstitution that follows AHSCT in AD

patients and how it can be extrapolated to Crohn's disease patients. Post-transplantation evidence of immune rejuvenation, with thymic rebound and improvement of regulatory mechanisms are shown in Crohn's disease patients, as well as increase in T-cell receptor (TCR) repertoire diversity in mucosal biopsies. The authors also discuss both established and potential effects of AHSCT on the innate immune system, which plays an important role in inflammatory bowel disease pathogenesis, and how innate cells may contribute to the high rate of Crohn's disease reactivations after transplantation.

Del Papa and Pignataro provide a very detailed and updated review on the mechanisms associated with vascular damage and repair in systemic sclerosis patients. The roles of mature endothelial cells and of endothelial progenitor cells (EPCs) are thoroughly described in the context of a disease characterized by diffuse microvasculopathy and endothelial damage. The authors discuss future EPC-based approaches, using either direct cell transplantation or pharmacological stimuli, aiming to promote endothelial repair. This important overview helps us understand possible pathogenic targets for future strategies involving cell therapy in systemic sclerosis.

Couri et al. and van Megen et al. contribute with their opinions on AHSCT for insulin-dependent diabetes mellitus (T1D). Although very consistent and evidence-supported, the opinions of these two groups differ slightly. The former discuss how AHSCT is able to promote temporary, but meaningful pancreatic beta cell preservation, and thus improve glycemic control. However, since the beneficial effects of AHSCT are transient, more intense immunosuppressive strategies may be warranted in future studies, possibly combined with cell-replacement approaches. On the less full side of the glass, van Megen et al. describe AHSCT as a still controversial issue. Ethical concerns such as transplant-associated risk, long-term toxicity, and enrolment of children are discussed, as well as the lack of substantial evidence for irrefutably beneficial results, despite the appeal of reducing long-term complications of T1D. The authors discuss the available information of immunological analyses before and after AHSCT and suggest a more personalized approach to enroll patients for transplantation.

Autologous HSCT for multiple sclerosis leads to abrogation of new clinical relapses and brain lesions. In parallel, there is selective reduction of Th17, but not Th1, cell population and activity. Darlington et al. demonstrate an increase in the kinetics of natural killer (NK) cell reconstitution, when compared to CD4+ T cells, in MS patients post-AHSCT. The resulting increased NK cell:CD4+ T cell ratio correlated with a decrease in Th17 responses. The authors suggest that rapid reconstitution of NK cells following AHSCT contribute to the suppression of Th17 re-emergence, highlighting the importance of NK cells in the post-transplantation setting.

Ben Nasr et al. report that *ex vivo* modulation of hematopoietic stem and progenitor cells with prostaglandins (PGs) increases their immunoregulatory properties by upregulating expression of the immune checkpoint-signaling molecule PD-L1. When tested in murine and human *in vitro* autoimmune assays, PG-modulated progenitor cells were shown to diminish the autoreactive T cell response. The use of PG-modulated progenitors may thus become an attractive and novel treatment for T1D, thus circumventing immunosuppression-related toxicity.

Two other contributions demonstrate that the programmed death-1 (PD-1) signaling pathway may control autoimmunity. T cells in a lymphopenic environment undergo LIP to fill the available "niche" as defined by (self) peptide:MHC (pMHC) complexes with which the TCRs interact and receive at least a weak "tonic" signal to promote T cell survival. The numbers of cells and diversity of the peripheral T-cell pool are controlled by intra and interclonal competition for resources, which together define T-cell "space." Ellestad et al. found that PD-1 controls pMHC-dependent tonic signals to T cells, independently of IL-7 signaling, at least when available pMHC is abundant. These data suggest that therapies aimed at reducing TCR signaling during the early phases of T cell reconstitution may be more effective than approaches that aim to limit homeostatic cytokine-mediated signals to T cells.

Finally, Ellestad et al. determine that PD-1 is upregulated on CD4+ T-cells undergoing the natural LIP characteristic of the neonatal period. Newly generated T cells lacking PD-1 maintained an enhanced autoimmune potential even after residence in a lymphoreplete periphery, emphasizing the importance of PD-1 in the establishment of peripheral tolerance. Neither Fas nor perforin-dependent killing mechanisms were required for autoimmunity, while host MHC-II expression was critical, suggesting that LIP-driven autoimmunity in the absence of PD-1 may primarily result from a CD4+ T-cell-mediated systemic cytokinemia. Their data suggest that even in a lymphoreplete adult host, peripheral newly generated T cells retain a potential for LIP-driven autoimmunity in the absence of PD-1.

Collectively, the articles from this Research Topic contribute to increase the knowledge of the field. Important aspects about the modulation of the immune system in autoimmune diseases are discussed, from cellular to more molecular approaches, and from bedside to bench, which in the future should reverse back to the bedside.

AUTHOR CONTRIBUTIONS

KM and MO wrote the initial draft of the editorial. AT and DF revised and approved the manuscript.

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ORIGINAL RESEARCH
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PD-1 Controls Tonic Signaling and Lymphopenia-Induced Proliferation of T Lymphocytes

Kristofor K. Ellestad[1,2], Jiaxin Lin[2,3], Louis Boon[4] and Colin C. Anderson[1,2,3,5]**

[1] Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, AB, Canada, [2] Alberta Diabetes Institute, University of Alberta, Edmonton, AB, Canada, [3] Department of Surgery, University of Alberta, Edmonton, AB, Canada, [4] Bioceros B.V., Utrecht, Netherlands, [5] Alberta Transplant Institute, University of Alberta, Edmonton, AB, Canada

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Edited by:
Joanna Davies,
San Diego Biomedical Research Institute,
United States

Reviewed by:
Anne Cooke,
University of Cambridge,
United Kingdom
Raymond John Steptoe,
The University of Queensland,
Australia
Deborah J. Fowell,
University of Rochester,
United States

***Correspondence:**
Kristofor K. Ellestad
ellestad@ualberta.ca;
Colin C. Anderson
colinand@ualberta.ca

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Recovery of the T lymphocyte compartment within a lymphopenic host by lymphopenia-induced proliferation (LIP) is regulated by inter- and intraclonal competition for limited resources, including homeostatic cytokines and peptide:MHC (pMHC) complexes with which the TCR can interact at least weakly to yield a tonic signal. Importantly, the process of LIP can synergize with other factors that promote T cell activation to drive inflammatory disease. While reconstitution of the lymphoid compartment of immune deficient Rag$^{-/-}$ mice by transfer of wild-type hematopoietic stem cells (HSC) does not generally result in an overt disease phenotype, transfer of HSC deficient in expression of the co-inhibitory molecule PD-1 results in severe systemic autoimmunity driven by newly generated T cells that emerge from the thymus into the periphery and undergo LIP. Importantly, autoimmunity does not appear to depend on a response to exogenous (i.e., gut flora-derived) antigens. PD-1 is well known to be upregulated during T cell activation in response to cognate antigens, but it is unclear whether PD-1 has a role in controlling LIP of T cells in the absence of cognate antigen, i.e., in response to tonic pMHC. We examined whether PD-1 controls LIP of newly generated T cells by controlling the response to tonic pMHC or the homeostatic cytokine IL-7. We found that PD-1-deficient T cells have a proliferative advantage over WT T cells during LIP and this effect is MHC-II dependent and independent of IL-7Rα signaling. Furthermore, our data suggest that signals through IL-7Rα can be dispensable for LIP and may instead be of increased importance for T cell survival in conditions of high competition for limited pMHC (e.g., post-LIP, in a lymphoreplete host). We hypothesize that autoimmunity post-PD-1$^{-/-}$ HSC transplant is the result of an overzealous T cell response to normally tonic self-pMHC precipitated by the synergy of LIP and PD-1 deficiency. Furthermore, potentiation of TCR signals in response to normally tonic self-pMHC may contribute to the success of PD-1 blockade in cancer immunotherapy.

Keywords: tolerance, autoimmunity, co-stimulation, co-inhibition, lymphopenia, PD-1, mouse models, IL-7

INTRODUCTION

The number of cells in and diversity of the peripheral T lymphocyte pool is controlled by intra- and interclonal competition for resources, which together define T cell "space" (1). Such resources include homeostatic cytokines like IL-7 and IL-15, but also peptide:MHC (pMHC) complexes; often self-pMHC, with which the TCR can interact and receive at least a weak "tonic" signal to promote

T cell survival. In lymphopenic hosts, recovery of the T cell compartment occurs *via* a process known as lymphopenia-induced proliferation (LIP), where resources are in excess and T cells expand to fill the available niche. While the term lymphopenia lacks a precise quantitative character, one can consider the extent to which LIP can occur, or "LIP potential" of a host, as a ratio of the available resources to the number of competitors for those resources (2). Thus, provision of competition for a particular pMHC can reduce LIP potential for other T cells that recognize the same pMHC (3–6). Similarly, Treg, which may be viewed as "super"-competitors for pMHC, can inhibit LIP (5, 7–14). Positive or negative regulation of the strength of the TCR signal a lymphocyte receives in response to a given pMHC, for example by blocking/reducing co-inhibition (15–19) or co-stimulation (9), respectively, can modulate LIP potential. Finally, a neonatal host might be viewed to have a low LIP potential due to small anatomic size (low absolute quantities of resources) as well as the recently described presence of an innate lymphoid cell population that may act as a sink for IL-7 (20).

Importantly, LIP is strongly associated with acquisition of an effector-memory phenotype in T cells, including the ability to rapidly mediate effector function (21), and in the context of concurrent infections or deficiencies of co-inhibitory pathways, LIP can result in overzealous T cell responses and autoimmune disease (2, 7, 14, 17, 19, 22–30). This association between LIP and inappropriate inflammation may be rationalized by considering that the conditions present in lymphopenia—including reduced competition for access to a given T cell's cognate or tonic pMHC, along with high concentrations of IL-7 and IL-15 [which have been suggested to potentiate TCR signals (31, 32)]—may conspire to increase the frequency with which T cells can productively interact with pMHC and generate an abnormally strong TCR signal when they do so. Systemic activation of sufficient numbers of T cells may result in immune pathology.

Reconstitution of the lymphoid compartment of an otherwise normal C57BL/6 (B6) $Rag^{-/-}$ mouse with B6 hematopoietic stem cells (HSC) does not typically result in the development of apparent autoimmune disease (19, 33). In stark contrast, we recently demonstrated that reconstitution of adult $Rag^{-/-}$ mice with HSC deficient in the co-inhibitory molecule PD-1 results in a rapid, severe, and frequently lethal systemic autoimmune disease soon after the first newly generated T cells emerge from the thymus (19). Severe and rapid autoimmunity can also result from transfer of PD-$1^{-/-}$ thymocytes to $Rag^{-/-}$ recipients, but not established peripheral cells from adult PD-$1^{-/-}$ mice [note, on the B6 background, PD-$1^{-/-}$ mice develop only a mild lupus-like autoimmune disease upon aging (34)]. The newly generated T cell population may have a higher average affinity for self-pMHC compared to established cells that developed in a lymphoreplete adult environment or an environment with low LIP potential (e.g., during early seeding of the lymphoid compartment in the neonate) and thus underwent more normal peripheral tolerance mechanisms (deletion, anergy, conversion to pTreg). One hypothesis to explain disease in our model is that in the "three-strikes" scenario of high LIP potential, PD-1 deficiency, and a periphery seeded with newly generated T cells, a stochastically greater number of the T cells on the higher end of the self-pMHC affinity spectrum receive a strong enough signal to acquire effector function giving rise to the observed systemic cytokinemia (19). On the other hand, when PD-1 is sufficient, its restraint on the magnitude of the TCR signal (35–37) limits the frequency of such spurious activation preventing a pathological systemic effect. Although multiple co-inhibitory molecules other than PD-1 have been shown to control LIP (15–18) and our data suggest that PD-1 does as well (19, 33), whether they do so *in vivo* by controlling TCR signals mediated by tonic self-pMHC is unknown. Indeed, PD-1 is known to be upregulated on *bona fide* activated T cells and it is unclear whether the generally weak, tonic signals provided by interaction with self-pMHC can drive its expression. Co-inhibitor deficient cells might instead come to predominate in LIP due to control of responses to homeostatic cytokines or completely independent of external signals. Furthermore, because of the polyclonal repertoire generated post-HSC transplant in our disease model, we cannot rule out that the response might be directed to foreign pMHC [although depletion of gut microbiota in PD-$1^{-/-}$ HSC recipients does not protect from disease (33)] or pMHC derived from tissue-restricted antigens against which developing PD-$1^{-/-}$ T cells failed to be appropriately negatively selected in the thymus. Herein, we examined whether PD-1 is able to control the response to tonic pMHC using transfer of monoclonal PD-$1^{+/+}$ or PD-$1^{-/-}$ male antigen (HY)-specific Marilyn $CD4^+$ TCR transgenic T cells (38) to MHC-II sufficient and deficient [MHC Class II transactivator (CiiTA) knockout (39)] lymphopenic hosts lacking the male antigen HY. We also examined whether PD-1 modulates signaling through IL-7Rα during LIP. We found that PD-1 controls pMHC-dependent tonic signals to T cells, independent of IL-7 signaling. Furthermore, our data suggest that IL-7 is particularly important for controlling T cell homeostasis during situations of high competition for limited pMHC but is not essential for LIP when available pMHC is abundant.

MATERIALS AND METHODS

Mice

B6.129S7-*Rag1*^{tm1Mom}/J (Rag1$^{-/-}$, Rag$^{-/-}$), B6.Cg-*Foxp3*$^{tm2(EGFP)Tch}$/J (FoxP3^{EGFP}), B6.SJL-Ptprca Pepcb/BoyJ (CD45.1), and B6.129S2-*Ciita*^{tm1Ccum}/J (CiiTA$^{-/-}$) mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and then bred at the University of Alberta. C57BL/6-*Pdcd1*$^{-/-}$ (backcrossed 11 generations to C57BL/6) were originally generated by Prof. T. Honjo and colleagues (34). FoxP3^{EGFP} × *Pdcd1*$^{-/-}$ mice were generated by crossing the above FoxP3^{EGFP} and B6-*Pdcd1*$^{-/-}$ mice and are referred to and used in the present manuscript simply as PD-$1^{-/-}$. $Rag^{-/-}$ × CiiTA$^{-/-}$ mice were generated by crossing the above $Rag^{-/-}$ and CiiTA$^{-/-}$ mice. Marilyn $Rag2^{-/-}$ $CD4^+$ anti-IA^b-HY TCR transgenic mice (called Marilyn herein) were originally generated by Lantz and colleagues (38) and were originally obtained from the NIAID exchange program. Marilyn-CD45.1, referred to herein as Marilyn-WT, were generated by crossing the above Marilyn mice with CD45.1 × $Rag2^{-/-}$ mice (Taconic). Marilyn-PD-$1^{-/-}$ mice were generated by crossing the above Marilyn mice with PD-$1^{-/-}$ mice and were maintained as $Rag2^{-/-}$. All Marilyn mice used as donors were female. Animals were cared

for in accordance with the guidelines of the Canadian Council on Animal Care and housed under clean conventional housing conditions at the University of Alberta Health Sciences and Laboratory Animal Services facilities (HSLAS).

Cell Preparations and Adoptive Transfer Experiments

For experiments involving transfer of thymocytes, recipient NK cells were depleted [to avoid potential NK-mediated killing of the input cells (40–42)] by treatment on days −4, −1, and +2 with 0.3 mg per mouse of anti-NK1.1 (PK136) injected intraperitoneally. In order to study the role of PD-1 in controlling LIP of newly generated T cells, which would not have been exposed to the periphery where they may have undergone peripheral tuning mechanisms, we chose to transfer minimally manipulated whole unfractionated thymocytes and monitor their proliferation *in vivo*. In mixed thymocyte transfer experiments, WT (CD45.1$^+$) and PD-1$^{-/-}$ (CD45.1−) Marilyn unfractionated thymocytes were counted and mixed in equal proportions prior to labeling and transfer. Labeling of mixed thymocyte populations with Celltrace violet (CTV, ThermoFisher) was performed using 5 μM CTV according to the manufacturer's protocols except with a final cell concentration in the labeling reaction of up to ~40×10^6 cells per mL. For subsequent *in vivo* use cells were washed once with PBS and resuspended in PBS on ice for immediate intravenous tail vein injection into recipient mice. Mixed cells for infusion into mice were analyzed by flow cytometry to determine the starting (day 0) ratio of PD-1$^{-/-}$: WT T cells calculated as %PD-1$^{-/-}$ T cells divided by % WT T cells. Initial ratios varied from 0.57 to 1.09. To facilitate combining data from multiple experiments, initial ratios were normalized to 1 by multiplying by a scaling factor which was applied to all subsequent PD-1$^{-/-}$:WT T cell ratio measurements within a given experiment. Anti-IL-7Rα treated mixed thymocyte recipients received twice weekly intraperitoneal injections of antibody (as described below) for the full course of the experiment.

For *in vitro* restimulation assays, cells were resuspended in E-DMEM (high glucose DMEM + 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 50 μM 2-mercaptoethanol, 10% FBS) at 2×10^6 cells/mL and seeded 200 μL per well into a 96 well, round bottom plate. A final concentration of 16 nM of phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) and 1.4 μM ionomycin (Sigma-Aldrich) were added for 2 h, at which time brefeldin A (eBioscience, 3 μg/mL) and monensin (eBioscience, 2 μM final) were added for a further 2 h prior to surface staining, fixation, intracellular staining, and analysis.

Definition of Disease and Data Analysis

Macroscopic signs of disease in thymocyte recipients included cachexia/weight loss (>15%), kyphosis (hunched appearance), ruffled fur, dermatitis, ocular lesions, and diarrhea. Recipient mice were no longer considered disease free when two or more of the above symptoms were evident, or if mice lost ≥20% body weight. Kaplan–Meier survival curve analysis of disease onset/incidence was performed using Graphpad Prism v5.0 software. Probability values reported for survival curve comparisons were calculated using the Mantel-Cox method. For thymocyte experiments, calculation of weight loss for disease determination was performed relative to weights at day 0 or day 1 relative to cell transfer. Unless otherwise indicated, animals were used between 6 and 20 weeks of age.

Antibodies and Flow Cytometry

For flow cytometric staining, fluorophore-labeled antibodies against the following markers were obtained from eBioscience (San Diego, CA, USA) unless otherwise indicated: CD4 (RM4-5), TCRβ (H57-597), CD8 (53-6.7), PD-1 (J43), CD5 (53-7.3), IL-7Rα/CD127 (A7R34), IFN-γ (XMG1.2), Bcl2 (10C4). Antibodies were used at manufacturer's recommended concentrations. Flow cytometric staining always used an Fc block cocktail to block nonspecific staining. Fc block cocktail consisted of 3 mL each of normal mouse, rat, and hamster serum, with addition of 0.3 mg of anti-CD16/32 antibody (clone 2.4g2, BioXCell). For intracellular staining, cells were fixed and permeabilized using the eBioscience FoxP3 Fixation/permeabilization buffer kit according to supplied protocols. Standard flow cytometric analysis was performed using a BD LSR II instrument. Flow cytometric data analysis was performed using FlowJo (Treestar software, Portland, OR, USA).

Anti-IL-7Rα treatment *in vivo* was carried out by biweekly intraperitoneal injection of 0.5 mg of anti-IL-7Rα clone A7R34 (BioXcell for polyclonal PD-1$^{-/-}$ thymocyte disease model experiments; or generated by us for mixed Marilyn experiments) or Rat IgG2a isotype control (2A3, BioXcell or generated by us) beginning on the day of cell transfer.

Statistical Analyses

For statistical analyses, we used Graphpad Prism software. Unless otherwise noted Student's *t*-test was used for comparisons. In the case of unequal variances, *t*-test was performed using Welch's correction. For comparisons of multiple groups, one-way ANOVA with Tukey's multiple comparison test was used.

RESULTS

PD-1 Controls LIP in Response to Tonic Self-pMHC-II Independent of IL-7 Signals

We have previously demonstrated that transfer of a mixture of Treg-depleted polyclonal WT and PD-1$^{-/-}$ CD4$^+$ T cells purified from the thymocyte or splenocyte population (including CD62L^{hi} selected cells) into lymphopenic Rag$^{-/-}$ hosts resulted in predominance of the PD-1$^{-/-}$ population in the periphery during LIP (33). In the same study, we also demonstrated that the proportion of WT and PD-1$^{-/-}$ cells could be equalized by treatment of hosts with anti-PD-L1, releasing the WT population from cell-intrinsic PD-1-dependent inhibition. These data indicated that the effect on population size was mediated through the PD-L1:PD-1 interaction. In order to examine whether PD-1 controls LIP in response to tonic self-pMHC signals in the absence of potential responses to conventional agonist cognate antigens by PD-1$^{-/-}$ cells within a polyclonal repertoire, we employed Marilyn male

antigen-specific CD4$^+$ TCR transgenic CD45.1 (CD45.1$^+$, "Marilyn-WT") mice as well as Marilyn PD-1$^{-/-}$ mice (CD45.2$^+$, Marilyn-PD-1$^{-/-}$) (43). We also generated Rag$^{-/-}$ and MHC Class II transactivator deficient (CiiTA$^{-/-}$) mice, which are largely MHC-II deficient (39) (CiiTA$^{-/-}$ × Rag$^{-/-}$ mice). We transferred a mixture (40×10^6 cells) of Marilyn-WT and Marilyn-PD-1$^{-/-}$ thymocytes labeled with CTV proliferation dye into NK-depleted Rag$^{-/-}$ females (tonic signals only) or males (systemic cognate HY antigen present) and CiiTA$^{-/-}$ × Rag$^{-/-}$ females or males, with or without biweekly anti-IL-7Rα treatment and monitored their relative proliferation and abundance over time (**Figure 1A**). NK depletion was carried out to maximize input cell survival as even syngeneic cells can be targets of NK cell killing (40–42).

Early after transfer (day 3), only barely detectable levels of proliferation had occurred within female Rag$^{-/-}$ recipients with none in the female Rag$^{-/-}$ anti-IL-7Rα-treated group (**Figure 1B**; Figure S1 in Supplementary Material), and no difference between the WT and PD-1$^{-/-}$ cells was apparent nor was there any change in the ratio of PD-1$^{-/-}$ to WT T cells in these recipients relative to the initial seeding ratio, regardless of anti-IL-7Rα treatment (**Figure 1D**). In contrast, in the presence of systemic cognate antigen all of the cells within the Rag$^{-/-}$ males, regardless of ant-IL-7Rα treatment, had proliferated extensively and had already almost completely diluted out their CTV dye (**Figure 1B**; Figure S1 in Supplementary Material). Proliferation had also clearly occurred albeit to a reduced extent within CiiTA$^{-/-}$ × Rag$^{-/-}$ male recipients, which may be due to small numbers of MHC-II sufficient thymic DC that were transferred along with the thymocytes or the known residual MHC-II expression in these knockouts (39, 44).

By day 5, PD-1$^{-/-}$ T cells had undergone significantly more proliferation than WT cells in Rag$^{-/-}$ female recipients (**Figure 1B**; Figure S1 in Supplementary Material) and the ratio of PD-1$^{-/-}$:WT cells started to increase (**Figure 1D**). Furthermore, in these recipients, we detected an MHC-II-dependent upregulation of PD-1 expression within the WT cell population at this time point among the extensively proliferated cells (**Figure 2A**), indicating that tonic pMHC signals are sufficient to upregulate PD-1. We also explored expression of another co-inhibitory receptor, CD5, a commonly used marker of TCR affinity and that has co-inhibitory function (45–47), and found it was modulated during LIP in response to tonic pMHC signals. Upon extensive proliferation, CD5 became up or downregulated on a substantial proportion of WT cells, while PD-1$^{-/-}$ cells had less downregulation of CD5 (**Figure 2B**), suggesting CD5 expression may help compensate for the lack of PD-1.

By 7 days post-cell transfer, the majority of cells within the Rag$^{-/-}$ female recipients had proliferated extensively and diluted their CTV labeling beyond detection limits, although significantly more proliferation had occurred in the PD-1$^{-/-}$ cell population in these recipients as judged by comparison of CTV MFI (**Figures 1B,C**; Figure S1 in Supplementary Material). Furthermore, the ratio of PD-1$^{-/-}$:WT cells in these animals increased approximately twofold relative to the input cell ratio which was significantly different from the female CiiTA$^{-/-}$ × Rag$^{-/-}$ recipients (2.03 ± 0.05 versus 1.18 ± 0.10, $p < 0.0001$, **Figure 1D**) in which the PD-1:WT ratio did not change and also in which the majority of the T cell population still had not proliferated (**Figures 1B,C**; Figure S1 in Supplementary Material). Importantly, this alteration in ratio was not affected by anti-IL-7Rα treatment of the hosts. During LIP 7 days post-mixed thymocyte transfer, the percentage of all cells that were TCRβ$^+$CD4$^+$ in blood of Rag$^{-/-}$ female recipients was not significantly affected by anti-IL-7Rα treatment; although, we did note a trend to reduced CD4 T cells with anti-IL-7Rα in the one experiment in which CD4 numbers were already quite high by day 7 (**Figure 2D**, green symbols). In contrast, in MHC-II-deficient female CiiTA × Rag$^{-/-}$ recipients, blockade of anti-IL-7Rα significantly reduced the frequency of CD4$^+$ T cells in the blood. Examination of the CTV dilution histograms suggested that anti-IL-7Rα treatment in the MHC-II sufficient female Rag$^{-/-}$ recipients appeared to primarily decrease the size of the unproliferated cell population (Figure S1 in Supplementary Material). In the presence of HY antigen in male Rag$^{-/-}$ recipients, by day 7 the ratio of PD-1$^{-/-}$:WT cells had increased more than sixfold relative to the input cell proportions, and this was also not affected by anti-IL-7Rα treatment (**Figure 1D**). Interestingly, the ratio of PD-1$^{-/-}$:WT cells did not change by day 7 in CiiTA$^{-/-}$ × Rag$^{-/-}$ male recipients despite robust expansion of input populations in these recipients; expansion that was potentially caused by residual MHC class II known to exist in the CiiTA knockout (39, 44). Male Rag$^{-/-}$ recipients had a significantly higher peripheral blood T cell abundance compared to females which was not significantly decreased by anti-IL-7Rα treatment (**Figure 2D**).

The increased proportion of PD-1$^{-/-}$ cells during tonic pMHC-stimulated LIP could have been due to increased proliferation or increased survival of proliferating cells. However, PD-1$^{-/-}$ cells did not have an increase of the pro-survival molecule Bcl2 (**Figure 2C**). In addition, Bcl2 expression, known to be stimulated by IL-7 signals (48), generally appeared to be depressed in response to anti-IL-7Rα treatment (**Figure 2C**) despite anti-IL-7Rα's lack of effect on both LIP (**Figure 1B**) and CD4 T cell numbers in MHC-II positive recipients (**Figure 2D**). However, IL-7Rα blockade together with a lack of MHC-II led to the strongest decrease in Bcl2 expression in both WT and PD-1$^{-/-}$ cells (**Figure 2C**), and this was associated with reduced CD4 T cell numbers (**Figure 2D**). Thus, IL-7 and Bcl2 appear important for CD4 T cells in the absence of tonic pMHC signals but not during tonic pMHC-stimulated LIP. Together, these data indicate that PD-1 controls signaling in response to tonic as well as cognate pMHC-II in a cell-intrinsic manner, which is independent of effects on IL-7 signaling.

Thus far, we have shown that PD-1 controls LIP to tonic pMHC signals. Whether tonic pMHC signals driven effector function (e.g., cytokine production) in addition to LIP is controlled by PD-1 is not known. We found no difference in the proportion of WT versus PD-1$^{-/-}$ T cells producing IFN-γ in response to *in vitro* restimulation at the d7 time point in the MHC-II sufficient female hosts, and in the absence of MHC-II IFN-γ was essentially undetectable (**Figure 2E**). Thus, while tonic pMHC signals are able to stimulate IFN-γ production, PD-1 appears important for controlling proliferation but not this effector activity triggered by tonic pMHC signals in LIP. However, it remains possible that PD-1 controls other effector functions of CD4 T cells during LIP.

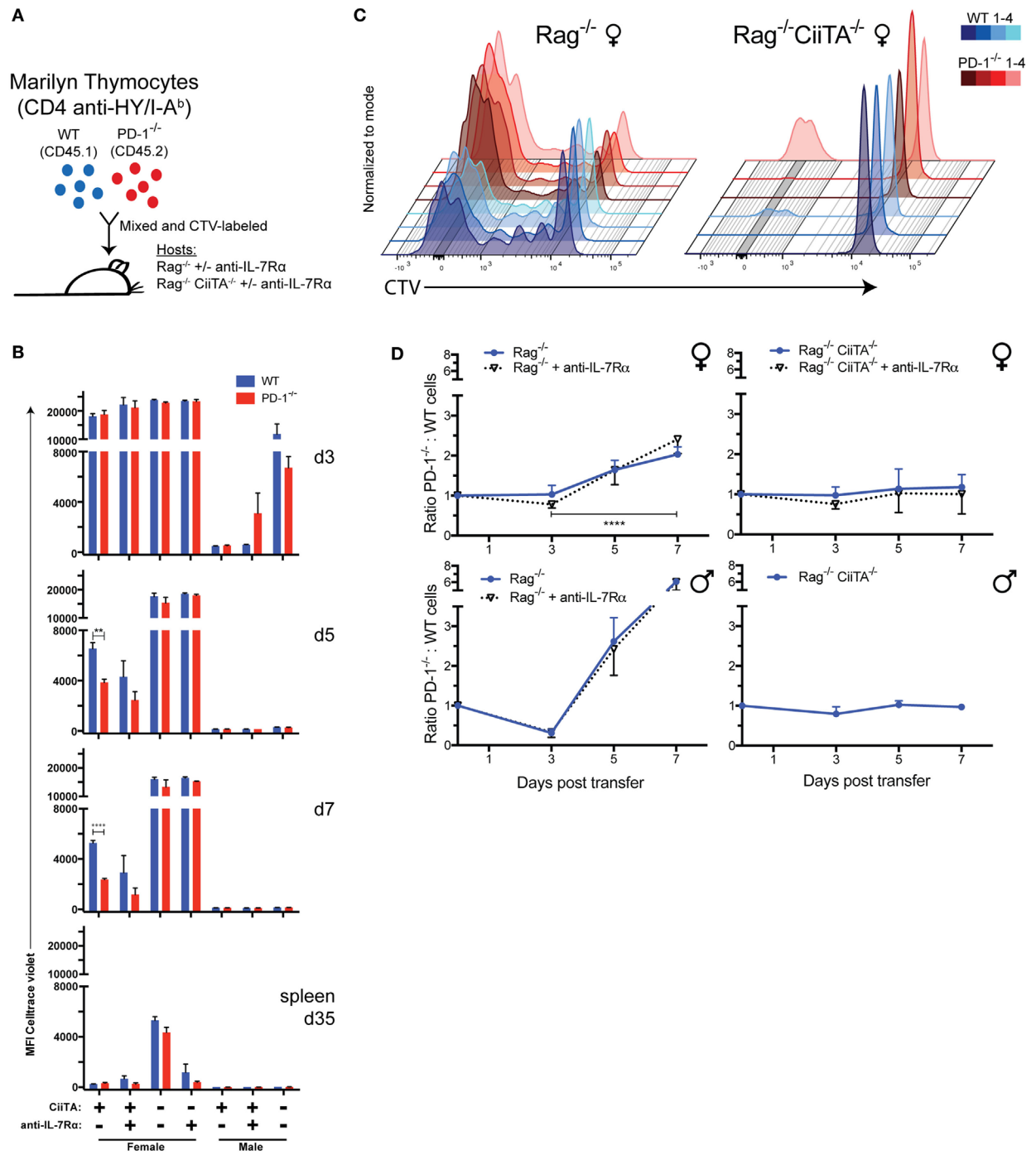

FIGURE 1 | Monoclonal CD4+ HY-specific PD-1$^{-/-}$ T cells out-proliferate and outnumber WT cells during LIP in response to tonic or cognate pMHC-II but independent of IL-7 signals. **(A)** 40 × 10^6 Celltrace violet (CTV)-labeled mixed Marilyn-WT (CD45.1+) and PD-1$^{-/-}$ (CD45.2+) thymocytes were transferred i.v. to Rag$^{-/-}$ or CiiTA$^{-/-}$ × Rag$^{-/-}$ male or female hosts with or without anti-IL-7Rα treatment. **(B)** Mean fluorescence intensity (MFI) of CTV in TCRβ+ CD4+ WT or PD-1$^{-/-}$ cells from blood collected at 3, 5, and 7 days and spleen at 35 days post-transfer (p.t.) ± SEM, n = 3–4 per group. **$p < 0.01$, ****$p < 0.0001$, Student's t-test. **(C)** Overlaid flow cytometry plots of CTV fluorescence at 7 days p.t. of mixed WT and PD-1$^{-/-}$ Marilyn thymocytes to Rag$^{-/-}$ and CiiTA$^{-/-}$ × Rag$^{-/-}$ female hosts. Four recipients from one experiment are depicted – with color darkness indicating recipient identity and blue and red hues representing WT and PD-1$^{-/-}$ cells, respectively, in a given recipient. **(D)** Ratio of PD-1$^{-/-}$ to WT CD4+ T cells in blood among indicated recipients at day 3–7 p.t. ± SEM. Data presented are combined from multiple independent experiments as follows: female Rag$^{-/-}$ (four independent experiments, n = 12, 16, 13 per group at days 3, 5, 7 respectively), female Rag$^{-/-}$ + anti-IL-7Rα (two independent experiments, n = 4, 8, 8 at days 3, 5, 7), female CiiTA$^{-/-}$ × Rag$^{-/-}$ (four independent experiments, n = 9, 11, 10 at days 3, 5, 7), female CiiTA$^{-/-}$ × Rag$^{-/-}$ + anti-IL-7Rα (two independent experiments, n = 3, 5, 5 at days 3, 5, 7), male Rag$^{-/-}$ (two independent experiments, n = 8), male Rag$^{-/-}$ + anti-IL-7Rα (two independent experiments, n = 7), male CiiTA$^{-/-}$ × Rag$^{-/-}$ (two independent experiments, n = 8, 7, 8 at days 3, 5, 7). ****$p < 0.0001$, Student's t-test, Rag$^{-/-}$ day 3 versus day 7. The starting ratio was normalized and set to a value of one with subsequent ratio measurements scaled accordingly.

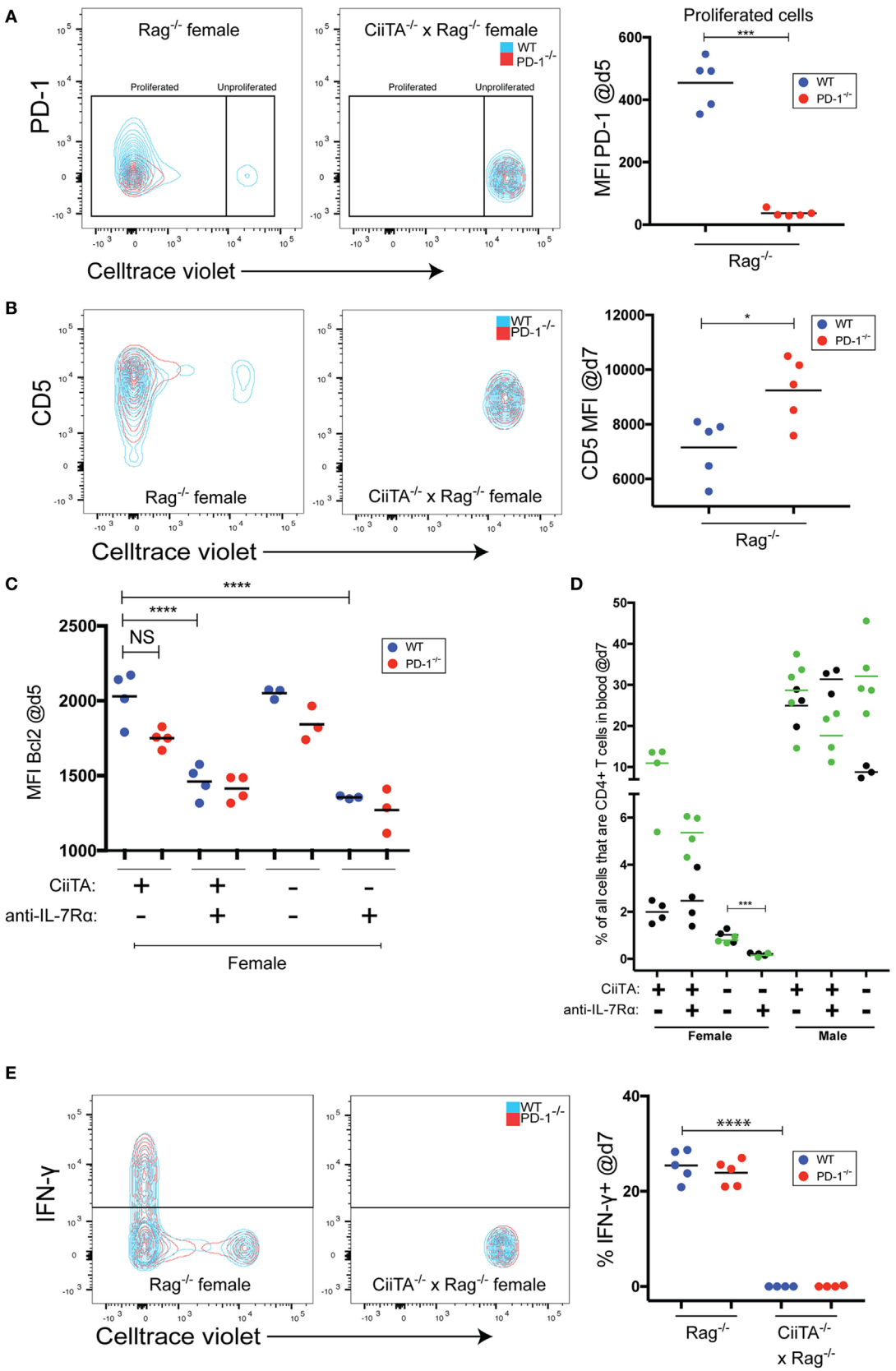

FIGURE 2 | Continued

FIGURE 2 | Continued
Tonic peptide:MHC signals control expression of co-inhibitory receptors, drive IFNγ expression, and limit the need for IL-7 signals during LIP. **(A)** (Left and center panels) Representative flow cytometry overlaid contour plots showing PD-1 expression versus. Celltrace violet (CTV) staining within the indicated recipients of 40×10^6 mixed Marilyn-WT and PD-$1^{-/-}$ thymocytes at day 5 p.t. in blood. (Right panel) Mean fluorescence intensity (MFI) of PD-1 staining within the proliferated WT or PD-$1^{-/-}$ cell populations from individual recipients. No significant difference in PD-1 expression was noted in unproliferated WT or PD-$1^{-/-}$ cells in CiiTA$^{-/-}$ × Rag$^{-/-}$ hosts. ***$p < 0.001$, Student's *t*-test. **(B)** (Left and center panels) Representative overlaid contour plots of CD5 expression versus CTV staining in the indicated recipients of mixed Marilyn-WT and PD-$1^{-/-}$ cells at day 7 p.t. in blood. (Right panel) MFI of CD5 staining within WT and PD-$1^{-/-}$ populations from individual recipients. *$p < 0.05$, Student's *t*-test. **(C)** Mean fluorescence intensity of Bcl2 staining in WT and PD-$1^{-/-}$ CD4$^+$ T cell populations in the blood at day 5 p.t. within the indicated recipients. ****$p < 0.0001$, One-way ANOVA with Tukey's Multiple comparison test. Data in **(C)** depicts individual biological replicates from one experiment. **(D)** Comparison of the % of all cells that were CD4$^+$ T cells in blood of indicated recipient groups at day 7 p.t. ***$p < 0.001$, Student's *t*-test. Two independent experiments are depicted with black (experiment 1) and green (experiment 2) symbols and means. **(E)** (Left and center panels) Representative overlaid contour plots of IFN-γ expression versus. CTV staining in indicated recipients at day 7 p.t. in *in vitro* restimulated splenocytes. (Right panel) % IFN-γ$^+$ cells within WT and PD-$1^{-/-}$ CD4$^+$ T cell populations from individual recipients. ****$p < 0.0001$, Student's *t*-test. For panels **(A,B,E)**, data presented are from individual recipients from one experiment that is representative of two independent experiments.

IL-7 Receptor Blockade Decreases T Cell Compartment Size in Conditions of Limiting pMHC

The findings that IL-7 signals appeared not to be important early during pMHC-stimulated LIP but were important when pMHC was limited (CiiTA deficient recipients), suggested the possibility that IL-7 becomes important when there is more competition for pMHC, such as in the post-LIP period. In splenocyte populations harvested at 5 weeks post-Marilyn thymocyte transfer, CTV labeling was virtually undetectable in all cells within the Rag$^{-/-}$ female recipients (**Figure 1B**; Figure S1 in Supplementary Material). We reasoned that LIP was largely complete by this time given that we saw no further increase in absolute splenic CD4$^+$ T cell numbers in mice harvested much later at day 76 post transfer (p.t.; data not shown). We, therefore, examined the phenotype and number of Marilyn cells late after cell transfer once T cell numbers had increased post-LIP and determined the effect of IL-7Rα blockade. A trend toward higher CD44 expression in PD-$1^{-/-}$ versus WT cells was noted at 5 weeks post-transfer, which reached statistical significance in the anti-IL-7Rα-treated MHC-II sufficient females (**Figure 3A**). Importantly IL-7Rα blockade itself did not significantly decrease CD44 expression among T cells in female or male recipients, but MHC-II deficiency in females significantly reduced memory phenotype acquisition (**Figure 3A**). We also noted that Marilyn T cells chronically exposed to agonist antigen present in male recipients had greatly reduced CD44 expression compared to female recipients where only tonic pMHC signals were present. In contrast to the day 7 time point (**Figure 2D**), at day 35 both MHC-II sufficient female and male recipients receiving anti-IL-7Rα treatment had markedly reduced absolute splenic T cell numbers compared to their untreated counterparts (**Figure 3B**). As expected, MHC-II deficient female hosts had significantly fewer splenic T cells compared to their MHC-II sufficient counterparts (**Figure 3B**), and this was further reduced by anti-IL-7Rα treatment with very few cells detectable overall in anti-IL-7Rα-treated MHC-II deficient female hosts. Although most cells in non-anti-IL-7Rα-treated MHC-deficient females appeared to have undergone proliferation by day 35 a significant proportion remained undivided (Figure S1 in Supplementary Material). In contrast, the very few detectable cells within anti-IL-7Rα-treated MHC-II-deficient females were almost completely proliferated cells, consistent with the previously noted decrease in the undivided cell population upon IL-7Rα blockade (Figure S1 in Supplementary Material). We did not note any differential expression of IL-7Rα expression between WT or PD-$1^{-/-}$ Marilyn T cells (**Figure 3C**) nor between cells in MHC-II sufficient or deficient female hosts although there was a trend toward lower IL-7Rα expression in the latter. Together, these data suggest that IL-7 signaling is dispensable for LIP in conditions of excess pMHC resources, but when the T cell compartment becomes more replete and competition for available pMHC resources increases or pMHC is otherwise limiting, IL-7 signals are critically important for survival of the T cell population.

In a Competitive Environment Blockade of IL-7Rα Ameliorates Morbidity Caused by PD-$1^{-/-}$ Thymocytes

TCR signals can downregulate IL-7Rα expression (e.g., **Figure 3C**) and have been reported to block, in an affinity-dependent manner, the response to IL-7-mediated survival signals (49, 50). Because we noted that IL-7Rα blockade had a marked effect on the size of the T cell compartment 5 weeks post-mixed Marilyn T cell transfer (**Figure 3B**) but not after only 7 days in class II sufficient recipients (**Figure 2D**), we reasoned that IL-7 signals perhaps became more important to T cell survival as competition for available pMHC became more intense (i.e., as the T cell compartment became more replete, and fewer cells could sufficiently access pMHC and receive a TCR signal). In order to explore this concept, we examined whether constraints on IL-7 signaling in high versus low competition settings would influence the development of autoimmunity. For this we used our model of disease following transfer of polyclonal PD-$1^{-/-}$ thymocytes to Rag$^{-/-}$ animals, which results most commonly in weight loss, kyphosis, diarrhea, dermatitis, and ocular lesions. We transferred either a low amount (10×10^6) or high amount (30×10^6) of PD-$1^{-/-}$ thymocytes to NK-depleted recipient animals. The recipients were treated biweekly with monoclonal anti-IL-7Rα or isotype control antibodies and monitored for weight changes and disease symptoms. Here, we noted a striking effect of anti-IL-7Rα treatment on loss of weight in the high-dose thymocyte recipient group, which was statistically significant from day 20 through to the termination of the experiment even though anti-IL-7Rα treatment was withdrawn at day 36 (**Figure 4A**). On the

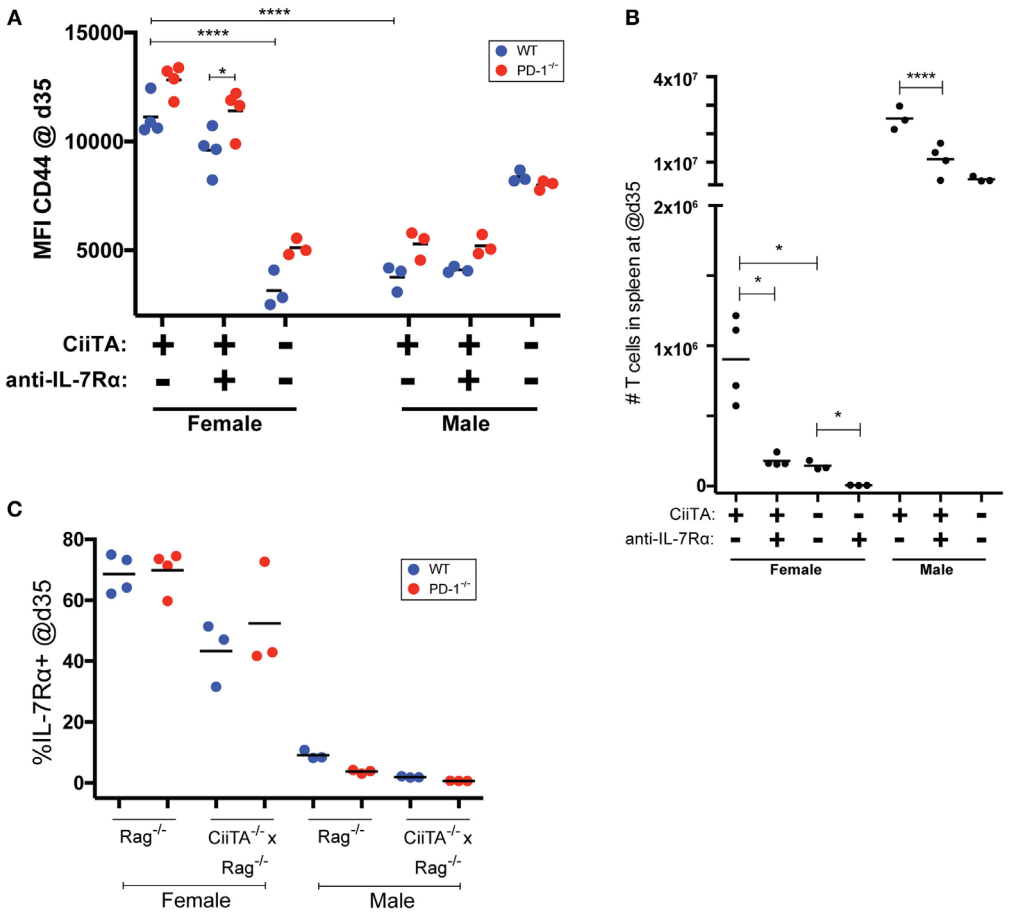

FIGURE 3 | IL-7Rα blockade decreases T cell compartment size post-LIP without preventing tonic peptide:MHC induced upregulation of CD44. **(A)** Mean fluorescence intensity (MFI) of CD44 expression in TCRβ+CD4+ WT or PD-1$^{-/-}$ cells in splenocytes from individual recipients collected at 35 days p.t. (post-LIP). *$p < 0.05$, ****$p < 0.0001$, One-way ANOVA with Tukey's multiple comparison test. **(B)** Absolute numbers of CD4+ T cells in spleen of recipient mice at day 35 p.t. *$p < 0.05$, Student's *t*-test, ****$p < 0.0001$ One-way ANOVA with Tukey's multiple comparison test. **(C)** Percent IL-7Rα+ cells within the WT and PD-1$^{-/-}$ CD4+ T cell populations in the indicated recipients' splenocytes at day 35 p.t. The data depict individual biological replicates from one experiment.

other hand, anti-IL-7Rα treatment of the low-dose thymocyte recipient group did not have any effect on the loss of weight (**Figure 4B**). Taken together, these data suggest that limitation of LIP potential by inhibiting the ability of T cells to respond to IL-7-mediated signals in situations of high but not low competition for finite pMHC can prevent the loss of weight associated with the pathology of LIP-driven autoimmunity.

DISCUSSION

The potential for LIP in a host depends on the resources available for T cells (tonic or cognate pMHC, homeostatic cytokines) relative to the level of competition for those resources (1). Importantly, LIP can drive inflammatory and autoimmune disease (7, 14, 17, 19, 22–30), and thus modulation of LIP can increase or decrease its potential to result in pathologic T cell activation. Several molecules with co-inhibitory function, such as BTLA, LAG-3, and TGFβRII have been shown to modulate T cell homeostasis and LIP (15–18), although to our knowledge none have demonstrated that such control of LIP *in vivo* is *via* modulating signals in response to pMHC, particularly tonic pMHC. Recently we described a severe systemic autoimmune disease mediated by newly generated PD-1$^{-/-}$ T cells in lymphopenic animals which could be blocked by the addition of competitors or reducing lymph node stroma (19), and which was not due to a deficiency in the generation or function of PD-1$^{-/-}$ Treg (33), suggesting that the co-inhibitory molecule PD-1 also plays an important role in the establishment of immune tolerance through control of LIP. This gave rise to our hypothesis that the disease in lymphopenic recipients of PD-1$^{-/-}$ newly generated T cells is mediated by inappropriate activation of T cells in response to normally tonic self-pMHC in the context of LIP. However, the possibility existed that the T cell response in our disease model was directed toward tissue-restricted antigens that were not appropriately negatively selected against in the thymus in the context of PD-1 deficiency, or due to altered population dynamics because of changes in positive selection (51). Furthermore, it was unclear whether the relatively low-affinity interactions with tonic pMHC can promote

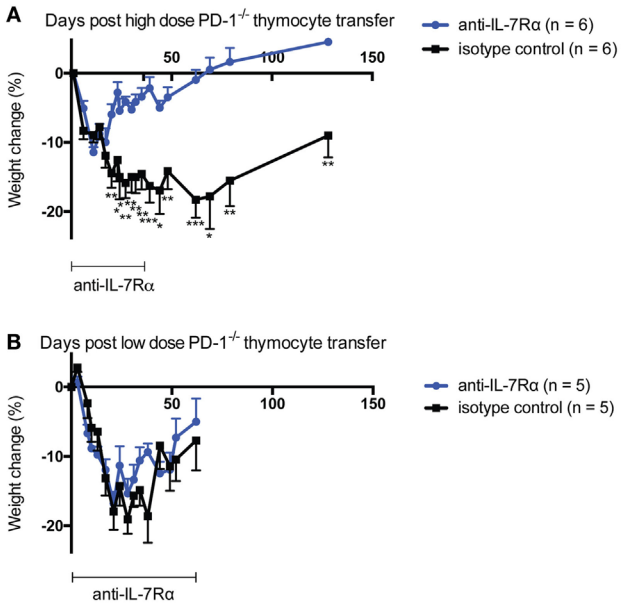

FIGURE 4 | IL-7Rα blockade ameliorates high- but not low-dose PD-1$^{-/-}$ thymocyte-mediated morbidity in lymphopenic recipients. Either high-dose [panel **(A)**, 30 × 10^6 total cells] or low-dose [panel **(B)**, 10 × 10^6 total cells] PD-1$^{-/-}$ unfractionated thymocytes were transferred to NK-depleted Rag$^{-/-}$ recipient animals with i.p. injection of 0.5 mg per mouse anti-IL-7Rα or isotype control twice weekly for the time periods indicated. Mice were monitored for disease symptoms and loss of weight. **(A)** Weight change in high-dose thymocyte recipients over the experimental period. **(B)** Weight change in low-dose thymocyte recipients over the experimental period. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Student's *t*-test. The data depict the mean ± SEM of individual biological replicates from one experiment.

upregulation of PD-1 on T cells for it to mediate its inhibitory function.

In female hosts, at least up to 7 days post transfer, PD-1$^{-/-}$ Marilyn T cells showed greater proliferation (**Figures 1B,C**; Figure S1 in Supplementary Material) and came to significantly outnumber the Marilyn-WT cells by ~2-fold and this was not affected by IL-7Rα blockade but was completely dependent on host MHC-II expression (**Figure 1D**). This supports our hypothesis that PD-1 deficiency enhances the TCR signaling response to tonic pMHC during LIP.

When we examined the expression of the anti-apoptotic molecule Bcl2 at 5 days post-cell transfer (a time during which rapid LIP was occurring), the PD-1$^{-/-}$ cells in female Rag$^{-/-}$ hosts showed a trend toward expressing less of this molecule (**Figure 2C**). Although we did not examine other IL-7/TCR signaling-associated anti-apoptotic molecules, such as Bcl-Xl and Mcl-1 (52), this suggests that any potential increased survival in the PD-1$^{-/-}$ population was not mediated by Bcl2. Importantly, examination of Bcl2 expression in T cells within all the recipient groups clearly indicated that Bcl2 expression was depressed in groups treated with anti-IL-7Rα and particularly in combination with pMHC deprivation in the CiiTA deficient hosts (**Figure 2C**). We also noted that at day 7, at which time cells were presumably still undergoing substantial LIP, IL-7Rα blockade did not reproducibly negatively impact the frequency of T cells in the blood in MHC-II sufficient hosts (**Figure 2D**). It is important to note that in contrast to studies that suggested that IL-7 signals were critical for the LIP of T cells (53, 54), anti-IL-7R treatment did not significantly inhibit proliferation in MHC-II sufficient hosts during the first week after thymocyte transfer when competition for pMHC would be expected to be low (**Figure 1B**; Figure S1 in Supplementary Material). The failure of T cells to undergo LIP in IL-7$^{-/-}$ × Rag$^{-/-}$ hosts (53, 54) is instead likely attributable to defects in lymph node structure and function as IL-7 is important for lymph node development (55). In contrast to our observation of no significant negative effect of anti-IL-7Rα treatment on the frequency of T cells in the blood of MHC-II sufficient recipients at day 7 post-transfer, IL-7Rα blockade led to significant decreases in absolute splenic T cell numbers by day 35 (**Figure 3B**). Together, these data suggest that by day 35, the recipients were more lymphoreplete and due to competition, tonic pMHC signals were no longer the most significant source for survival signals for the T cells and hence IL-7Rα blockade could now have a marked effect.

Despite its now clear role in controlling LIP in response to tonic pMHC-II signals, it was unclear whether PD-1 would be upregulated during LIP stably and sufficiently such that it would be detectable by flow cytometric staining. Indeed, at day 5 post-transfer, PD-1 expression was detectably increased by tonic pMHC signals in the WT T cell population but only among the highly proliferated cells (**Figure 2A**). The lack of obvious PD-1 expression on non- or intermediately proliferated cells (i.e., CTV mean fluorescence intensity ≥10^3, **Figure 2A**) might suggest that regulation of LIP (and even perhaps normal primary T cell activation in a lymphoreplete host) by PD-1 may not require its high-level surface expression on T cells, or might occur primarily after many rounds of division in the highly proliferated cells. PD-1 upregulation may vary depending on the strength of the TCR:pMHC interaction experienced by a given T cell. Thus, the extent of PD-1 upregulation evoked upon LIP of Marilyn T cells in response to tonic pMHC may differ significantly from that of a polyclonal population (19) owing to a greater diversity of interaction affinities in the latter. Consistent with this notion, in the experiments described herein we noted that PD-1 expression levels were markedly lower among highly proliferated cells in female compared to male Rag$^{-/-}$ recipients of Marilyn T cells (data not shown).

CD5 is a negative TCR signaling regulator commonly considered as a marker of TCR affinity set during thymic T cell selection processes as well as a reliable marker of TCR activation (45–47). CD5 expression has been correlated with the propensity for T cells from various TCR transgenic backgrounds to undergo LIP in a lymphopenic host (56–59). We found that highly proliferated cells stimulated by tonic pMHC in both the WT and PD-1$^{-/-}$ Marilyn T cell populations contained subpopulations that had either up- or downregulated CD5 expression – with significantly higher overall CD5 expression in the PD-1$^{-/-}$ group (**Figure 2B**). Potentially, CD5 is acting here as a readout of the extent of the TCR signal received by cells undergoing LIP. One could also speculate that the PD-1$^{-/-}$ T cells are under pressure to maintain or increase CD5 expression due to a lack of co-inhibition from

PD-1. However, pragmatically speaking these findings suggest that CD5 expression can change considerably in a monoclonal T cell population during LIP, which raises important questions about the validity of its use as a marker of T cell affinity/avidity for thymic pMHC as commonly seen in the literature, particularly if the cells under analysis have been exposed to a lymphopenic environment and have undergone LIP.

Compared to its effects on LIP in response to tonic pMHC signals, the effect of PD-1 deficiency on LIP of Marilyn T cells was even more pronounced in response to cognate pMHC in male Rag$^{-/-}$ hosts (**Figure 1D**) and it was also IL-7 independent. In CiiTA$^{-/-}$ hosts, APC within the eye and brain can use a CiiTA-independent pathway to express MHC-II in response to IFN-γ and TNF-α (44), and it is possible that low numbers of MHC-II sufficient thymic APC were co-transferred with the Marilyn thymocytes. While robust proliferation occurred in male CiiTA$^{-/-}$ × Rag$^{-/-}$ hosts, interestingly no appreciable increase in the PD-1$^{-/-}$:WT ratio was seen by day 7 post transfer. While further studies are needed to fully understand this difference, two possibilities seem likely. Either the residual MHC class II expression in the CiiTA$^{-/-}$ recipients is limited to specific APC types that present antigen but do not engage PD-1 or alternatively it is simply the quantity of antigen exposure that determines whether PD-1 controls the response. We have previously proposed that chronic antigen signaling, as would occur with widely distributed pMHC ligands, leads to upregulation of co-inhibitory signals (60). From this viewpoint, the intermittent (not chronic) exposure to sparse pMHC in female or male CiiTA$^{-/-}$ × Rag$^{-/-}$ recipients would not lead to co-inhibitory signals such as those through PD-1.

Although we did not note any difference in IL-7Rα expression between WT and PD-1$^{-/-}$ T cells (**Figure 3C**), we did note that compared to female hosts, the IL-7Rα-expressing T cell populations in male recipients were considerably less numerous which is consistent with these cells constantly receiving strong TCR signals from high-affinity cognate pMHC interactions and downregulating IL-7Rα. Chronic high-level stimulation may also underlie the decreased CD44 expression noted in male MHC-II sufficient hosts (**Figure 3A**), a phenomenon previously observed in chronic viral infection (61). Indeed, the much larger size of the T cell compartment within male versus female hosts (**Figure 3B**) stems from the presence of pMHC that can provide a higher affinity signal (i.e., cognate pMHC) and could be viewed as a richer source of resources. Thus a HY containing host was able to support a much larger population of T cells.

Consistent with our interpretation that increased competition for pMHC signals in more lymphoreplete conditions would create greater dependence on IL-7Rα signals, we found that IL-7Rα blockade had a robust effect on preventing and reversing weight loss only when high numbers of PD-1$^{-/-}$ thymocytes were transferred (**Figure 4A**). IL-7 has previously been associated with the promotion of IL-3 and GM-CSF expression in human T cells (62). Very recently, it was reported that IL-7 could promote the development of a unique subset of GM-CSF and IL-3-producing T cells ("Th-GM") in mice and this was associated with encephalitogenicity in the experimental autoimmune encephalomyelitis model (63). Based on these reports, we considered that the effects of IL-7Rα blockade might be attributable to inhibition of GM-CSF expression and, therefore, we explored this *in vivo via* administration of anti-GM-CSF antibody to lymphopenic recipients of PD-1$^{-/-}$ HSC. We found a small, statistically insignificant effect of anti-GM-CSF blockade in this experiment on disease incidence with no significant effect on loss of weight (Figure S2 in Supplementary Material). These data suggest that the effect of blockade of IL-7Rα is unlikely to be solely mediated through effects on GM-CSF. Our findings support the concept that in situations of high competition for pMHC, limiting LIP potential by blocking the response to homeostatic cytokines like IL-7 can help to ameliorate the wasting aspect of systemic autoimmunity.

Our finding that the co-inhibitory molecule PD-1 controls LIP by modulating the response to tonic self-pMHC signals *in vivo* lends support to our hypothesis that the disease in lymphopenic recipients of PD-1$^{-/-}$ HSC or newly generated T cells results from exaggerated responses to normally tonic self-pMHC signals. These data are consistent with the concept that establishment of peripheral tolerance involves a tuning process (64), a tuning that is regulated at least in part by PD-1. Furthermore, the effects of PD-1 deficiency on control of LIP were independent of IL-7Rα-mediated signaling, and IL-7-mediated signals were largely irrelevant to the size of the overall T cell compartment while LIP potential was still high and rapid LIP in response to abundant pMHC was occurring within the hosts. These data suggest that in LIP-associated inflammatory disorders, such as immune reconstitution inflammatory syndrome in HIV patients (25, 65), or graft-versus-host disease, therapies aimed at reducing TCR signaling during early phases of reconstitution may be more effective and should take priority over approaches that aim to limit homeostatic cytokine-mediated signals to T cells. Furthermore, the ability of PD-1 to control tonic signals in response to self-pMHC may partially underlie the effectiveness of PD-1 blockade in tumor immunotherapy—for example, by enhancing survival and activation of T cells in the context of limiting neoantigen-derived pMHC in the tumor microenvironment or by promoting tumor clearance through the enhancement of collateral damage (43).

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Canadian Council on Animal Care. The protocol was approved by the University of Alberta Health Sciences Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

KE designed, performed research and data analysis, and wrote and critically edited the manuscript. JL performed research and data analysis and critically edited the manuscript. LB provided reagents and critically edited the manuscript. CA designed research, performed data analysis, and critically edited the manuscript.

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SUPPLEMENTARY MATERIAL

The supplementary material for this article can be found online at http://www.frontiersin.org/article/10.3389/fimmu.2017.01289/full#supplementary-material.

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Prior to Peripheral Tolerance, Newly Generated CD4 T Cells Maintain Dangerous Autoimmune Potential: Fas- and Perforin-Independent Autoimmunity Controlled by Programmed Death-1

Kristofor K. Ellestad[1,2], Govindarajan Thangavelu[2,3], Yohannes Haile[2], Jiaxin Lin[2,3,4], Louis Boon[5] and Colin C. Anderson[1,2,3,4]**

[1] Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, AB, Canada, [2] Alberta Diabetes Institute, University of Alberta, Edmonton, AB, Canada, [3] Department of Surgery, University of Alberta, Edmonton, AB, Canada, [4] Alberta Transplant Institute, University of Alberta, Edmonton, AB, Canada, [5] Bioceros B.V., Utrecht, Netherlands

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Federica Casiraghi, Istituto Di Ricerche Farmacologiche Mario Negri, Italy

***Correspondence:**
Kristofor K. Ellestad
ellestad@ualberta.ca;
Colin C. Anderson
colinand@ualberta.ca

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Lymphopenia can result from various factors, including viral infections, clinical interventions, or as a normal property of the fetal/neonatal period. T cells in a lymphopenic environment undergo lymphopenia-induced proliferation (LIP) to fill the available "niche" as defined by peptide–MHC and homeostatic cytokine resources. We recently reported systemic autoimmunity following reconstitution of the lymphoid compartment of Rag1$^{-/-}$ mice with PD-1$^{-/-}$ hematopoietic stem cells or by transfer of thymocytes, but not splenocytes, suggesting that programmed death-1 (PD-1) plays a crucial role in controlling recent thymic emigrants (RTE) and preventing autoimmunity upon their LIP. However, it is unclear whether RTE residing within the periphery of a lymphoreplete host maintain enhanced autoimmune generating potential or if this property only manifests if RTE experience a lymphopenic periphery immediately after export from the thymus. Furthermore, it is unclear which of a variety of T cell effector mechanisms generate pathology when control of RTE by PD-1 is lacking. Herein, we determined that PD-1 is upregulated on CD4 T cells undergoing the natural LIP characteristic of the neonatal period. Newly generated T cells lacking PD-1 maintained an enhanced autoimmune potential even after residence in a lymphoreplete periphery, emphasizing the importance of PD-1 in the establishment of peripheral tolerance. Neither Fas nor perforin-dependent killing mechanisms were required for autoimmunity, while host MHC-II expression was critical, suggesting that LIP-driven autoimmunity in the absence of PD-1 may primarily result from a CD4 T cell-mediated systemic cytokinemia, a feature potentially shared by other autoimmune or inflammatory syndromes associated with immune reconstitution and LIP.

Keywords: tolerance, autoimmunity, co-stimulation, co-inhibition, lymphopenia, checkpoint inhibitor, cytokine storm, graft versus host

Abbreviations: LIP, lymphopenia-induced homeostatic proliferation; HSC, hematopoietic stem cells; pTreg, peripherally generated regulatory T cell; Treg, regulatory T cell; SP, single positive; RTE, recent thymic emigrants; pMHC, peptide–MHC; Tcon, conventional T cell; PD-1, programmed death-1; MFI, mean fluorescence intensity; WT, wild type.

INTRODUCTION

Thymic selection processes can be viewed as the first "filtration" step on the developing T cell repertoire. Although these processes serve to remove the majority of strongly self-reactive T cells from the developing repertoire or convert them to thymic Treg, some self-reactive conventional T cell (Tcon) clones escape (1). This is, for example, evidenced by the ability to induce autoimmune diseases, such as myelin-oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis in mice (2). Peripheral tolerance mechanisms, such as tuning, anergy, deletion, or conversion to peripherally generated Treg (pTreg), are thus important for the establishment and maintenance of immune tolerance and can be viewed as a second filter on the peripheral T cell repertoire.

Aside from the naturally lymphopenic neonatal period associated with immune system development (3), lymphopenia also occurs in a variety of clinical settings. Hematopoietic cell transplantation, T cell depleting therapy for solid organ transplantation, cancer chemotherapy, as well as HIV infection are all associated with lymphopenia of varying severity. In the lymphopenic state, cells undergo a process known as lymphopenia-induced proliferation (LIP), which is facilitated and regulated in its extent by the availability of "resources" for T cells that together define T cell "space" (4). These include peptide–MHC complexes (derived from self or otherwise) that can mediate at least a weak, "tonic" signal through the TCR, as well as homeostatic cytokines, such as IL-7 and IL-15. Regulatory T cells (Treg) (5–13), co-stimulatory molecules such as CD28 (7, 14), as well as molecules with known co-inhibitory activity [e.g., BTLA (15), LAG-3 (16), TGFβRII (17, 18)] can modulate the kinetics of LIP and the maximum size of the T cell compartment. In addition, LIP can promote autoimmune disease and LIP of TGFβRII$^{-/-}$ T cells results in autoimmunity (17).

Programmed death-1 (PD-1), a co-inhibitory receptor expressed on activated T cells, enters the immune synapse upon T cell:APC interaction and is known to recruit the phosphatase SHP2 upon receptor ligation which can dampen proximal TCR signaling cascades (19–21) or co-stimulatory signals through CD28 (22). In contrast to the C57BL/6 PD-1$^{-/-}$ mouse, which displays a relatively mild phenotype characterized by development of a lupus-like disease with spontaneous arthritis and glomerulonephritis upon aging (23), we have shown that reconstitution of the lymphoid compartment of lymphopenic adult Rag1$^{-/-}$ animals *via* transfer of PD-1$^{-/-}$ hematopoietic stem cells (HSC) leads to a rapid, severe, and lethal systemic autoimmune disease soon after the first newly generated T cells, or recent thymic emigrants (RTE), emerge into the periphery (24). The disease is associated with infiltration of CD4 and CD8 T cells into multiple organs, including heart, liver, and kidney, although Rag1$^{-/-}$ K$^{b-/-}$D$^{b-/-}$ animals remain fully susceptible suggesting that MHC-I-restricted CD8 T cells are dispensable for disease. Significantly elevated levels of several pro-inflammatory cytokines and chemokines in serum (24) as well as elevated pro-inflammatory cytokine transcripts in infiltrated organs (25) are also associated with disease. Macroscopically the disease is characterized by kyphosis, cachexia, diarrhea, and skin and ocular lesions. Interestingly, PD-1$^{-/-}$ HSC reconstitution of day 1 Rag$^{-/-}$ neonates results in a drastically reduced incidence of disease (24), suggesting that limited T cell "space" due to small anatomic size (e.g., of lymph nodes) or other factors can limit the aberrant activation of T cells promoted by LIP. Indeed lymph node-deficient Rag$^{-/-}$γc$^{-/-}$ or irradiated LTα$^{-/-}$ hosts were also resistant to disease after PD-1$^{-/-}$ HSC transfer (24). Transfer of PD-1-deficient thymocytes to adult Rag1$^{-/-}$ mice likewise results in autoimmunity; however, transfer of splenocytes from mature PD-1$^{-/-}$ mice does not result in disease. These data suggest that the RTE/newly generated T cell population, which has not yet been subject to peripheral tolerance mechanisms, has greater autoimmune potential than established peripheral T cells and that PD-1 is critically important for controlling their activity during LIP. However, several lines of evidence suggest that newly generated T cells have properties that promote tolerance (26). It is not clear whether newly generated T cells in an adult retain a heightened potential for the generation of autoimmunity after their emergence into the periphery or whether exposure of newly generated T cells to a lymphoreplete environment leads to their rapid tolerization. Herein, we have taken advantage of the Rag2pGFP transgenic (Tg) mouse strain in which GFP is expressed during early T cell development and remains detectable as a marker of newly generated lymphocytes after their emergence into the periphery (27, 28). PD-1$^{-/-}$ peripheral newly generated T cells or established T cells were purified from adult mice and tested for their ability to drive autoimmunity upon transfer into lymphopenic hosts. We found that purified peripheral PD-1$^{-/-}$ newly generated T cells are similar to thymocytes in their ability to drive systemic autoimmunity upon transfer to lymphopenic hosts. Using lymphopenic hosts lacking Fas or MHC-II expression, or PD-1$^{-/-}$ donors lacking perforin expression, we also show that host MHC-II expression is required for disease after PD-1$^{-/-}$ HSC transfer, and that Fas- and perforin-dependent killing mechanisms are dispensable for disease. Taken together, our data suggest that even in a lymphoreplete adult host, peripheral newly generated T cells retain a heightened potential for LIP-driven autoimmunity in the absence of PD-1, which is mediated by CD4 T cells.

MATERIALS AND METHODS

Mice

B6.129S7-*Rag1*^{tm1Mom}/J (Rag1$^{-/-}$, Rag$^{-/-}$), B6.Cg-*Foxp3*$^{tm2(EGFP)Tch}$/J (FoxP3^{EGFP}, used in the present manuscript as WT), B6.MRL-*Fas*^{lpr}/J (Fas^{lpr}), C57BL/6-*Prf1*^{tm1Sdz}/J (Prf1$^{-/-}$), and B6.129S2-*Ciita*^{tm1Ccum}/J (CiiTA$^{-/-}$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Days 13–14 gestation pregnant C57BL/6-CD45.1 (B6-CD45.1) mice were purchased from NCI (Frederick, MD, USA). C57BL/6-*Pdcd1*$^{-/-}$ (backcrossed 11 generations to C57BL/6) were originally generated by Prof. T. Honjo and colleagues (23). FoxP3^{EGFP} × *Pdcd1*$^{-/-}$ mice were generated by crossing the above FoxP3^{EGFP} and B6-*Pdcd1*$^{-/-}$ mice and are referred to in the present manuscript simply as PD-1$^{-/-}$. PD-1$^{-/-}$ × Prf1$^{-/-}$ mice were generated by crossing the above PD-1$^{-/-}$ and Prf1$^{-/-}$ mice without any selection for the FoxP3^{EGFP} transgene. Rag$^{-/-}$ × CiiTA$^{-/-}$ mice were generated by

crossing the above $Rag^{-/-}$ and $CiiTA^{-/-}$ mice. $Fas^{lpr} \times Rag^{-/-}$ mice were generated by crossing the above Fas^{lpr} and $Rag^{-/-}$ mice. B6.Rag2pGFP (Rag2pGFP) mice (27, 28) were kindly provided by Pamela Fink (University of Washington, Seattle, WA, USA). Rag2pGFP × $PD\text{-}1^{-/-}$ mice were generated by crossing Rag2pGFP mice with the above B6-*Pdcd1*$^{-/-}$, with screening and selection of breeders for high GFP expression. Marilyn $Rag2^{-/-}$ $CD4^+$ anti-HY/I-A^b TCR Tg mice (called Marilyn herein) were generated by Lantz and colleagues (29) and were originally obtained from the NIAID exchange program. Cells from Marilyn mice were tracked based on their expression of CD4, CD45.2, and a Vβ6 TCR and lack of CD45.1. Animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care and housed under clean conventional housing conditions at the University of Alberta Health Sciences and Laboratory Animal Services facilities (HSLAS).

Cell Preparations and Adoptive Transfer Experiments

For experiments involving transfer of thymocytes or peripheral T cells, recipient NK cells were depleted [to avoid potential NK-mediated killing of the input cells (30–32)] by treatment on days −4, −1, and +2 with 0.3 mg per mouse of anti-NK1.1 (PK136) injected intraperitoneally. Thymocytes or splenocytes for injection were prepared by disruption in HBSS (Gibco) + 2% fetal bovine serum (FBS, Sigma-Aldrich) through a 70-µm nylon cell strainer. Cells were centrifuged at ~300 × *g* for 5–10 min at room temperature, and red blood cell lysis was performed by resuspending cells in ACK lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA) with incubation for 5 min, followed by addition of ~10 volumes of HBSS + 2% FBS, and two cycles of centrifugation at ~300 × *g* for 5–10 min, and resuspension in PBS with no additives. If cells were prepared for further manipulation (e.g., staining and sorting), they were instead washed and resuspended in HBSS + 2% FBS. For experiments involving adoptive transfer of HSC, fetal liver cells (FLC; embryonic days 14–15) were used as a source of HSC. On ice, fetal livers were disrupted by repeated trituration through a 5-mL serological pipet, followed by filtration through a 70-µm nylon mesh filter basket. Cells were then centrifuged at ~300 × *g* for 10 min at 4°C, and resuspended according to their intended further use. FLC were used either fresh or frozen. For immediate use for *in vivo* transfers, FLC were resuspended at 50×10^6 cells/mL in PBS. For freezing, cells were resuspended at 2×10^8 cells/mL in 90% FBS + 10% DMSO, frozen in a −1°C per minute rate-controlled cell freezing apparatus in a −80°C freezer and transferred to the vapor phase of a liquid nitrogen tank for long-term storage. 1.5×10^7 fresh or frozen FLC were transferred intravenously to the indicated recipients. In islet transplantation experiments, female $Rag^{-/-}$ recipients with an established islet transplant received a mixture of 4:1 female B6-CD45.1 and female Marilyn FLC (total of $10–15 \times 10^6$ cells). Sex of fetuses was determined by PCR as described (33).

Definition of Disease and Data Analysis

Macroscopic signs of disease in HSC/thymocyte/peripheral cell recipients included cachexia/weight loss (>15%), kyphosis (hunched appearance), ruffled fur, dermatitis, ocular lesions, and diarrhea. Recipient mice were no longer considered disease free when two or more of the above symptoms were evident, or if mice lost ≥20% body weight. For thymocyte experiments, calculation of weight loss for disease determination was performed relative to weights at day 0 or day 1 relative to cell transfer. For HSC experiments, calculation of weight loss was determined relative to initial weight measurements taken prior to day 30. Unless otherwise indicated, animals were used at 8–16 weeks of age.

Islet Transplantation

Diabetes was induced in recipient mice *via* treatment with streptozotocin (Sigma-Aldrich) at 185–190 mg/kg. Recipients were considered to be diabetic after two consecutive blood glucose measurements of >20 mM using a OneTouch Ultra glucometer (Lifescan Canada, Burnaby, BC, Canada). Pancreatic islets were isolated from male or female $Rag^{-/-}$ mice as previously described (34). Diabetic recipients were transplanted with 500 islets placed under the kidney capsule and thereafter monitored for return to normoglycemia. All recipients returned to normoglycemia within 48 h of islet transplantation and remained normoglycemic until they were given FLC between 3 and 6 months after islet transplantation; post fetal liver injection some mice with male islets became hyperglycemic (rejected the islet transplant) as described in the Section "Results."

Antibodies, Flow Cytometry, and Fluorescence-Activated Cell Sorting (FACS)

For flow cytometric staining and sorting, fluorophore-labeled antibodies against the following markers were obtained from eBioscience (San Diego, CA, USA) unless otherwise indicated: CD4 (RM4-5), TCRβ (H57-597), CD8α (53-6.7), PD-1 (J43), Ki-67 (SolA15), CD44 (IM7), CD19 (1D3), Granzyme B (NGZB), CD45.1 (A20), CD45.2 (104), Vβ6 (RR4-7). Antibodies were used at manufacturer's recommended concentrations. Flow cytometric staining always used an Fc block cocktail to block nonspecific staining. Fc block cocktail consisted of 3 mL each of normal mouse, rat, and hamster serum, with addition of 0.3 mg of anti-CD16/32 antibody (clone 2.4g2, BioXCell). Fixation and permeabilization were performed using the eBioscience FoxP3 Fixation/Permeabilization buffer kit (Thermo Fisher) according to the manufacturer's protocols. For cell sorting, a BD Influx cell sorter was used controlled with Spigot software (Beckton Dickinson, Franklin Lakes, NJ, USA). Briefly, for sorting source cells were stained and resuspended in HBSS + 20% FBS + 10 mM HEPES and sorted directly into FBS supplemented with 10 mM HEPES. Standard flow cytometric analysis was performed using a BD LSR II instrument. Flow cytometric data analysis was performed using FlowJo (Treestar software, Portland, OR, USA).

Statistical Analysis

Statistical analysis was performed using Graphpad Prism software. Details of statistical tests used are provided in figure legends.

However, in general, for comparisons of two groups, Student's *t*-test was used. In cases of comparison of groups with unequal variances, Welch's correction was applied. Unless otherwise noted, for multiple group comparisons, one-way ANOVA with Tukey's multiple comparison test was used. Disease onset/incidence was compared by the Kaplan–Meier method. Probability values reported for survival curve comparisons were calculated using the Mantel–Cox method.

RESULTS

PD-1 Expression Is Tied to Lymphopenia-Induced Proliferation in Newly Generated T Cells

Programmed death-1 expression appears to be critical for the generation of immune tolerance during LIP of newly generated T cells. We previously showed that newly generated T cells in a lymphopenic setting (i.e., in a lymphopenic recipient of WT HSC) had a high proportion of PD-1 expressing T cells that diminished over time (24). Whether the higher PD-1 expression on newly generated T cells was dependent on exposure to the lymphopenic environment or is an intrinsic property of newly generated T cells even in lymphoreplete mice has not been fully assessed. We, therefore, examined PD-1 expression in the steady state in both TCRβ$^+$ thymocytes and peripheral splenocytes from 10- to 12-week-old adult Rag2pGFP animals (**Figures 1A,B**). Unexpectedly, peripheral newly generated CD4 single positive (SP) splenocytes expressed very little PD-1 while approximately 15% of established or "mature" GFP$^-$ CD4 SP T cells were found to be PD-1 positive (**Figure 1A**). Likewise, mean fluorescence intensity of PD-1 staining was significantly higher in the CD4 SP GFP$^-$ cells compared to the GFP$^+$ population (**Figure 1B**). Neither established nor newly generated T cells within the splenic CD8 SP population expressed appreciable levels of this co-inhibitor. Within the thymocyte population, approximately 90% of the CD4 SP and 85% of the CD8 SP were GFP$^+$ (data not shown), with the remainder presumably representing mature cells that had recirculated from the periphery back to the thymus, although it is conceivable that at least a subset could represent cells that failed to exit the thymus for longer than 3 weeks post-VDJ recombination (28). GFP$^+$ thymic CD4 and CD8 SP cells were slightly enriched for PD-1 positivity (3.1 and 2.6% positive, respectively, **Figure 1A**) compared to their splenic counterparts (both 1.3%), although this difference was not statistically significant. Similar to what was seen in splenocytes, the thymic CD4 SP GFP$^-$ population contained a sizeable population of PD-1$^+$ cells (26%), and thus, these more established cells had higher overall PD-1 expression compared to the GFP$^+$ newly generated T cell population (**Figures 1A,B**). Unlike their splenic counterparts, there was a trend toward increased PD-1 positivity and overall PD-1 expression in the thymic CD8 SP GFP$^-$ cells compared to the GFP$^+$ cells although this difference was not statistically significant (**Figures 1A,B**).

Thus far, our data indicated that in the steady state, RTE of adult lymphoreplete mice do not express high levels of PD-1 (**Figure 1**) while adoptive transfer of RTE-generating HSC to lymphopenic mice did lead to high PD-1 expression (24). We, therefore, asked whether heightened PD-1 expression is peculiar to LIP in adoptive transfer or whether it might occur naturally (without adoptive transfer) during the LIP triggered by the lymphopenic state of the neonatal period (3). We examined PD-1 expression on peripheral lymphocytes of 10-day-old Rag2pGFP neonatal mice. More than 97% of thymic CD4 SP cells are GFP$^+$ in young Rag2pGFP mice (Figure S1B in Supplementary Material), and all splenic T cells in 10-day-old neonates would be considered to be newly generated based on elapsed time from initial development and, therefore, might also be expected to be GFP$^+$. However, a significant percentage (~30% of CD4, ~65% of CD8) of the splenic T cells had lost detectable GFP fluorescence in 10-day-old neonates (**Figure 1C**). The association between loss of GFP in CD4 T cells and acquisition of a CD44^{high} memory phenotype, as well as an association of CD44 with Ki-67 expression, a marker of cycling cells (**Figure 1D**, upper panels) suggests that this was due to these cells having undergone multiple rounds of LIP, diluting GFP. Importantly, loss of GFP expression, high CD44 expression, and high Ki-67 expression were all associated with elevated PD-1 expression (**Figure 1D**, bottom panels): more than 90% of PD-1-expressing CD4 T cells were Ki-67 high. Very few CD8 T cells expressed PD-1 in the neonates at this age and PD-1 expression was not associated with heightened Ki-67 expression in these cells (Figure S1A in Supplementary Material).

Together, these data indicate that while PD-1 is upregulated on T cells following LIP or a period of residency in the periphery (possibly due to encounter with antigen), only very low/barely detectable levels of PD-1 are expressed on newly generated adult T cells. Thus, the heightened PD-1 expression on newly generated T cells seen in lymphopenic HSC recipients is not an intrinsic characteristic of these cells but is LIP-induced. The naturally occurring LIP of the neonatal period also upregulated PD-1 on newly generated CD4 T cells, suggesting PD-1 is involved in establishing tolerance under physiologic conditions (i.e., not just under conditions of cell transfer).

Steady State Peripheral Newly Generated T Cells in Adult Mice Maintain Heightened Autoimmune Potential Relative to Established T Cells

Our previous studies showed that newly generated T cells exported from the thymus directly into a lymphopenic environment are critically dependent on PD-1 to establish tolerance and prevent autoimmunity. In contrast, PD-1 was not needed to maintain tolerance after transfer of peripheral T cells from PD-1$^{-/-}$ adult animals, which would largely be comprised of established cells. However, it is unknown whether steady-state newly generated T cells in the periphery of immunocompetent adult mice retain this heightened potential for autoimmunity or if instead the peripheral tolerance process is rapid and newly generated T cells are immediately tolerized upon export to a lymphoreplete environment. In order to test the hypothesis that steady-state peripheral RTE/newly generated T cells had

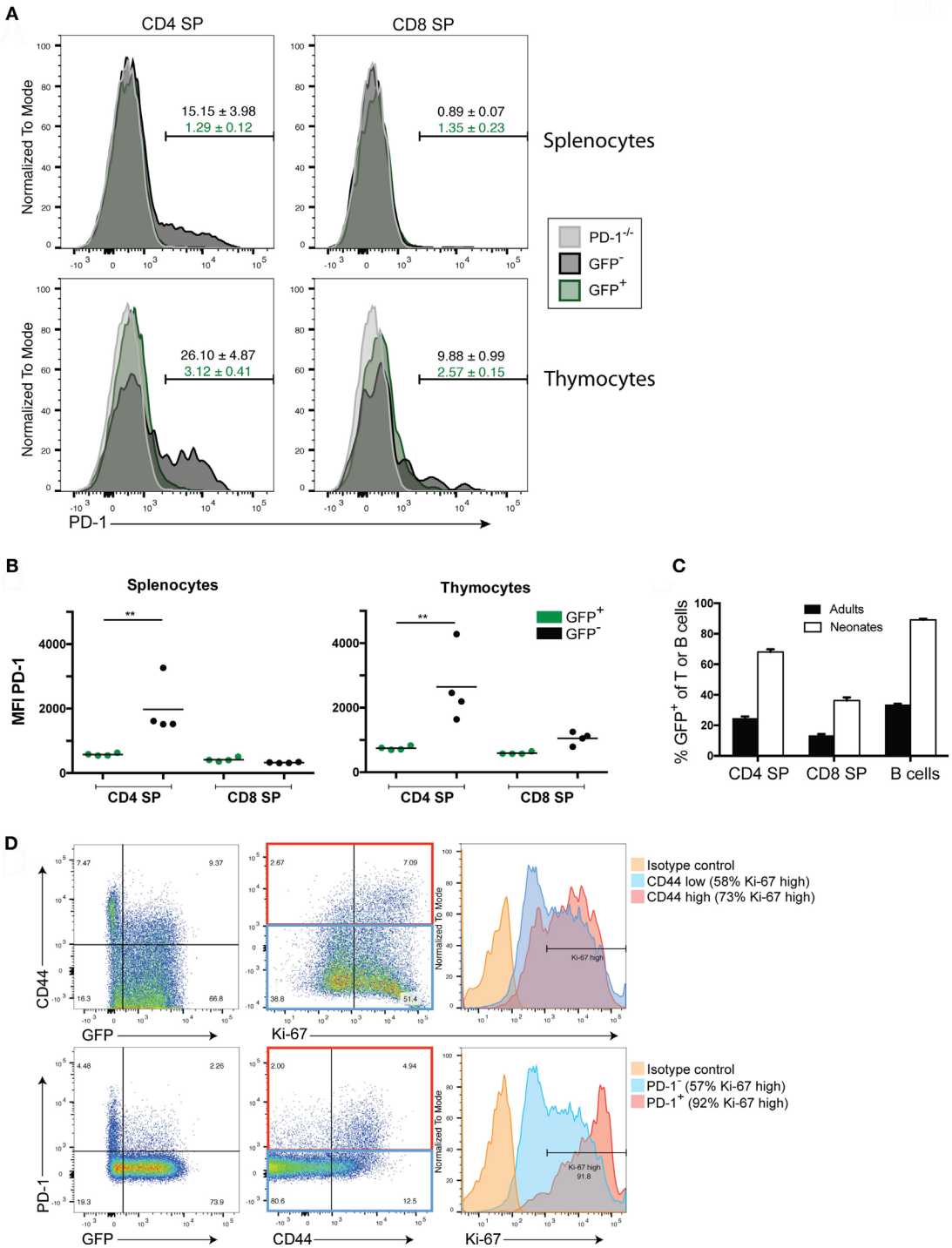

FIGURE 1 | Newly generated T cells in adults lack programmed death-1 (PD-1) expression while in neonates their expression of PD-1 is linked to LIP. **(A)** Representative flow cytometric analysis of splenocytes and thymocytes from 10- to 12-week-old adult Rag2pGFP mice for PD-1 expression among TCRβ$^+$ CD4 or CD8 single positive (SP) cells that are GFP$^+$ (newly generated T cells) or GFP$^-$ (mature cells). T cells from PD-1$^{-/-}$ mice were also stained as a control for background. Values above gates are the group average %PD-1 positive cells within the mature GFP$^-$ population (black text) or GFP$^+$ population (green text), ±SEM. **(B)** Mean fluorescence intensity (MFI) of PD-1 staining in CD4 or CD8 SP, GFP$^+$ or GFP$^-$ T cells from Rag2pGFP splenocytes or thymocytes from the experiment depicted in **(A)**. $^{**}p < 0.01$, one-way ANOVA with Tukey's multiple comparison test. **(C)** Comparison of percent GFP$^+$ cells among peripheral splenocytes in the indicated cell populations in adult vs. neonatal mice ± SEM. $n = 8$ per group. **(D)** Representative ($n = 3$) flow cytometry plots of the indicated markers in splenic TCRβ$^+$ CD4 SP T cells from 10-day-old Rag2pGFP neonates. Histogram (right) analyses of Ki-67 on CD44 high or PD-1 high cells and CD44 low/negative cells or PD-1 low/negative cells employed gates as shown in the red and blue rectangles (middle dot plots), respectively. Data presented are from samples that were either permeabilized and stained with antibodies to TCRβ, CD4, CD8, PD-1, CD44, and Ki-67, or non-permeabilized and stained with antibodies to TCRβ, CD4, CD8, PD-1, and CD44 (to allow analysis of GFP).

increased ability to drive autoimmunity in a lymphopenic host, we generated B6 Rag2pGFP x PD-1$^{-/-}$ mice and purified the GFP^{+} or GFP^{-} T cell populations from splenocytes of adult animals by FACS. Purified cells or thymocytes containing an equivalent number of SP T cells were transferred to Rag$^{-/-}$ recipient animals and disease was monitored. Approximately 20 days after transfer, mice that received either PD-1$^{-/-}$ thymocytes or purified GFP^{+} newly generated T cells began to develop autoimmune disease while the recipients of established cells were relatively spared (**Figure 2A**, left panel). Similarly, while all recipient mice lost some weight beginning almost immediately after cell transfer until around 2 weeks post-transfer, after this point PD-1$^{-/-}$ established T cell recipients began to regain their lost weight while the thymocyte and newly generated T cell recipients clearly and significantly diverged from the established cell recipients and continued to lose weight (**Figure 2A**, right panel). Despite the clearly higher propensity of newly generated vs. established cells to drive autoimmunity, it should be mentioned that a low proportion of the GFP^{-} established T cell recipients also met the criteria for disease albeit at a much later time point relative to the other groups (**Figure 2A**). Approximately, 40% of the purified GFP^{+} newly generated cells were CD4 SP and 56% were CD8 SP (a CD4:CD8 ratio of ~0.7:1), whereas the purified GFP^{-} population contained approximately 80% CD4 SP and 17% CD8 SP (CD4:CD8 ratio ~4.7:1, **Figure 2B**). While our experiments were designed to test whether the newly generated T cells as a whole have heightened autoimmune potential, the differences in the proportions of CD4 vs. CD8 SP cells in newly generated vs. established T cells could potentially contribute to the differences in their ability to drive disease.

T Cells with Specificity for a Neo-Self Antigen Can Persist in the Periphery with a Slow Decline in Frequency Over time

The finding that newly generated T cells have a greater potential for autoimmunity than established peripheral T cells appears incongruent with several reported aspects of RTE biology (26) such as reduced effector functions or an increased propensity to convert to pTreg (25, 35, 36) compared with established T cells. These studies raise the question of how newly generated T cells could have a greater capacity to cause autoimmune disease. There are at least two "filters" against self-reactivity, namely central and peripheral tolerance. We surmised that newly generated T cells may have a greater potential for generating autoimmunity because they have yet to pass through the second filter, peripheral tolerance. Autoimmunity caused by newly generated T cells during LIP might be due in part to a greater frequency of T cells with self-specific receptors than are present in the established T cells that have undergone peripheral tolerance mechanisms, such as deletion or conversion to pTreg. In order to explore the concept that potentially dangerous self-reactive cells could persist for a significant length of time in a lymphoreplete periphery post immune reconstitution of lymphopenic mice and be held in check by peripheral tolerance mechanisms without resulting in overt immune pathology, we used the HY antigen expressed by an established graft as a model neo-self antigen (33). We gave mixed Marilyn (anti-male antigen, HY/IA^{b}) and WT B6 female HSC to Rag$^{-/-}$ female mice bearing an established islet graft from male or control female Rag$^{-/-}$ donors and monitored subsequent graft rejection and the percentage of cells that were Marilyn over time post immune reconstitution (**Figure 2C**). We found that reconstitution with a mixture of Marilyn and WT HSC (1:4 Marilyn to WT) led to a small but significant fraction (<30%; 5 of 17) of recipients rejecting the transplant. This rejection appeared to be associated with the appearance in the periphery of a high ratio of Marilyn to WT T cells and earlier export of Marilyn T cells into the periphery compared to WT T cells (data not shown). Nevertheless, the vast majority of recipients tolerated the peripheral male islet transplant. In these male islet graft recipients, we compared the frequency of Marilyn T cells (CD45.2^{+}, CD45.1^{-}, CD4^{+}Vβ6^{+}) to recipients of a control female islet graft. The data in **Figure 2D** show that the male islet transplant, given 3–4 months prior to a 1:4 (Marilyn:WT) mixture of HSC, causes a slow reduction in frequency of neo-self antigen-specific Marilyn T cells. The mice with male but not female islets were tolerant to the male antigen, as they accepted a male skin graft given 4–6 months post HSC (**Figure 2E**). Thus, peripheral CD4 tolerance was associated with a decreased frequency of neo-self antigen-specific T cells over time, with newly generated T cells having a higher frequency of CD4 T cells specific to the neo-self antigen.

Host MHC Class II but Not Fas Is Required for Disease upon Transfer of PD-1$^{-/-}$ HSC

We previously showed that adult Rag1$^{-/-}$ K$^{b-/-}$D$^{b-/-}$ mice were fully permissive for the development of autoimmune disease following transfer of PD-1$^{-/-}$ HSC (24); importantly, no perturbations in the time to disease onset or severity were reported compared to Rag$^{-/-}$ hosts, as would be reasonably expected if the disease were CD8 T cell independent. To delineate the importance of CD4 T cells in this disease model, we generated Rag$^{-/-}$ and MHC Class II transactivator-deficient (CiiTA$^{-/-}$) mice, which are largely deficient in MHC-II expression (37). We transferred PD-1$^{-/-}$ HSC to Rag$^{-/-}$ or Rag$^{-/-}$ × CiiTA$^{-/-}$ hosts and monitored the mice for signs of disease. In addition, we examined potential effector mechanisms of disease. Two canonical pathways of T cell killing, namely the FasL-Fas and perforin-dependent pathways, have been described (38). In order to begin to elucidate which, if any, of these pathways are involved in mediating autoimmunity in the PD-1$^{-/-}$ HSC model, we generated a double mutant Fas^{lpr} × Rag$^{-/-}$ mouse, which lacks functional Fas expression (39). While Rag$^{-/-}$ mice developed systemic autoimmune disease around day 44 after transfer, Rag$^{-/-}$ × CiiTA$^{-/-}$ were completely spared (**Figure 3A**). Similarly, the pronounced weight loss encountered in the Rag$^{-/-}$ recipient group was not seen in the Rag$^{-/-}$ × CiiTA$^{-/-}$ recipients and indeed the latter group actually gained weight for much of the experiment (**Figure 3B**) despite CD8 T cell and B cell development by day 46 post-transfer (**Figure 3C**). These data indicate that MHC-II expression in the host is required for disease after PD-1$^{-/-}$ HSC transfer, suggesting disease is dependent on CD4 T cells. Transfer of

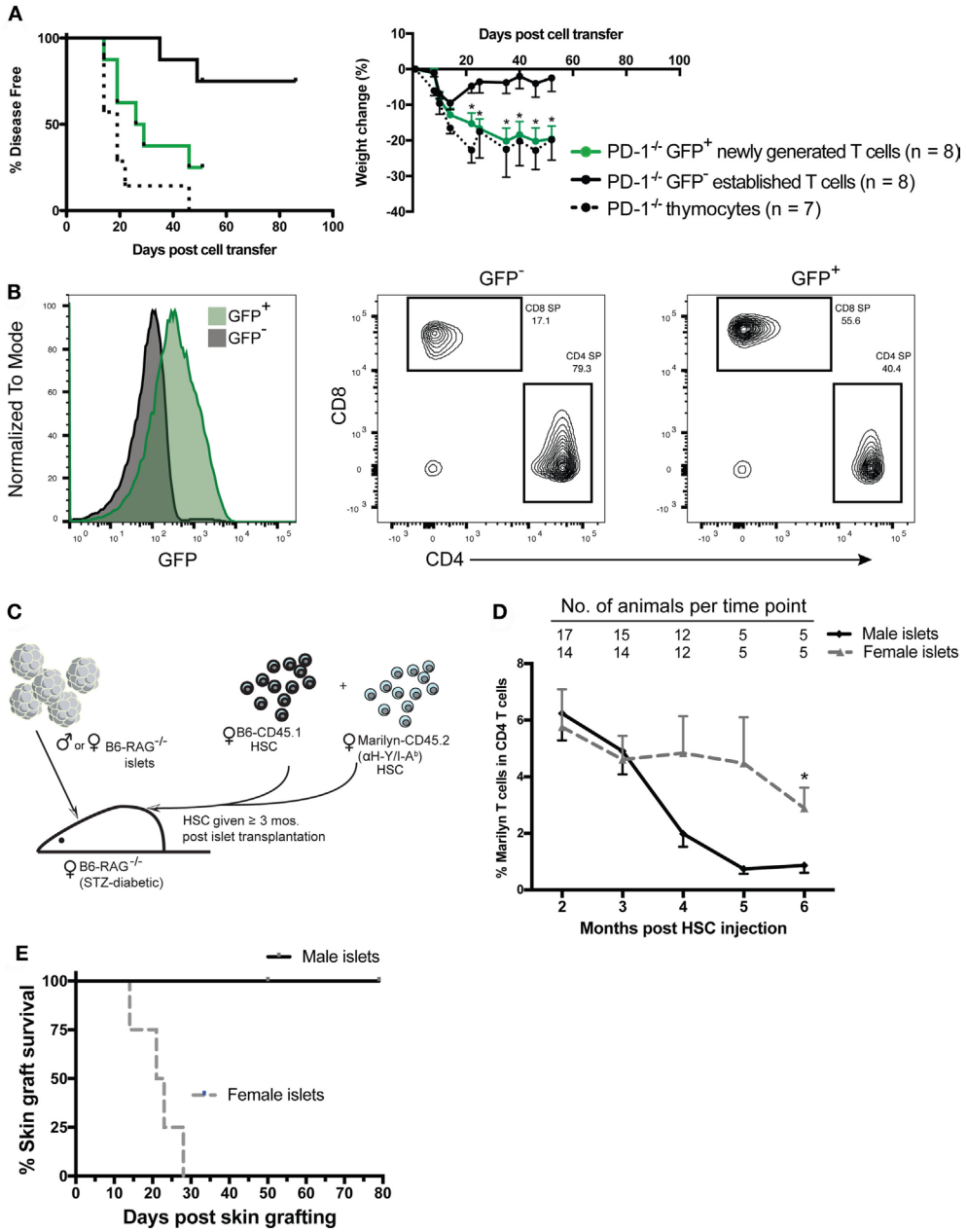

FIGURE 2 | Peripheral PD-1$^{-/-}$ newly generated T cells preferentially drive autoimmunity in lymphopenic recipients. CD4 and CD8 single positive (SP), GFP$^+$, and GFP$^-$ cells were sorted from ~6-week-old Rag2pGFP × PD-1$^{-/-}$ splenocytes. 1 × 10^6 sorted CD4 and CD8 SP GFP$^+$ or GFP$^-$ cells, or unfractionated thymocytes containing an equivalent number of CD4 and CD8 SP cells (~8–9 × 10^6) were injected into NK depleted adult Rag$^{-/-}$ recipients and mice were monitored for disease symptoms. **(A)** *Left panel:* disease incidence in recipients of thymocytes, GFP$^+$ newly generated T cells or GFP$^-$ established T cells. Survival curve comparison demonstrated a significant difference between the three groups with $p = 0.003$; PD-1$^{-/-}$ GFP$^+$ newly generated cell recipient vs. established cell recipient curves $p = 0.02$; PD-1$^{-/-}$ thymocyte recipient vs. PD-1$^{-/-}$ GFP$^+$ newly generated cell recipient curves, p = NS. Data are combined from two independent experiments, starting n values are indicated in the legend. *Right panel:* weight changes in recipients of indicated cells from two independent experiments ± SEM. Weight observations for which similar time points were available between experiments (±2 days) were combined and used for this analysis. *$p < 0.05$, GFP$^+$ newly generated T cells vs. GFP$^-$ established T cells, one-way ANOVA with Tukey's multiple comparison test. **(B)** Representative GFP expression (left panel) and CD4 and CD8 SP proportions (right 2 panels) in purified cell populations used in **(A)**. **(C)** Diagram of experimental approach using an established minor mismatched transplant as a model peripheral neo-self antigen and anti-donor (HY) TCR transgenic (Tg) Marilyn T cells to track the frequency of CD4 T cells specific to the "neo-self" antigen over time. Male (or control female) islet transplants were allowed to heal ≥3 months into streptozotocin (STZ)-induced diabetic female Rag$^{-/-}$ recipients prior to immune reconstitution *via* transfer of female hematopoietic stem cells (HSC) from CD45 congenic wild-type (B6-CD45.1) and monoclonal TCR Tg mice. **(D)** Frequency of Marilyn T cells (CD4$^+$ CD45.2$^+$ CD45.1$^-$ Vβ6$^+$) within total CD4 T cells in the peripheral blood of recipients with male vs. female islets that maintained normoglycemia is shown (mean, SEM, and n for each time point is shown). *$p < 0.05$, Student's t-test. **(E)** Six months post fetal liver cell injection, male skin grafts were transplanted to normoglycemic Rag$^{-/-}$ recipients that had either a male ($n = 5$) or female ($n = 4$) islet transplant and grafts were monitored for rejection. Survival curves are significantly different with $p = 0.018$, Mantel–Cox test.

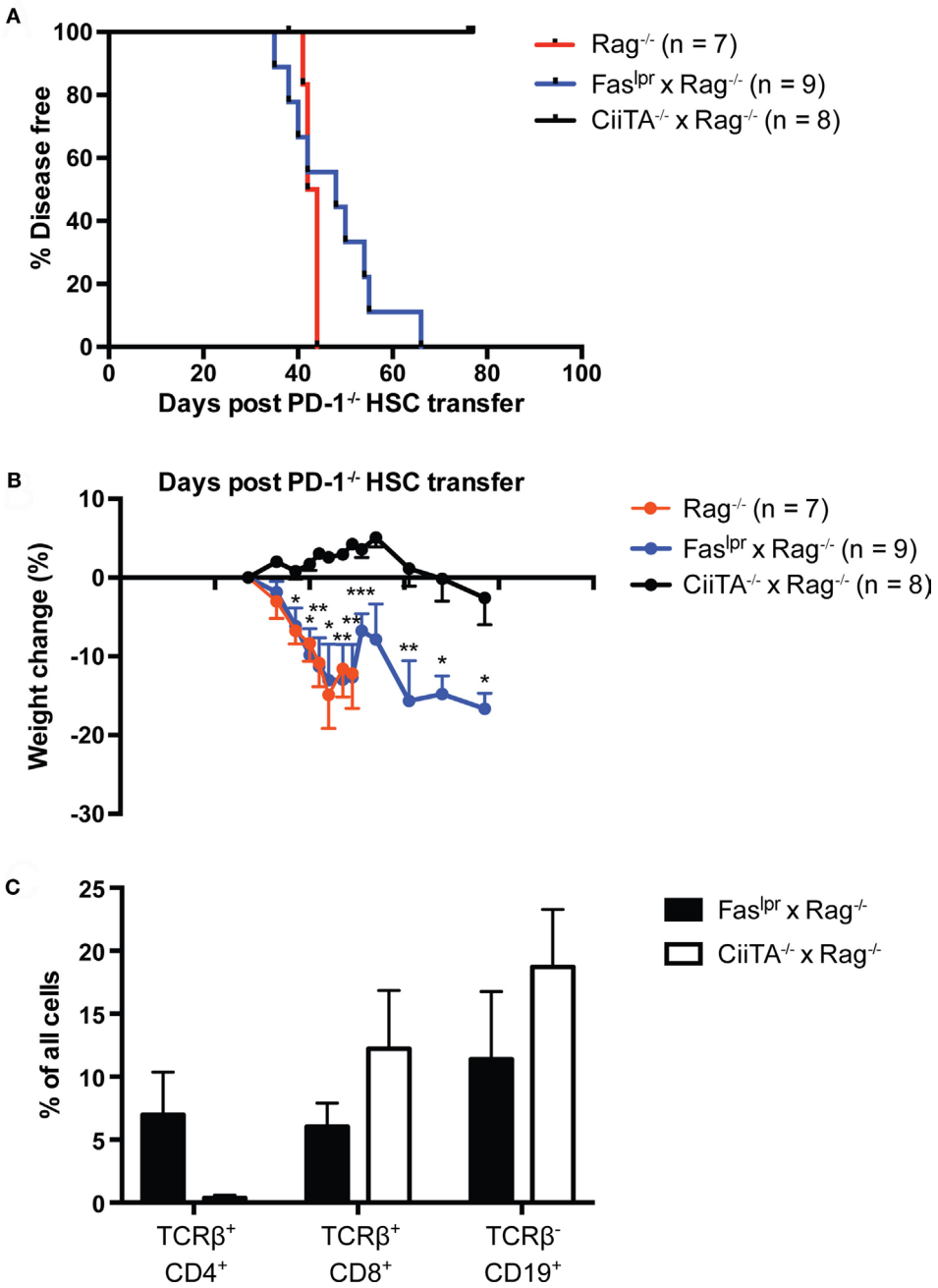

FIGURE 3 | Systemic autoimmunity in lymphopenic PD-1$^{-/-}$ hematopoietic stem cells (HSC) recipients requires host MHC Class II but not Fas. PD-1$^{-/-}$ HSC were transferred to Rag$^{-/-}$, Fas^{lpr} × Rag$^{-/-}$, or CiiTA$^{-/-}$ × Rag$^{-/-}$ recipients and mice were monitored for disease symptoms. **(A)** Kaplan–Meier survival curve analysis of disease incidence among HSC recipients—groups are significantly different with $p = 0.0002$. Data are combined from two independent experiments, with starting numbers per group indicated in the legend. **(B)** Weight changes in recipients relative to day 27 post-transfer, combined from two independent experiments, ±SEM. Weight observations for which similar time points were available between experiments (±2 days) were combined. For each timepoint, only data from groups with $n > 2$ are presented. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Fas^{lpr} × Rag$^{-/-}$ vs. CiiTA$^{-/-}$ × Rag$^{-/-}$, one-way ANOVA with Tukey or Kruskal–Wallis with Dunn's multiple comparison test (days 27–49) or Student's *t*-test (day > 51). **(C)** % of all peripheral blood cells that were CD4+ or CD8+ T cells or CD19+ B cells in Fas^{lpr} × Rag$^{-/-}$ and CiiTA$^{-/-}$ × Rag$^{-/-}$ recipients from one experiment in **(A,B)** above, measured at day 46 post-HSC transfer.

PD-1$^{-/-}$ HSC to Fas^{lpr} × Rag$^{-/-}$ double mutants revealed that host Fas expression was completely dispensable for the generation of autoimmunity in this model (**Figures 3A,B**). No statistically significant difference in the survival curves of the Rag$^{-/-}$ and Fas^{lpr} × Rag$^{-/-}$ recipient groups was detected. Similarly, weight loss in Fas^{lpr} × Rag$^{-/-}$ hosts was indistinguishable from that seen in the Rag$^{-/-}$ hosts (**Figure 3B**). Thus, host Fas expression is not required for disease post PD-1$^{-/-}$ HSC transfer.

Perforin Expression in the Lymphoid Compartment Is Not Required for LIP-Driven Autoimmunity after Transfer of PD-1$^{-/-}$ Thymocytes

Next, we examined whether perforin expression in lymphocytes was required for disease following transfer of PD-1$^{-/-}$ thymocytes to a lymphopenic host. We generated perforin knockout (Prf1$^{-/-}$) and PD-1$^{-/-}$ mice. The double knockout animals we were able to generate were the F1 progeny of heterozygous crosses and never successfully bred in our facility as homozygous double knockouts (unpublished observations). We transferred thymocytes from the Prf1$^{-/-}$ x PD-1$^{-/-}$ or PD-1$^{-/-}$ mice to Rag$^{-/-}$ recipients and monitored the animals for disease and weight loss. Prf1$^{-/-}$ × PD-1$^{-/-}$ thymocyte recipients all developed autoimmune disease between days 13 and 21 post-transfer, while the PD-1$^{-/-}$ thymocyte recipients had a relatively slightly delayed course of disease (**Figure 4A**, $p = 0.01$). Furthermore, weight loss (**Figure 4B**) was significantly greater in the Prf1$^{-/-}$ × PD-1$^{-/-}$ thymocyte recipients, and in general the severity of symptoms, particularly diarrhea, appeared greater in this group compared to PD-1$^{-/-}$ thymocyte recipients. In addition to these findings, we also performed two experiments to question whether Prf1$^{-/-}$ was required for disease in the HSC transfer model. In the first experiment, Prf1$^{-/-}$ × PD-1$^{-/-}$ T cell depleted bone marrow cells were transferred to Rag$^{-/-}$ hosts. In the second, Prf1$^{-/-}$ HSC were transferred to Rag$^{-/-}$ hosts treated intraperitoneally with 200 µg/mouse monoclonal blocking anti-PD-1 antibody (clone J43) every 2 days from day 25 until termination. In both of these experiments, recipients developed autoimmunity (data not shown) further supporting the notion that perforin expression in T cells is not required for disease in this model.

Because of the potential for functional redundancy between the Fas–FasL and perforin-dependent killing pathways, we also tested whether adoptive transfer of cells from diseased Rag$^{-/-}$ recipients of Prf1$^{-/-}$PD-1$^{-/-}$ thymocytes to Fas^{lpr} × Rag$^{-/-}$ hosts would result in the development of autoimmunity. Indeed, splenocytes + lymph node cells from the diseased Prf1$^{-/-}$PD-1$^{-/-}$ thymocyte recipients generated robust development of autoimmunity when transferred either to Rag$^{-/-}$ or Fas^{lpr} × Rag$^{-/-}$ recipients (disease onset: day 18, 22, >22 vs. 13, 18 × 3, 22, respectively; p = NS). Although both recipient groups lost weight, by day 22 post-adoptive transfer the Fas^{lpr} × Rag$^{-/-}$ hosts had lost significantly more weight than the Rag$^{-/-}$ recipients (25 vs. 15%, **Figure 4C**), suggesting that Fas in the host may actually play an immunoregulatory role. Taken together, these data show that neither perforin expression in T cells nor Fas expression in the host are required for LIP- and newly generated T cell-driven autoimmunity in the context of PD-1 deficiency.

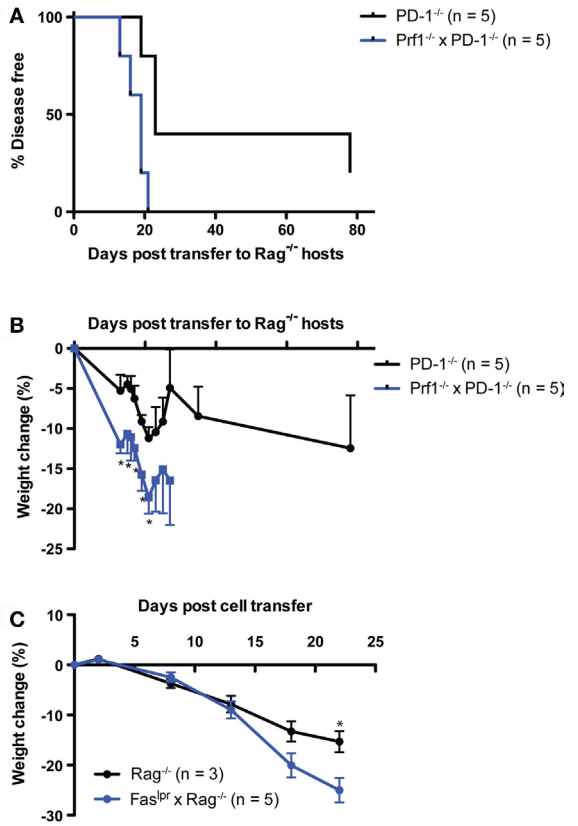

FIGURE 4 | Systemic autoimmunity after PD-1$^{-/-}$ thymocyte transfer to lymphopenic recipients is independent of both perforin and Fas-mediated killing. 10 × 10^6 Thymocytes from PD-1$^{-/-}$ or Perforin$^{-/-}$ × PD-1$^{-/-}$ (Prf1$^{-/-}$ × PD-1$^{-/-}$) animals were transferred i.v. to NK depleted adult Rag$^{-/-}$ recipients and mice were monitored for disease symptoms. **(A)** Kaplan–Meier survival curve analysis of disease incidence among individual recipients of the indicated thymocytes. Survival curve comparison demonstrated a significant difference between the two groups with $p = 0.01$. **(B)** Weight change among the recipients in **(A)**, ±SEM. Data are from one experiment, starting n values are indicated in the legend. **(C)** Splenocytes and inguinal lymph node cells were harvested from three diseased Prf1$^{-/-}$ × PD-1$^{-/-}$ thymocyte recipients from the experiment depicted in **(A)** at day 28 post-transfer, and 20 × 10^6 cells were transferred i.v. into NK depleted, adult Rag$^{-/-}$ or Fas^{lpr} × Rag$^{-/-}$ recipients. One Fas^{lpr} × Rag$^{-/-}$ recipient received 10 × 10^6 cells. Weight change among cell recipients, ±SEM. *$p < 0.05$, Student's t-test. Data are from one experiment with starting n values in the legend.

DISCUSSION

Mechanisms of T cell homeostasis function to maintain a diverse repertoire of sufficient size for effective immune surveillance of the host. Reconstitution of the T lymphocyte compartment of a lymphopenic host by HSC transplant or transfer of T cells results in LIP as the cells expand to fill the available niche defined by available peptide–MHC (pMHC) and cytokine "resources." The relative abundance of resources in a host with respect to the numbers of competitors for those resources can be considered as a way to define the "severity" of lymphopenia in that host or its "LIP-potential" (40). Importantly, LIP is strongly associated with the promotion of autoimmune or inflammatory disease (5, 13, 17, 24, 40–49). Such promotion of autoimmunity may result from potentiation of TCR signaling by high concentrations of homeostatic cytokines like IL-7 and IL-15 (50, 51) and/or unimpeded access to self-pMHC due to decreased competition from other Tcon or Treg. Thus, perturbations which would be expected to increase the LIP potential of a host, such as

decreased Treg, increased homeostatic cytokines, or decreases in co-inhibitory signals would be expected to increase the potential for such autoimmunity, and *vice versa*. Newly generated T cells during LIP appear particularly predisposed to the generation of autoimmunity when PD-1 mediated co-inhibitory signals are compromised (24). Newly generated T cells have not been subjected to peripheral tolerance mechanisms and thus may contain an increased proportion of strongly self-reactive cells compared to established T cells that arose in a host with low LIP potential upon initial seeding of its peripheral T cell compartment (i.e., during the neonatal period) and were tolerized. Indeed, in the current studies, we showed that a high frequency of CD4 T cells specific to a peripheral neo-self antigen can persist for an extended period in a lymphoreplete adult host, declining slowly with time without overt immune-mediated pathology (i.e., lack of rejection of the neoantigen expressing graft; **Figures 2C–E**). In addition, we have recently shown that PD-1 contributes to control of tonic pMHC signaling, which may tune signaling thresholds to establish a general T cell homeostasis needed for peripheral tolerance (52). Altogether our data suggests that PD-1 is more critical for control of newly generated CD4 T cells because of increased self-specific T cells in this population as well as a need to set response thresholds to tonic pMHC signals in all CD4 T cells. Although this view is consistent with recent findings from Jiang and colleagues, in which they showed PD-1 was important in reducing the number of high-affinity self-specific CD4 T cells (53), their conclusion differs substantially from our own. While they concluded that PD-1 is needed to maintain tolerance, both their data and ours are instead consistent with the view that PD-1 is needed to establish tolerance, with PD-1 playing relatively little or no role in the maintenance of tolerance once it is established. On the other hand, much of the published literature describing newly generated T cells/RTE suggests that they have functional properties geared toward promotion of tolerance (26). However, we found that newly generated PD-1$^{-/-}$ T cells isolated from the periphery of adult animals were indeed capable of generating autoimmune disease (particularly cachexia, kyphosis, and diarrhea) similar to PD-1$^{-/-}$ thymocytes, and much more efficiently than established T cells which contained approximately 1.3% contaminating GFP^{+} cells (**Figure 2A**). These data indicate that steady state newly generated T cells have a dangerous autoimmune potential even when their initial export from the thymus is into a lymphoreplete environment.

Tolerance first becomes established in mice during the neonatal period. Compared to adult Rag$^{-/-}$ mice, we consider that prior to/during initial seeding of the lymphoid compartment neonatal mice would, by virtue of anatomic size (small lymph nodes, reduced overall resources) and competition by specialized populations of innate lymphoid cells for IL-7 (54) have significantly lower LIP potential. This is supported by our finding that neonatal Rag$^{-/-}$ hosts rarely developed severe autoimmunity after reconstitution with PD-1$^{-/-}$ HSC (24). A lymphoreplete adult WT host on the other hand would be considered to have lower LIP potential than either an adult Rag$^{-/-}$ or a WT neonate (i.e., the ranking of LIP potential would be adult Rag$^{-/-}$ > neonatal WT > adult WT) (40). We hypothesize that the mild but significant LIP potential in neonates creates a situation in which establishment of tolerance in the nascent T cell population by PD-1 is important, and the absence of PD-1-mediated control of neonatal LIP may set the stage for the lupus-like autoimmunity that manifests later in life in PD-1$^{-/-}$ animals.

Although PD-1 is critical for tolerance in newly generated T cells, and PD-1 was expressed on neonatal T cells undergoing LIP, we did not, in the steady state, detect PD-1 expression above background on the peripheral newly generated T cell population of adult Rag2pGFP mice and only low expression in thymic GFP^{+} T cells (**Figure 1**). In contrast, both the peripheral CD4 SP and thymic CD4 and CD8 SP established (GFP^{-}) T cell population contained sizeable populations that robustly expressed PD-1. Previous data from our lab assessing PD-1 expression in steady state RTE (i.e., in WT lymphoreplete mice) used CD24 as the marker to define RTE and the data suggested RTE have higher PD-1 (24). However, based on our current data with the Rag2pGFP mouse and the fact that only a small fraction of newly generated T cells expressed CD24 and established T cells can also express CD24, we conclude that steady-state adult newly generated T cells do not have increased PD-1. Together with our finding that PD-1 can regulate LIP and the response to tonic pMHC signals (25, 52), these observations suggest that PD-1 is upregulated during LIP as a negative feedback mechanism. Finally, it is worth noting that because a significant portion (10–15%) of thymic T cells are established cells based on lack of GFP expression, consistent with the previously described ability of peripheral T cells to recirculate back to the thymus (55), the use of the term "recent thymic emigrants" to describe GFP^{+} cells in the Rag2pGFP Tg model as is common in the literature is somewhat imprecise as presumably some cells emerging from the thymus in an adult are also GFP^{-}. Therefore, although all peripheral GFP^{+} cells are RTE, all RTE are not necessarily GFP^{+}.

One potential explanation for our finding that purified peripheral newly generated T cells efficiently caused disease upon transfer to lymphopenic hosts while established cells did not is that the relative proportions of CD4 and CD8 T cells differed significantly between these populations. Based on the present data including the lack of disease in CiiTA$^{-/-}$ hosts despite generation of CD8 T cells and B cells (**Figure 3C**) and our previous findings that MHC Class I-deficient lymphopenic hosts were fully disease permissive, we conclude that CD4 T cells in this setting are the key effectors of autoimmune pathology. Given that the purified established T cell population contained a greater frequency of CD4 vs. CD8 T cells, their inability to drive disease (and conversely the ability of GFP^{+} PD-1$^{-/-}$ newly generated cells to do so) cannot be explained by insufficient numbers of CD4 T cells.

While CD4 T cells are most commonly considered as "helpers" of the immune response, numerous studies have suggested that they can in some circumstances acquire cytolytic effector function *via* upregulation of killing mechanisms typically associated with CD8 cytotoxic T lymphocytes such as the perforin and Fas-ligand (FasL) pathways (56). Our finding that neither Fas^{lpr} × Rag$^{-/-}$ recipients of PD-1$^{-/-}$ HSC, nor recipients of Prf1$^{-/-}$ × PD-1$^{-/-}$ thymocytes were spared from disease

(**Figures 3** and **4A,B**) suggests that both of these canonical T cell effector pathways are dispensable for LIP-driven autoimmunity in the setting of PD-1 deficiency. Furthermore, our finding that disease in Prf1$^{-/-}$ × PD-1$^{-/-}$ thymocyte recipients was exacerbated compared to PD-1$^{-/-}$ thymocyte recipients (**Figures 4A,B**) suggests that perforin-dependent effector pathways may actually play an immunoregulatory role during LIP. This is perhaps not surprising given that perforin has been reported to be important for contraction of the CD8 population following infection (57, 58), and as a mediator of suppression by Tr1 cells (59). Similarly, weight loss of increased severity in the Fas^{lpr} × Rag$^{-/-}$ compared to Rag$^{-/-}$ recipients of adoptively transferred Prf1$^{-/-}$ PD-1$^{-/-}$ cells (**Figure 4C**) suggested that Fas may also play an immunoregulatory role during LIP. This might also be anticipated given the lymphoproliferative disease characteristic of the Fas and Fas-ligand-deficient *lpr* and *gld* mice (60) which is partially attributable to defects in Fas-mediated killing of antigen-presenting cells (61). The latter experiment also ruled out functional redundancy between Fas–FasL and perforin-dependent killing pathways. We did consider the possibility that Granzyme B produced by T cells activated during LIP might act independently of perforin, for example through the mannose-6-phosphate receptor (62). However, examination of Granzyme B expression in WT vs. PD-1$^{-/-}$ HSC recipients at the peak of disease (day 45) demonstrated no significant difference in splenic CD4 or CD8 T cell populations although a small trend toward increased expression in PD-1$^{-/-}$ HSC recipient CD8 T cells was noted (Figure S2 in Supplementary Material).

The broad upregulation of a number of pro-inflammatory cytokines in PD-1$^{-/-}$ HSC recipients (IFN-γ, IL-13, TNF-α, IP-10, MIG, MCP-1, VEGF) (24) along with the lack of requirement for either perforin- or Fas-dependent killing pathways for autoimmunity in this model suggest that the immune pathology seen is primarily a CD4 T cell and "cytokine-storm" dependent phenomenon. Several similarities exist between the LIP-driven autoimmunity described herein and certain clinical syndromes, such as immune reconstitution inflammatory syndrome (IRIS) in HIV patients experiencing a rebound of the CD4 T cell compartment after treatment with antiviral drugs (44, 63) or in chronic GVHD post-allogeneic bone marrow transplantation (64). Notably a mouse model of IRIS was recently described in which transfer of purified CD4 T cells to *Cryoptococcus*-infected lymphopenic mice yields weight loss and systemic inflammatory disease associated with cytokine dysregulation (45). In addition, the striking increases in the chemokines IP-10 and MIG described previously in our model (24) are also observed in clinical chronic GVHD (65). Indeed, the term "cytokine-storm" was originally used to describe the syndrome of cytokine dysregulation (particularly IL-6, IL-1, and TNF-α) which is associated with and greatly contributes to GVHD pathology (66, 67). One could make the argument that our model of LIP-driven autoimmunity after transfer of PD-1$^{-/-}$ newly generated T cells to a lymphopenic host might actually be viewed as a model of syngeneic GVHD, similar to the autoimmunity that occurs when cyclosporine A (CsA) is discontinued post bone marrow transplantation. However, broad-spectrum antibiotics prevented CsA-induced syngeneic GVHD (68) but did not prevent disease in our model (25). Instead, the stimulus for PD-1-deficient RTE appears to involve autoantigens, potentially including the low-affinity interactions with self-peptide MHC that otherwise generate only tonic survival signals (40). Thus, beyond the insights it offers into the role of co-inhibitory molecules in establishing tolerance, further characterization of this model may lead to translatable insights to treat cytokine-driven systemic autoimmunity and inflammatory disease including after therapies involving immune reconstitution.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Canadian Council on Animal Care. The protocol was approved by the University of Alberta Health Sciences Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

KE designed, performed research and data analysis, and wrote and critically edited the manuscript. GT, YH, and JL designed and performed research and data analysis and critically edited the manuscript. LB provided reagents and critically edited the manuscript. CA designed research, performed data analysis, and critically edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2018.00012/full#supplementary-material.

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Challenges and Opportunities for Biomarkers of Clinical Response to AHSCT in Autoimmunity

Kristina M. Harris[1], Tingting Lu[1], Noha Lim[1] and Laurence A. Turka[1,2]*

[1] *Immune Tolerance Network, Bethesda, MD, United States,* [2] *Center for Transplantation Sciences, Massachusetts General Hospital, Boston, MA, United States*

Autoimmunity represents a broad category of diseases that involve a variety of organ targets and distinct autoantigens. For patients with autoimmune diseases who fail to respond to approved disease-modifying treatments, autologous hematopoietic stem cell transplantation (AHSCT) after high-dose immunosuppressive therapy provides an alternative strategy. Although more than 100 studies have been published on AHSCT efficacy in autoimmunity, the mechanisms that confer long-term disease remission as opposed to continued deterioration or disease reactivation remain to be determined. In a phase II clinical trial, high-dose immunosuppressive therapy combined with autologous $CD34^+$ hematopoietic stem cell transplant in treatment-resistant, relapsing-remitting multiple sclerosis (RRMS) resulted in 69.2% of participants achieving long-term remission through 60 months follow-up. Flow cytometry data from the 24 transplanted participants in the high-dose immunosuppression and autologous stem cell transplantation for poor prognosis multiple sclerosis (HALT-MS) trial are presented to illustrate immune reconstitution out to 36 months in patients with aggressive RRMS treated with AHSCT and to highlight experimental challenges inherent in identifying biomarkers for relapse and long-term remission through 60 months follow-up. AHSCT induced changes in numbers of CD4 T cells and in the composition of CD4 and CD8 T cells that persisted through 36 months in participants who maintained disease remission through 60 months. However, changes in T cell phenotypes studied were unable to clearly discriminate durable remission from disease reactivation after AHSCT, possibly due to the small sample size, limited phenotypes evaluated in this real-time assay, and other limitations of the HALT-MS study population. Strategies and future opportunities for identifying biomarkers of clinical outcome to AHSCT in autoimmunity are also discussed.

Keywords: autoimmunity, multiple sclerosis, immune cell reconstitution, biomarkers, immune tolerance, flow cytometry, T cells

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****Correspondence:***
Kristina M. Harris
kharris@immunetolerance.org

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Abbreviations: AHSCT, autologous hematopoietic stem cell transplantation; RRMS, relapsing-remitting multiple sclerosis; HALT-MS, high-dose immunosuppression and autologous stem cell transplantation for poor prognosis multiple sclerosis; SSc, systemic sclerosis; SLE, systemic lupus erythematosus; T1D, type 1 diabetes; NIH, National Institutes of Health; ITN, immune tolerance network; MRI, magnetic resonance imaging; EDSS, Expanded Disability Status Scale; TCR, T cell receptor; BCR, B cell receptor; RTE, recent thymic emigrants; PBMC, peripheral blood mononuclear cell; CNS, central nervous system; CSF, cerebrospinal fluid.

INTRODUCTION

Autologous hematopoietic stem cell transplantation (AHSCT) therapy utilizes immunoablation and immune reconstitution from hematopoietic progenitors. For more than two decades, AHSCT has been studied as a therapeutic approach for severe autoimmunity, including multiple sclerosis (MS), systemic sclerosis (SSc), systemic lupus erythematosus (SLE), Crohn's disease, type 1 diabetes (T1D), and rheumatoid arthritis (RA) (1). The rationale for AHSCT for treating aggressive autoimmunity is that immunoablative therapy diminishes the pool of self-reactive immune cells and allows engrafted stem cells to generate a new, potentially self-tolerant, immune repertoire (2). Mechanistic studies have revealed qualitative changes in the reconstituted immune system after AHSCT that favor immunoregulation over pro-inflammatory signatures (2, 3). The tolerogenic properties of the reconstituted immune system likely result from coordinated interactions of various immune competent cells with regulatory potential. This dynamic and collective process of immune reprogramming is thought to underlie AHSCT's mode of action.

Although more than 100 studies have been published, the mechanisms that confer long-term disease remission with AHSCT in autoimmunity, as opposed to limited short-term benefit and early disease recurrence, are not well understood. Restoration of regulatory immune networks may contribute to durable treatment; however, functional assessments of the reconstituted immune system compared to pretherapy profiles are needed to understand how AHSCT limits or controls autoreactive lymphocytes during and after repopulation. Currently, it is unclear whether relapse after AHSCT is driven by residual autoreactive memory cells that escaped depletion and are resistant to regulatory mechanisms in the renewed immune system or to re-emergence of a *de novo* autoreactive population, possibly reflecting a genetic predisposition to disease. In this perspective, we present data from the high-dose immunosuppression and autologous stem cell transplantation for poor prognosis multiple sclerosis (HALT-MS) trial to illustrate past and present approaches to address this question and discuss experimental challenges and strategies for identifying the biomarkers of clinical response to AHSCT in autoimmunity.

IMMUNE TOLERANCE NETWORK (ITN) HALT-MS TRIAL EXPERIENCE

High-dose immunosuppression and autologous stem cell transplantation for poor prognosis MS was a phase II clinical trial conducted by the ITN that investigated the efficacy of AHSCT in treatment-resistant patients with relapsing-remitting multiple sclerosis (RRMS) (4). Twenty-four participants underwent AHSCT and were evaluated through 60 months posttransplant for event-free survival, defined as survival without death or disease activity. Progression-free survival, clinical relapse-free survival, and magnetic resonance imaging (MRI) activity-free survival were 91.3, 86.9, and 86.3%, respectively, indicating that AHSCT without maintenance disease-modifying therapy was effective for inducing durable remissions of active RRMS for at least 5 years (4). The primary mechanistic objectives for the HALT-MS trial were to determine the impact of AHSCT on the diversity of T cell receptor (TCR) repertoires in reconstituted peripheral blood and intrathecal compartments and to assess the treatment effect on pro-inflammatory versus regulatory T cell phenotypes in peripheral blood.

Here, we present flow cytometry data from HALT-MS to demonstrate the characteristics of immune reconstitution in RRMS patients through 36 months post-AHSCT and to highlight potential confounders that interfere with identification of biomarkers for relapse and long-term remission. Of the 24 transplanted participants in HALT-MS, 3 experienced clinical relapse, 2 showed disease progression by increased Expanded Disability Status Scale, and 2 had increased MRI through 60 months follow-up. Results are displayed as mean values for the long-term remission group ($n = 15$–17) with individual lines for the seven participants who experienced disease reactivation at different times through 60 months follow-up to help illustrate the obstacles these variances impose on our biomarker efforts. We hypothesized that favorable changes in the balance of pro-inflammatory and regulatory/naive/hyporesponsive T cell phenotypes in reconstituted peripheral blood would be associated with long-term remission after AHSCT. Paired statistical comparisons pretherapy to posttherapy were restricted to the long-term remission group because of the sample size and heterogeneity of the group that experienced disease reactivation during the 60 months post-AHSCT follow-up.

Most differences in circulating lymphocytes occurred early after AHSCT at 1–2 months. Absolute numbers of CD8 and CD4 T cells and B cells diminished, while $CD56^{hi}$ precursor NK cells expanded after AHSCT (**Figure 1**). Numbers of CD8 T cells and B cells returned between 2 and 6 months post-AHSCT (**Figures 1A,C**), whereas CD4 T cells and CD4/CD8 ratios remained significantly decreased at 36 months post-AHSCT compared to pretherapy (**Figures 1B,D**). Numbers of CD56dim cytotoxic NK cells declined at 6 months post-AHSCT and remained reduced from pretherapy at 36 months. Early during reconstitution, CD4 and CD8 T cells reflected a bias toward memory phenotypes with reduced proportions of $CD27^{+}CD45RO^{-}$ naive cells (**Figures 2A,B**). Following their early increase, $CD27^{+}CD45RO^{+}$ central memory cells decreased and stayed significantly lower than pretherapy proportions at 36 months (**Figures 2C,D**). $CD27^{-}CD45RO^{+}$ effector memory cells transiently increased early post-AHSCT (**Figure 2E,F**). $CD31^{+}CD45RA^{+}CD45RO^{-}$ CD4 recent thymic emigrants (RTEs) increased after their initial decline and remained significantly elevated at 36 months post-AHSCT, indicative of thymic renewal (**Figure 2G**). $CD27^{-}CD45RO^{-}$ long-term memory CD8 T cells transiently diminished and reached pretherapy proportions at 36 months post-AHSCT (**Figure 2H**). A relative expansion of potentially senescent (5) $CD28^{-}CD56^{-}CD57^{+}$ CD8 T cells (**Figure 2I**) was observed after AHSCT at the expense of cytotoxic $CD28^{-}CD56^{+}CD57^{+}$ CD8 T cells (**Figure 2J**). These results are consistent with previous immunophenotyping studies in patients with MS and other autoimmune diseases treated with AHSCT (6–11).

Potential T cell biomarkers of response to AHSCT have been reported in MS and T1D (6, 7), including the expansion

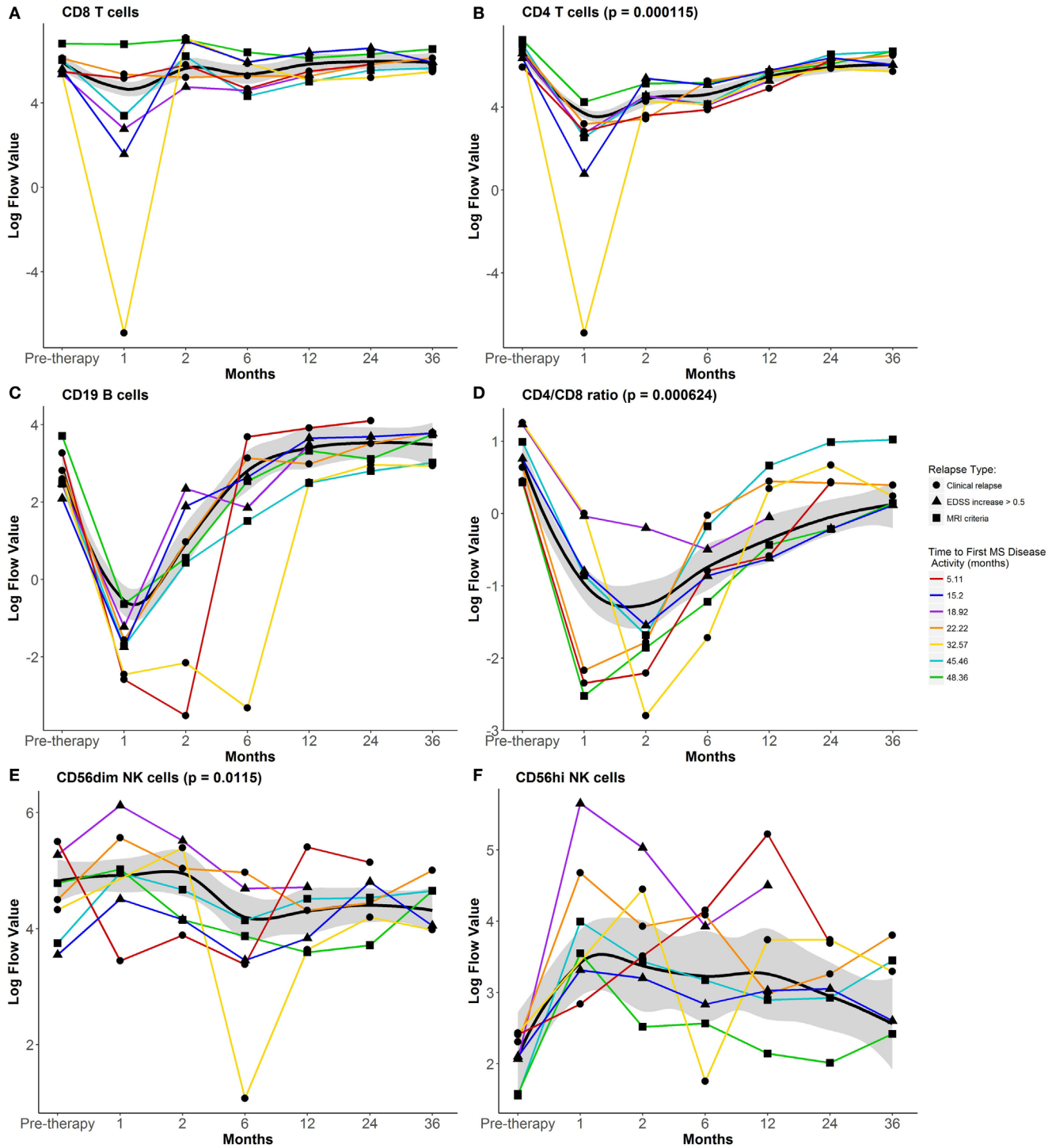

FIGURE 1 | Impact of autologous hematopoietic stem cell transplantation (AHSCT) on numbers of circulating lymphocyte populations through 36 months follow-up. Absolute cell numbers per microliter of whole blood were analyzed for **(A)** CD8 T cells, **(B)** CD4 T cells, **(C)** CD19 B cells, **(E)** $CD56^{dim}$ NK cells, and **(F)** $CD56^{hi}$ NK cells. **(D)** CD4/CD8 ratios were calculated from absolute cell numbers **(A,B)**. Flow data were plotted after log transformation for normalization of these variables. Data shown are mean values for the group that maintained remission through 60 months post-AHSCT. The black line represents the Loess Regression fitted curve with a span = 0.7, and its 95% confidence band colored in gray. Paired *t*-test was used to examine persistent changes at 36 months from pretherapy numbers within the group that maintained remission through 60 months post-AHSCT (n = 15). CD4 T cells, CD4/CD8 T cell ratio, and $CD56^{dim}$ NK cells were reduced from pretherapy numbers at 36 months post-AHSCT. Individual lines for the seven participants who experienced disease reactivation prior to 60 months post-AHSCT are plotted using different symbols to indicate the type of disease activity and different colored lines to indicate the time to first multiple sclerosis (MS) disease activity. For additional details including flow cytometry data without log-transformation, see https://www.itntrialshare.org/HALTMS_fimmu_fig1.url.

of regulatory CD4 (Foxp3^{+}CD127^{lo}) and CD8 (CD28^{-}CD57^{+} or PD-1^{+}) T cells. However, T cell phenotypes that clearly discriminate disease reactivation from long-term remission post-AHSCT have not been identified in the immunophenotyping studies presented here or previously published for HALT-MS (10). Overall reconstitution patterns appeared similar for participants who did and did not achieve remission through 60 months post-AHSCT. While a few dramatic deviations from the mean

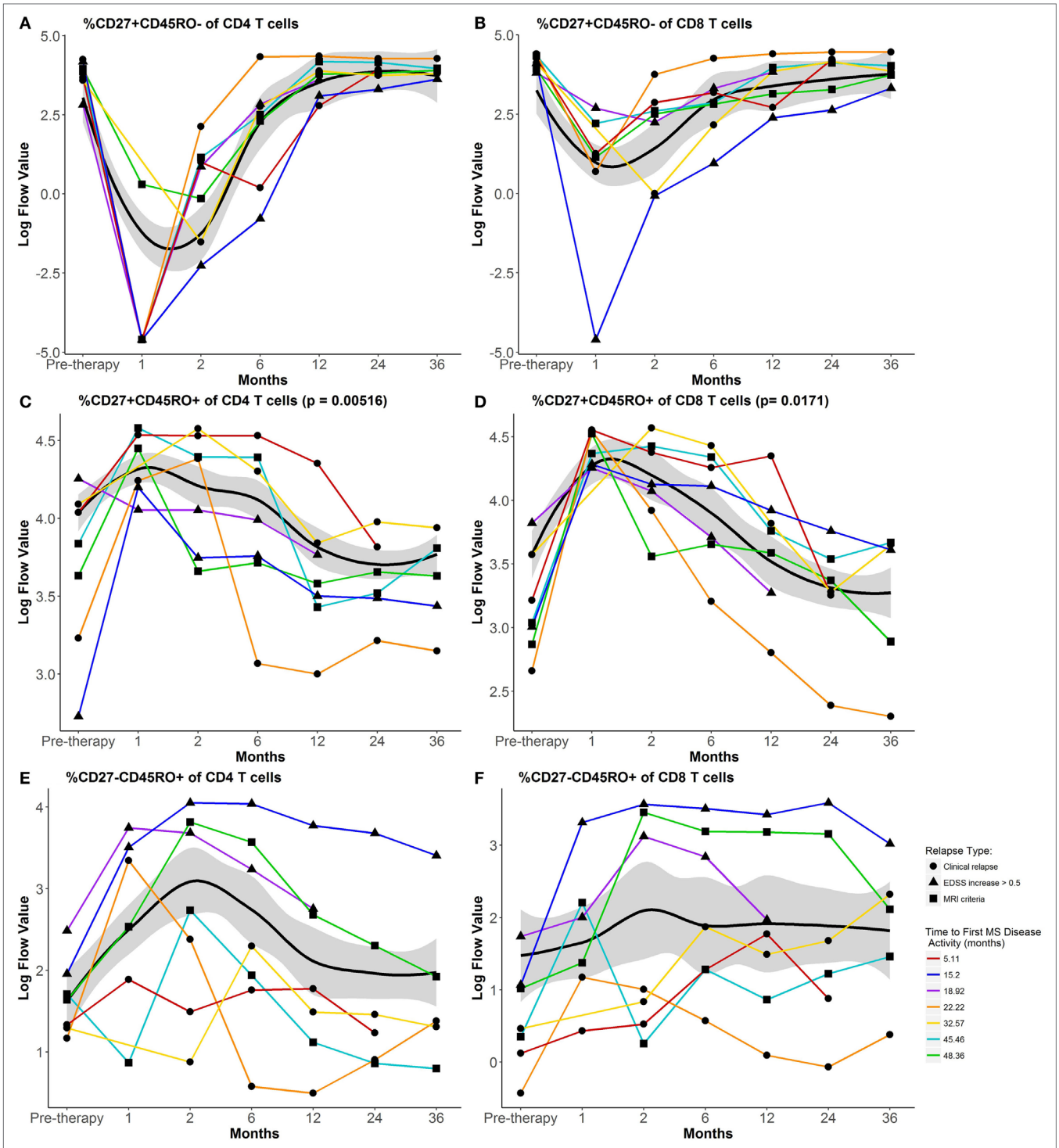

FIGURE 2 | Continued

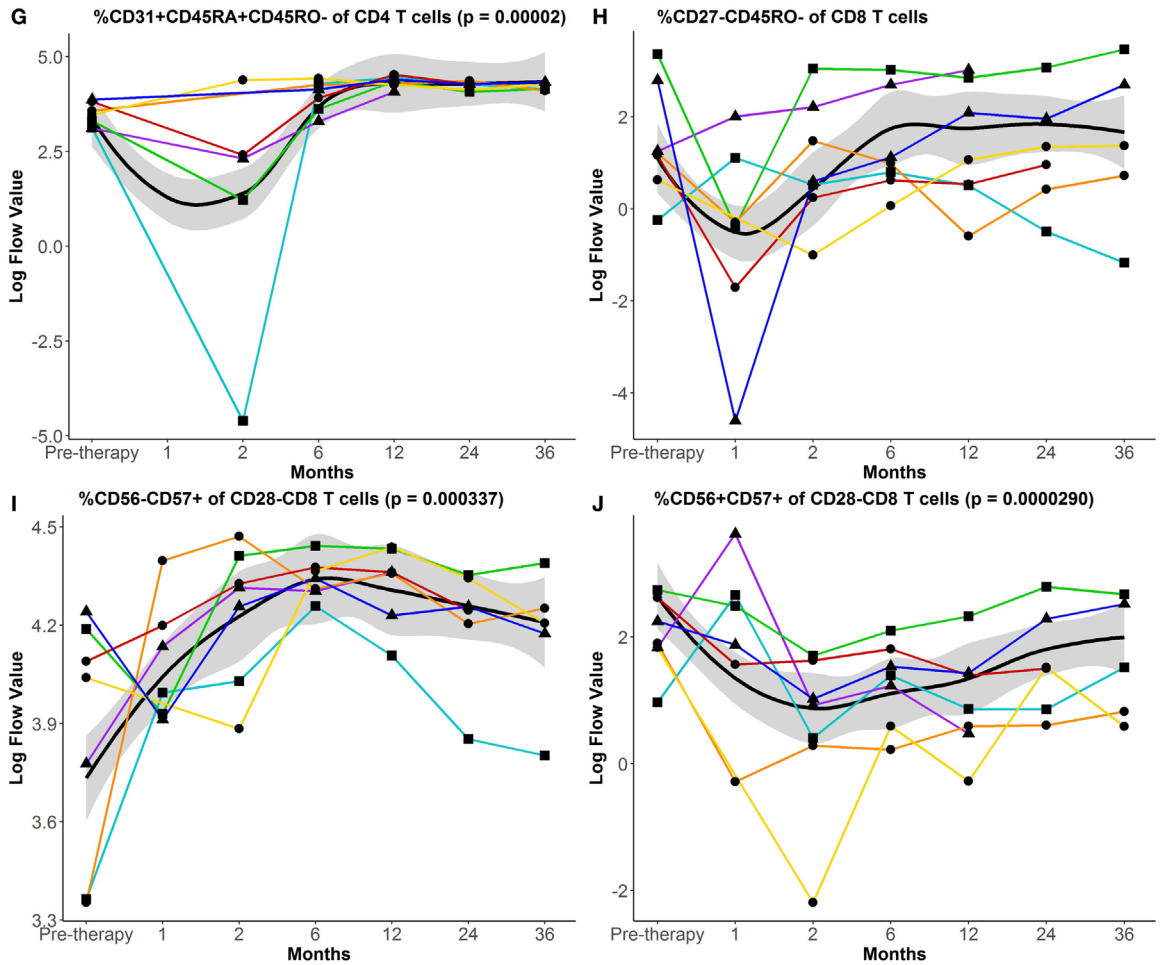

FIGURE 2 | Impact of autologous hematopoietic stem cell transplantation (AHSCT) on the composition of circulating CD4 and CD8 T cells through 36 months follow-up. Percentages of $CD27^{+}CD45RO^{-}$ naive CD4 **(A)** and CD8 **(B)** T cells, $CD27^{+}CD45RO^{+}$ central memory CD4 **(C)** and CD8 **(D)** T cells, $CD27^{-}CD45RO^{+}$ effector memory CD4 **(E)** and CD8 **(F)** T cells, **(G)** $CD31^{+}CD45RA^{+}CD45RO^{-}$ CD4 recent thymic emigrants (RTEs), **(H)** $CD27^{-}CD45RO^{-}$ long-term memory CD8 T cells, **(I)** $CD56^{-}CD57^{+}CD28^{-}$ CD8 T cells, and **(J)** $CD56^{+}CD57^{+}CD28^{-}$ CD8 T cells. Flow data were plotted after log transformation for normalization of these variables. Data shown are mean values for the group that maintained remission through 60 months post-AHSCT. The black line represents the Loess Regression fitted curve with a span = 0.7, and its 95% confidence band colored in gray. Paired *t*-test was used to examine sustained changes at 36 months from pretherapy numbers within the group that maintained remission through 60 months post-AHSCT (n = 15). AHSCT induced persistent changes in relative proportions of central memory and RTE phenotypes in reconstituted CD4 T cells. In reconstituted CD8 T cells, sustained alterations in proportions of central memory, $CD56^{-}CD57^{+}CD28^{-}$ (senescent) and $CD56^{+}CD57^{+}CD28^{-}$ (cytotoxic) phenotypes were observed. Individual lines for the seven participants who experienced disease reactivation before 60 months post-AHSCT are plotted using different symbols to indicate the type of disease activity and different colored lines to indicate the time to first multiple sclerosis (MS) disease activity. For additional details including flow cytometry data without log-transformation, see https://www.itntrialshare.org/HALTMS_fimmu_fig2.url.

of the long-term remission group were observed for individuals who did not maintain remission, no consistent changes in cell subtypes studied characterized all or any of the three categories of disease reactivation. These changes could reflect true biology or could be spurious findings unrelated to disease activity or adverse events. Two of the seven participants were in remission at the time of this analysis and relapsed by MRI activity later on; therefore, it is possible that relevant biomarkers had not yet re-emerged. Interpretation is limited by the sample size and various times to disease reactivation, particularly since specimens were not collected temporal to disease activity before rescue medication was given. Expansion of regulatory CD4 T cells ($Foxp3^{+}CD25^{hi}$, $CD25^{hi}CD127^{lo}$) has been reported in AHSCT trials in MS, SSc, SLE, and T1D (6, 7, 12–14); however, an equivalent phenotype was not analyzed when this "real-time" flow analysis was initiated at the start of the HALT-MS trial. This highlights one of the major limitations of real-time assays for biomarker research; they are often outdated by the end of the trial. Highly sophisticated immunophenotyping studies are in progress to assess reconstitution of T and B cell phenotypes relevant to MS and autoimmunity post-AHSCT; however, our response to biomarker efforts will still be confounded by the small sample size and suboptimal collection

schedule of participants who experienced disease reactivation after AHSCT during the 60 months follow-up in HALT-MS. These challenges and others are discussed below.

CHALLENGES OF BIOMARKER DISCOVERY FOR CLINICAL RESPONSE TO AHSCT

Clinical Trial Design and Disease Heterogeneity

Nearly all studies evaluating AHSCT in autoimmunity have been open-label, single-arm, observational phase I/II trials (1, 2). Although highly efficacious, the nature of these trials imposes challenges on biomarker research. These include small numbers of transplanted participants, fewer subjects in potentially unbalanced and subjective binary outcome groups, and the lack of appropriate controls. Heterogeneity within a given autoimmune disease can manifest as a spectra of disease severity, duration, clinical manifestations, and tissue targets. Biological diversity and treatment history of a study population can also influence participant responses at each step of the transplantation procedure, including stem cell mobilization, conditioning efficacy, stem cell engraftment, and immune reconstitution. The timing and type of prior immunotherapy can alter the immunological profile pretherapy with implications on biomarkers that predict or correlate with response to AHSCT. Inconsistencies between transplantation protocols can influence the range, degree, and kinetics of immune cell depletion and reconstitution. For example, autoimmune activity may persist if depletion of autoreactive lymphocytes is incomplete or re-establish with the graft, depending on the conditioning regimen and grafted stem cells (i.e., $CD34^+$ selected or not) (15). Some of these interindividual variables can and should be mitigated during protocol development with careful planning of inclusion/exclusion criteria for the study.

Specimen Collection

Since the primary objective of AHSCT is to achieve durable remission of autoimmunity without immunosuppressive agents, a long-term follow-up period is required for the assessment of clinical and scientific goals. This provides researchers the opportunity to monitor the dynamic process of immune reconstitution for biomarker discovery. It also magnifies potential variances between biomarker studies as standardized schedules for specimen collection post-AHSCT were lacking. Guidelines for immune monitoring and biostorage of AHSCT-treated autoimmune patient specimens have been published to provide a consistent framework for future biomarker research (16). Specimens should be collected at two time points pretherapy, at regular intervals post-AHSCT, and proximal to the first disease-related event prior to disease-modifying treatment. The latter is essential for identifying changes in biomarkers associated with durable versus short-term remission to AHSCT.

Serial samples of whole blood, peripheral blood mononuclear cells (PBMCs), plasma, serum, saliva, and urine specimens can be readily obtained in sufficient volumes for biomarker studies of AHSCT in autoimmunity. In contrast, sequential sampling of disease-relevant tissues may not be possible or may be limited due to ethical constraints and the practicalities of trial conduct. Preanalytical variations can be introduced by sample types, timing, technique, and collection devise; handling and storage conditions, including stabilizing agents, temperature, duration, and freeze–thaw cycles; and documentation of specimen data (17). Since few patients are transplanted at each site in multicenter trials, these variables can adversely affect overall findings and reproducibility of biomarker studies. Therefore, standard and optimized protocols must be followed for each step of specimen collection. Centralized processing and testing of high-quality, banked specimens at designated, well-qualified laboratories is strongly recommended. Cryopreserved specimens are particularly useful because they permit focused design and execution of biomarker studies at the end of the trial, when all end points have been identified. Banked specimens allow simultaneous testing of all visits from a participant using state-of-the-art technologies in a single laboratory. This helps minimize interassay deviations, avoids testing immune parameters and technologies that may become obsolete during the trial, and eliminates interlaboratory variability. All of which can confound detection of changes in biomarkers that are rare such as autoreactive and regulatory lymphocytes. Although banked specimens are preferred for mechanistic studies, it is beneficial to perform a reliable full blood count analysis on fresh specimens when feasible to enumerate major leukocyte populations for interpreting immunological changes induced by AHSCT in banked specimens.

Experimental Design

Mechanistic studies of AHSCT in autoimmunity have focused on disease-specific autoimmune parameters in the context of general immune reconstitution using immunophenotyping and transcriptional analyses. Despite rapidly evolving technologies and variation between cohorts, this global approach has revealed similar patterns of immune reconstitution and qualitative changes in PBMC in different autoimmune diseases (3, 18–20). This supports the hypothesis that AHSCT reprograms the self-destructive immune system toward a tolerant state; however, more sophisticated functional studies and analytical approaches are required for discovery and validation of biomarkers of response to AHSCT.

Different types of autoimmunity are associated with distinct immunological signatures; therefore, biomarker(s) of response to AHSCT may be disease specific and require different assays. The prevailing view is that both B cells and T cells are important in SSc, SLE, and MS, whereas the role of B cells in T1D is not clear (2). Previous studies indicated that myelin-specific CD4 T cell responses are initially limited in MS patients after AHSCT, but return to pretherapy levels after immune reconstitution (21, 22). It is not evident whether these autoreactive CD4 T cells re-emerged through incomplete immune ablation or were generated *de novo* and if they are even relevant to disease. Reliable *ex vivo* assays for phenotyping and sorting disease-relevant central nervous system (CNS) antigen-reactive T cells for molecular assays pretherapy to posttherapy are highly desirable because they could determine whether functional differences of reconstituted T cells

are associated with clinical response. However, validated assays for MS clinical trials that do not require *in vitro* manipulation are still in development, in part because CNS antigen-reactive T cells are rare in blood of MS patients, and disease-relevant CNS antigens need to be clarified. This approach could be used in other diseases where the autoantigen is known, and *ex vivo* tetramer reagents are available, such as in T1D (7).

In the absence of a validated assay for assessing autoreactive T cells in HALT-MS, we used TCR repertoire analysis of CD4 and CD8 T cells in blood and the cellular fraction of cerebrospinal fluid (CSF) to better understand how AHSCT shapes adaptive immunity in the reconstituted immune system. CSF is the compartment in closest proximity to the CNS parenchyma that might reflect immune pathology in MS. The impact of AHSCT on TCR repertoire diversity was investigated during the HALT-MS trial, and distinct effects on circulating CD4 and CD8 T cells were identified. The majority of CD4 TCR clones arose *de novo*, and there was expansion of pre-existing CD8 clones through 12 months post-AHSCT (23). Reconstitution of a new and diverse TCR repertoire in blood has been reported in other trials of AHSCT in autoimmunity; however, it is difficult to compare with our findings because of a number of technical differences (11, 24, 25). Additional TCRβ sequencing analyses are in progress to evaluate the impact of AHSCT on the pre-existing TCR repertoire in CSF and to determine whether AHSCT changes the pattern of clonal sharing between CSF and blood in HALT-MS.

Analytical Approach and Biomarker Validation

Overwhelming amounts of mechanistic data can now easily be generated and integrated with relevant clinical data for hypothesis-based and unbiased biomarker discovery approaches. This presents a major challenge for determining the right data sets to include and the appropriate statistical models needed to address key scientific questions (26). Different data sets and analytical approaches are necessary for addressing the two primary aims shared by all mechanistic studies of AHSCT, which include identification of biomarkers that predict, and those that correlate with, response outcomes. Biomarkers that predict outcome can be used to stratify patient populations for improved efficacy of AHSCT. However biomarkers refractory to, or induced by, AHSCT that correlate with poor outcome may help identify therapeutic targets for more durable remission with sequential administration of approved agents. For example, a small study by Capobianco et al. indicated that natalizumab after AHSCT could be an effective strategy for RRMS patients who continue to deteriorate after AHSCT therapy (27).

Before putative biomarkers that correlate with clinical response can help guide clinical practice, they must be validated and shown to be clinically meaningful. This is the biggest challenge for biomarker efforts because well-matched cohorts may not be available due to inconsistencies in trial designs and specimen collection (17) or in response to safety concerns like high morbidity in the T1D trial of AHSCT (28). Validation cohorts should involve prospective, controlled, randomized trials with standardized specimen collection, reliable assays, bioinformatics, and data sharing.

OPPORTUNITIES FOR BIOMARKERS OF RESPONSE TO AHSCT

High-dose immunosuppression and autologous stem cell transplantation for poor prognosis MS demonstrated that it is possible to achieve durable remission in patients with treatment-resistant RRMS using AHSCT therapy (4). We speculate that a combination of persistent changes in T cell numbers and composition that support increased thymic output, TCR repertoire renewal, and immune senescence and/or regulation over central memory or cytotoxicity contribute to long-term remission in HALT-MS. Still, functional and transcriptional studies of autoreactive, pro-inflammatory, and tolerogenic T and B cell subsets collected at optimal time points from sufficient numbers of participants in long-term remission and disease reactivation groups are needed to show that AHSCT induces qualitative changes in favor of immune tolerance that persist in patients achieving durable remission. In addition, TCR/B cell receptor sequencing in CSF and blood are necessary to identify clonotypes associated with disease reactivation and to determine whether renewal/diversification of the reconstituted adaptive immune system contributes to long-term remission post-AHSCT. HALT-MS can provide clues about some of these important questions; however, validation of putative response biomarkers will require larger patient numbers, independent cohorts, and appropriate controls.

The success of the HALT-MS trial has provided the foundation for a clinical trial in the development by the National Institutes of Health and the ITN that will compare AHSCT with best available approved therapy in the treatment of RRMS. This trial presents a unique opportunity to follow-up on TCR repertoire and immunophenotyping studies from HALT-MS in a larger, controlled study. The primary mechanistic objective of the follow-up trial is to understand the mechanisms that distinguish AHSCT from the high-efficacy approved agents in the control arm. Quality specimens from peripheral blood and CSF will be interrogated using state-of-the-art technologies that become available and validated for analysis of primary end point outcomes to corroborate proposed and discover new, biomarkers of response to AHSCT compared to high-efficacy disease-modifying therapies. This knowledge will confirm the treatment rationale and help refine future protocols, so patients with aggressive RRMS can achieve durable remission in the absence of ongoing immunosuppression.

In summary, HALT-MS uncovered a number of challenges for identifying biomarkers of clinical response to AHSCT, many of which can be avoided in future trials through thoughtful trial design, specimen collection, experimental planning, bioinformatics, and data sharing. To achieve this, the ITN brings together a multidisciplinary team of clinicians, regulatory officials, research scientists, and biostatisticians through partnerships between academia, government, industry, and other research consortia. Data sharing is available through ITN TrialShare,[1] an online resource that allows users open access to our clinical trial and mechanistic

[1] www.itntrialshare.org.

data for reuse and independent analysis to provide new concepts or insights that expand our knowledge of AHSCT in RRMS and beyond (29).

MATERIALS AND METHODS

HALT-MS Subjects

Clinical data from the HALT-MS participants have been reported previously (4) and are available through ITN TrialShare.[2]

Flow Cytometry

By using a stain-lyse method, peripheral blood cells shipped ambient overnight to the ITN Flow Cytometry Core at RPCI were stained with 5-color monoclonal antibody panels using anti-human CD3-PECy7, CD4-PERCP, CD4-APC, CD8-PERCP, CD31-FITC, CD45RA-APC, CD45RO-PE, CD27-FITC, CD28-APC, CD57-FITC, CD56-PE, CD19-PECy7 (all from BD Biosciences). Blinded samples were acquired on a Canto A flow cytometer (BD Biosciences), then gated, and analyzed using FlowJo Software (Tree Star Inc.).

Statistical Considerations

Flow data were log-transformed for statistical analysis because they were not normally distributed. Paired *t*-test was used to compare changes from baseline at 36 months within the durable remission group. Analyses were performed in SAS 9.4 (SAS Institute, Cary, NC, USA), and graphics were plotted in R Version 3.4.1 (30).

DATA AND MATERIALS AVAILABILITY

Data sets for these analyses are accessible through ITN Trial Share, a public website managed by the Immune Tolerance Network (www.itntrialshare.org/HALTMS_fimmu.url).

[2]https://www.itntrialshare.org/study/Studies/ITN033AI5YR/Study%20Data/dataset.view?datasetId=502.

ETHICS STATEMENT

The HALT-MS study [a Phase II Study of High-Dose Immunosuppressive Therapy (HDIT) Using Carmustine, Etoposide, Cytarabine and Melphalan (BEAM) + Thymoglobulin, and Autologous CD34+ Hematopoietic Stem Cell Transplant (HCT) for the Treatment of Poor Prognosis Multiple Sclerosis] was sponsored by NIAID and conducted by the ITN (ITN033AI) (ClinicalTrials.gov NCT00288626). This study was carried out in accordance with the recommendations of NIAID with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the by the IRB at each of the clinical sites.

AUTHOR CONTRIBUTIONS

LT contributed to concept development and experimental design. NL collected data. NL and TL helped with data analysis and visualization. KH interpreted the data and wrote the manuscript. All authors made contributions to the final manuscript prior to submission.

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REVIEW
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Regenerating Immunotolerance in Multiple Sclerosis with Autologous Hematopoietic Stem Cell Transplant

Jennifer C. Massey[1,2,3,4], Ian J. Sutton[2,4], David D. F. Ma[1,3,4] and John J. Moore[1,3,4]*

[1] Haematology and Bone Marrow Transplantation, St Vincent's Hospital Sydney, Darlinghurst, NSW, Australia, [2] Neurology, St Vincent's Hospital Sydney, Darlinghurst, NSW, Australia, [3] Centre for Applied Medical Research, St Vincent's Hospital Sydney, Darlinghurst, NSW, Australia, [4] St Vincent's Clinical School, University of New South Wales, Sydney, NSW, Australia

Multiple sclerosis (MS) is an inflammatory disorder of the central nervous system where evidence implicates an aberrant adaptive immune response in the accrual of neurological disability. The inflammatory phase of the disease responds to immunomodulation to varying degrees of efficacy; however, no therapy has been proven to arrest progression of disability. Recently, more intensive therapies, including immunoablation with autologous hematopoietic stem cell transplantation (AHSCT), have been offered as a treatment option to retard inflammatory disease, prior to patients becoming irreversibly disabled. Empirical clinical observations support the notion that the immune reconstitution (IR) that occurs following AHSCT is associated with a sustained therapeutic benefit; however, neither the pathogenesis of MS nor the mechanism by which AHSCT results in a therapeutic benefit has been clearly delineated. Although the antigenic target of the aberrant immune response in MS is not defined, accumulated data suggest that IR following AHSCT results in an immunotolerant state through deletion of pathogenic clones by a combination of direct ablation and induction of a lymphopenic state driving replicative senescence and clonal attrition. Restoration of immunoregulation is evidenced by changes in regulatory T cell populations following AHSCT and normalization of genetic signatures of immune homeostasis. Furthermore, some evidence exists that AHSCT may induce a rebooting of thymic function and regeneration of a diversified naïve T cell repertoire equipped to appropriately modulate the immune system in response to future antigenic challenge. In this review, we discuss the immunological mechanisms of IR therapies, focusing on AHSCT, as a means of recalibrating the dysfunctional immune response observed in MS.

Keywords: multiple sclerosis, autologous hematopoietic stem cell transplantation, T cell receptor repertoire, immune tolerance, lymphopenia-induced proliferation, alemtuzumab, cladribine

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****Correspondence:***
Jennifer C. Massey
jennifer.massey@svha.org.au

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INTRODUCTION

Multiple sclerosis (MS) is an inflammatory condition of the central nervous system (CNS) that affects over 2.3 million people worldwide and is the commonest cause of non-traumatic neurological disability in young adults, with a median age of onset of 34 years (1). In the majority of patients, the disease follows a relapsing and remitting course [relapsing-remitting multiple sclerosis (RRMS)], with up to 80% of individuals entering a secondary progressive (SPMS) phase of the

disease associated with gradual neurological decline. While the transition from RRMS to SPMS remains difficult to define, it typically occurs between 10 and 20 years from first MS symptoms (2). The early phase of the disease is marked by episodic inflammation. Once patients have reached the progressive disease phenotype, degeneration becomes the predominant feature of the disease and fails to respond to immunotherapy. Longitudinal studies have demonstrated the median time from disease onset to a requirement of a cane to walk 100 m is 27.9 years (3) and a recent meta-analysis reported a 2.8-fold increase in mortality ratio in MS, predominately in patients with Expanded Disability Status Scale (EDSS) scores >6, as a consequence of bulbar or respiratory muscle dysfunction (4). Suppression of inflammatory activity is the cornerstone of MS treatment and the introduction of disease-modifying therapy (DMT) for MS in the mid-1990s demonstrated that immune modulation could reduce the rate of clinical relapses and accompanying magnetic resonance imaging (MRI) changes of inflammation (5), in turn leading to a reduction in the rate of accumulation of disability. It is well established that early initiation of immunotherapy following first presentation of RRMS is associated with improved long-term outcomes (6). The introduction of immunosuppressive strategies in the management of MS has resulted in improved treatment efficacy (7), although it is becoming increasingly clear that the requirement for long-term maintenance dosing is associated with a small but significant risk of opportunistic infection, most notably progressive multifocal leukoencephalopathy, and possibly malignancy (8–11). Immune reconstitution (IR) therapies encompass a heterogeneous group of pulsed lympho- or myeloablative treatments designed to transiently or permanently induce an immune "reset." Over the last decade, there has been increasing interest in high-efficacy IR therapies, including chemotherapeutics, monoclonal antibodies such as alemtuzumab, and autologous hematopoietic stem cell transplantation (AHSCT) in autoimmune disease (AID), represented by a surge in clinical trials (12–15) and reviews in the literature (15–19). This has been underpinned by an understanding that appropriate application of IR therapies can induce long-term disease remission, potentially avoiding the need for further treatment.

Immunoablation followed by AHSCT has been utilized as a therapeutic intervention in aggressive AID over the past two decades, following proof of principle experiments using animal models of disease (20–26) and reports of co-incidental improvement of AID symptoms in patients undergoing transplantation for hematological malignancies (27, 28). Therapeutic trials of total body irradiation (TBI) or cyclophosphamide and busulfan (29) with allogeneic bone marrow transplantation (BMT) in rats with experimental allergic encephalomyelitis (EAE), a rodent model of CNS inflammation, and syngeneic grafting experiments (pseudoautologuous HSCT) showed that transplantation can induce disease remission, prevent relapses and enhance recovery from paresis (30, 31). Given the preferable safety record of autologous transplantation in comparison to allografting, particularly in the non-malignant setting, autologous HSCT was deemed acceptable for clinical trials in AID by the mid-1990s. The pivotal report of feasibility of AHSCT in MS was published in 1997 (32), following a cohort of 15 patients with progressive disease who underwent transplantation from 1995. Since then, over 25 phase I and II clinical trials have been published, expanding our understanding of the role of AHSCT in MS (32–42). It is increasingly recognized that AHSCT is more efficacious and associates with a lower mortality rate when applied earlier in the inflammatory phase of the disease than later in the progressive phase (42), at which time there is minimal residual inflammation and significant disability increases the risks of the procedure. However, immunoablation strategies are not without complication and associate with infertility and a dramatic increase in the short-term rate of cerebral atrophy, which is a correlate of acquired neurological disability. It is anticipated that a number of randomized controlled trials planned for the near future (18, 43) will establish the role of AHSCT in the management of treatment-refractory and aggressive MS. Continued investigation into the immunobiological changes occurring following application of AHSCT in MS is needed to facilitate the development of IR therapies with a lower treatment associated mortality and morbidity risk.

MS PATHOGENESIS

Multiple lines of evidence have established that a significant proportion of the disability acquired in patients with MS arises as a result of inflammation mediated by an antigen-specific immune response. Genetic studies have consistently confirmed that the MHC class II HLA-DRB1 gene, HLA-DRB1*15:01 is the most important risk factor for the development of MS (44–48), increasing the risk of disease threefold (49). Molecular studies have identified shared clonal populations of T cells between the peripheral blood, cerebrospinal fluid (CSF) and CNS of MS patients (50, 51) and oligoclonal immunoglobulins (Igs) are identified in the CSF, but not serum, of >95% of MS patients (52). T cell receptor (TCR) analysis of MS lesions post mortem has identified clones across anatomically distinct regions (53) with "silent" nucleotide exchanges (different nucleotides coding for the same amino acid) within the V-CDR3-J region, supporting the concept of T cell recruitment to a common antigenic driver of disease. Additionally, genome-wide association studies have identified more than 150 single-nucleotide polymorphisms (SNPs), common genetic variants (54) linked with MS. The majority of these encode for cytokines, cytokine receptors, transcription factors, and costimulatory molecules of the adaptive immune system, indicating that the immune phenotype is critical to disease susceptibility.

The concept of MS as a prototypical AID, where self-reactive lymphocytes induce inflammation in response to myelin or myelin cross-reactive epitopes (55) is based on EAE disease models (56–58) and supported by the presence of myelin-reactive Th1 and Th17 cells (59–61) and their cytokines in the CNS and CSF of MS patients (55). However, while myelin antigens are attractive candidates for an autoreactive immune response, it is accepted that the antigenic target(s) of the clonally expanded lymphocytes in MS remains unknown at present. Inadequacies in the widely accepted theory of MS resulting from CNS-specific autoreactive lymphocytes has been exemplified by historical studies in to the association between infectious agents and MS (62) and recent work investigating the role of human

endogenous retroviruses in triggering a pathogenic immune response within the CNS (63, 64). EAE has successfully served as a model of CNS inflammation for development of certain disease-modifying therapies (58), but while widely cited as "an animal model of MS" EAE should not be viewed as such, since induction of EAE results in widespread inflammatory change that lacks specificity for oligodendrocyte injury and the perivascular and sub-pial distribution of the neuropathological changes observed in EAE (65) are quite distinct from the pathology that is observed in MS (66).

Both EAE and therapeutic trials of natalizumab, a humanized IgG4 monoclonal antibody against a4 integrin that blocks T cell trafficking into the CNS, have clearly demonstrated that a dysregulated immune response can be highly damaging to the brain and spinal cord. Moreover, the brain itself has long been recognized as an immune privileged site and an immunologically tolerogenic environment, raising important questions as to how pathological immune responses are generated and sustained in MS. In this regard, critical neuropathological evidence (67–70) has demonstrated that the earliest pathological change in an MS lesion is oligodendrocyte apoptosis and microglial activation in the absence of lymphocytic infiltration, with secondary demyelination (71). The recent identification of long hypothesized efferent CNS lymphatic pathways (72) and seminal allogeneic transplant experiments in the 1940s (73) demonstrate that the tolerogenic environment in the brain can be overcome by peripheral activation of an immune response. It is therefore plausible that CNS injury in MS arises as the result of peripheral activation of an aberrant adaptive immune response to an undefined antigen associated with oligodendroctye apoptosis (**Figure 1**).

Although we are yet to define the antigenic target in MS or understand disease induction and how the immune system regulates the inflammatory changes that associate with the early relapsing-remitting disease course, it has been established that in addition to primary oligodendrocyte loss there is also marked axonal injury within the acute lesion (74). Over time, disability due to axonal injury accumulates and acute bouts of inflammation that associate with clinical relapses become less frequent (**Figure 2**). These observations favor the concept that IR therapies such as AHSCT should be applied early in the disease course when inflammatory changes are most prominent and prior to the accrual of irreversible neuroatrophy. In order to best understand the mechanisms underpinning IR therapies, it is essential to

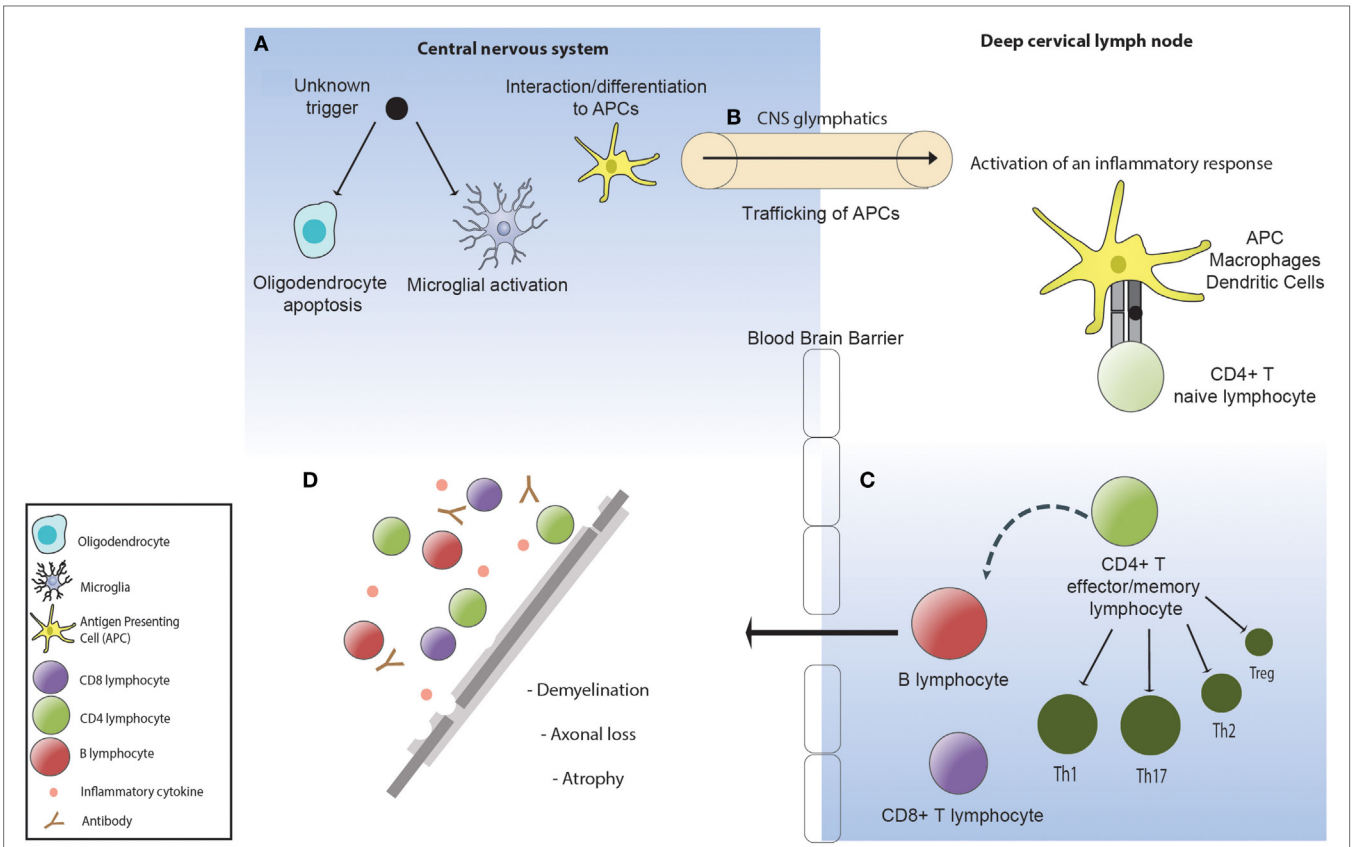

FIGURE 1 | **(A)** An unknown factor triggers oligodendrocyte apoptosis. Microglia are activated and phagocytose the apoptotic oligodendrocytes. **(B)** Phagocytic cells containing oligodendrocyte myelin breakdown products traffic to the deep cervical lymph nodes *via* the central nervous system (CNS) lymphatics (glymphatics) where they activate an inflammatory immune response directed toward the undefined antigenic target of disease. **(C)** The inflammatory response in multiple sclerosis is defined by a dominance of Th1 and Th17 lymphocytes, pro-inflammatory cytokines, and impaired suppressor activity of Tregs. **(D)** Activated lymphocytes re-enter the CNS where they become re-activated and recruit local and systemic immune populations resulting in demyelination and subsequent axonal loss.

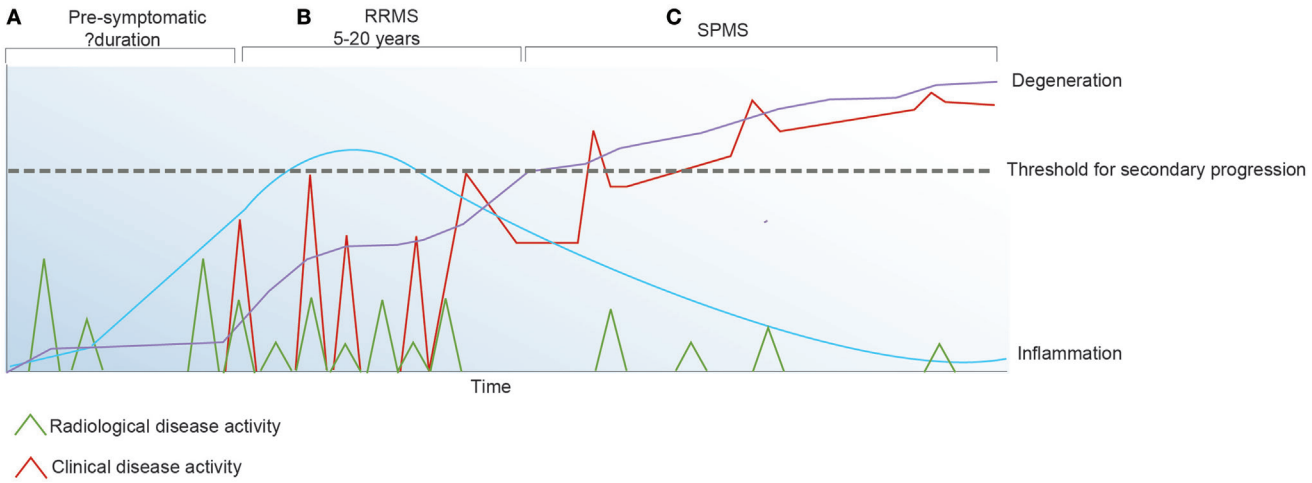

FIGURE 2 | Inflammatory activity in multiple sclerosis (MS) may be detected clinically and/or radiologically. **(A)** The pre-symptomatic phase of the disease is defined by radiologically apparent relapses in the absence of clinical symptoms. **(B)** Following the first symptomatic demyelinating event, clinical and radiological relapses continue to occur. **(C)** Secondary progressive (SP) MS is defined by irreversible accumulation of disability due to chronic axonal loss which associates with ongoing brain atrophy and minimal inflammatory change on magnetic resonance imaging.

consider factors maintaining immune homeostasis in health and disease.

THE LYMPHOCYTE "STEADY STATE"

The circulating T lymphocyte pool is generated in early life *via* thymic development of T cells. Random and imprecise intra-thymic rearrangements of TCRA and TCRB genes generate rich TCR diversity (75) estimated to exceed 10^{15}, with a circulating αßTCR repertoire in the range of 2.5×10^7 (76). As thymocytes proliferate and mature into T cells, they undergo a series of distinct steps defined by changes in the expression of the TCR and the co-receptors, CD4 and CD8. T cells expressing the CD4 co-receptor are capable of interacting with MHC class II molecules present on antigen presenting cells, while CD8 expressing cells may be stimulated by any cell expressing MHC class I molecules. In health, approximately 50% of the circulating lymphocyte pool are T cells, with a dominance of $CD4^+$ to $CD8^+$ in a roughly 2:1 ratio. Naïve CD8 T cells emerging from the thymus are predestined to become cytotoxic cells, while $CD4^+$ cells become "helper" lymphocytes whose fate is further determined during their first encounter with antigen. Mature naïve $CD4^+$ or $CD8^+$ cells survive in interphase for weeks to months in response to tonic TCR signals—weak, but significant stochastic interactions with self-peptide/MHC in the presence of IL-7 (77). Survival of these cells is determined by threshold "tuning," which modulates the intensity of TCR signaling required for cell activation and proliferation (78).

Naïve T (TN) cells are defined conventionally by a host of receptors which facilitate lymphocyte entry to secondary lymph node organs, allowing these cells to interact with cognate antigen presented by APCs, resulting in conversion to a memory phenotype (78, 79). Activation of T lymphocytes enables splicing of pre-mRNA encoding CD45, a receptor-linked protein tyrosine phosphatase essential for TCR activation, resulting in expression of the prototypical antigen experienced T cell marker CD45RO (80). Antigen-specific proliferation is not only reliant on high affinity binding between MHC and the TCR, but co-stimulation in the context of IL-2, IL-4, IL-7, and IL-15 (79). In addition to conversion of phenotype from naïve to memory in response to antigen, a proportion of naïve cells undergo steady-state proliferation into memory-phenotype cells, in response to self-peptide, presumably with a lower degree of interaction than that required to trigger auto-inflammation (79). Following interaction with antigen, two major subsets of T memory (T_M) cells develop (**Table 1**); those maintaining receptors enabling homing to lymphoid organs are known as central memory lymphocytes (T_{CM}), with the alternate being effector T cells (T_E) (79, 81–84). It has been further postulated that T_{CM} cells may differentiate into effector memory (T_{EM}) or T_E cells on re-exposure to antigen (83, 85). More recently, a long-lived human memory T-cell population, termed T stem cell memory (T_{SCM}) cells have been described (83). This population display functional attributes of naïve cells,

TABLE 1 | Model of T cell subsets by differentiation and phenotypical markers.

	T naïve	T stem cell memory	T central memory	T effector memory	T terminal effector
CD45RA	+	+	−	−	+
CD45RO	−	−	+	+	−
CCR7	+	+	+	−	−
CD62L	+	+	+	−	−
CD28	+	+	+	+/−	−
CD27	+	+	+	+/−	−
IL-7Rα	+	+	+	+/−	−
CD95	−	+	+	+	+
IL2Rβ	−	+	+	+	+
CD58	−	+	+	+	+
CD57	−	−	−	+/−	+

with enhanced self-renewal and multipotent capacity to derive T_{CM}, T_{EM}, and T_E cells, in addition to memory characteristics including increased proliferative capacity and the ability to rapidly release cytokines on activation (82, 83). Phenotypically, this population express both traditional naïve and memory markers. It has been hypothesized that these T_{SCM} cells may be the earliest antigen experienced cells, and may serve as a reservoir of memory lymphocytes (82, 86). Costimulatory molecules CD27 and CD28 are present on memory cells; however, expression is lost as cells become terminally differentiated (82). In fact, terminally differentiated lymphocytes may even revert to a CD45RA phenotype, eventually transitioning to functional senescence or apoptosis (87, 88) defined by the expression of CD57 and CD58. Phenotypic, functional, and gene expression studies of T cell subsets suggest a non-linear continuum of lymphocyte development from T naïve to T stem cell memory, T central and effector memory, and finally terminal effector populations (83, 89), where less differentiated cells give rise to more differentiated progeny in response to antigenic or lymphopenic-induced proliferation requirements (79, 81–84).

This model of "progressive differentiation" proposes that depending on the strength and quality of stimulatory signals, naïve lymphocytes are driven toward progressive stages of differentiation (78). More differentiated cells are under mounting feedback pressure to further differentiate or undergo apoptosis, culminating in the generation of terminally differentiated effector T cells which are prone to replicative senescence, reversing clonal population kinetics from growth to decline (87). Support for this model includes *ex vivo* analysis of virus-specific T cells, and *in vitro* differentiation studies (81, 90–92). In humans, progressive differentiation and replicative senescence has been associated with the generation of short lived T effector cells, expressing a $CD8^+CD28^-CD57^+$ phenotype (93). These cells demonstrate reduced replicative potential, decreased telomere length, and a gene expression profile suggestive of a state of senescence (87, 88, 94), although immunosuppressive functions of this population have also been described (95). *In vitro* models have identified that $CD4^+$ lymphocytes appear less prone to basal proliferation than $CD8^+$ cells (96, 97), presumably relating to the ability of $CD8^+$ lymphocytes to be activated by MHC class I molecules, present on all nucleated cells. Additionally, an elevated threshold for entering the cycle of progressive differentiation may serve as a mechanism to preserve the $CD4^+$ population from senescence (98); however, the purpose of such variability remains unclear.

Interaction with cognate antigen and T cell activation results in ≈1,000 fold increase in size of the antigen-specific lymphocyte pool; however, it is estimated that less than 10% of cells activated after antigenic challenge survive in the memory pool (78) due to a process known as attrition (99), where a proportion of the expanded T cell pool are sacrificed to ensure "space" for newly developing T cells.

In addition to a retraction of clonal populations, response to antigen is also modulated over time. Studies in HIV, *M. tuberculosis* and Epstein–Barr virus (EBV) have informed our understanding of T cell anergy that occurs in the context of antigen persistence (100). Repeated antigen exposure has been shown to generate T cells that respond less intensely, producing lower levels of cytokine response than acutely stimulated effectors (101). Antigenic clearance is not required to stop clonal expansion of T_N cells. Instead, a protracted immune response can trigger a refractory state by adaptive "tuning" whereby ongoing antigenic stimulation may result in an activation-resistant state (78).

The ability to modulate response to antigen over time is a defining feature of adaptive immunity. Maintenance of immune homeostasis and prevention of auto-reactivity is mediated by regulatory T cells (Tregs), the primary drivers of peripheral tolerance due to their ability to suppress other cells in the immune system. The majority of Tregs are thymically derived (tTregs); cells that have been primed following their thymic interactions with self-peptide to constitutively express transcription factors (most notably FoxP3) that enable their suppressive function (102). Tregs may also differentiate from naïve conventional T cells under certain conditions, likely relating to TCR stimulation, cytokine environment, and associated epigenetic priming in the periphery (102, 103). Peripheral Tregs may be specific for both self and pathogen (103). Naïve Tregs are those which are yet to encounter cognate antigen in the periphery, while effector or activated Tregs have received strong antigenic stimulation evidenced by markers of proliferation and enhanced suppressive function (104). Activated Treg numbers fluctuate in the context of infection and appear to be modulated in order to thwart collateral damage to tissues exposed to chronic immune stimulation (105). Expanding literature reports a deficiency in the number and/or function of Tregs in autoimmune and inflammatory diseases, enabling a dysregulated immune response to antigen (106–111). This may reflect both a failure in induction of peripherally derived Tregs and inadequacies of thymic Treg production.

Thymic involution, an important aspect of immunosenescence, causes a decline in the output of conventional and regulatory naïve T cells from the thymus (112). It is therefore unsurprising that high-throughput TCR sequencing studies have shown that the TCRβ repertoire is most diverse in samples of umbilical cord blood and progressively decreases with age (113) so that in later life, the decline in naïve T cells and expansion of antigen experienced clones leads to a consistent decrease in TCR diversity (114). Furthermore, compensatory auto-proliferation in response to thymic involution has been postulated in the immunogenesis of inflammatory disorders (115–117). Molecular studies have confirmed that end-differentiated effector CD8 T cells, likely responsive to latent viruses such as cytomegalovirus (CMV), are expanded with advancing age, contributing to the decrease of TCR richness in memory, as opposed to naïve T cell pools (85, 118).

The natural history of MS is manifest by dissipation of CNS inflammation over time. Akin to the anergy that develops in response to chronic infection, it is possible that the later phase of MS, where inflammation is absent, relates to a "tuning" of the immune response. AHSCT, through the ablation of lymphocytes, induces IR bound by the principles of lymphocyte homeostasis outlined above. In addition to ablation of pathogenic clonal cells and the induction of thymopoiesis, it is proposed the AHSCT induces a recalibration of the immune system modifying the context in which antigen is first encountered and accelerating the induction of immunotolerance in the absence of relapse-associated disability.

AHSCT IN MS

In the context of MS, AHSCT encompasses a heterogeneous procedure involving five key stages: (i) mobilization of $CD34^+$ hematopoietic stem cells (HSCs), (ii) HSC collection and preservation, (iii) immunoablative conditioning, (iv) HSC infusion, and (v) post-transplant care. Variations in AHSCT protocols exist between transplant units and no consensus exists regarding the optimal treatment regimen for MS (119). Mobilization of HSCs is undertaken by administering a marrow stimulating agent such as granulocyte colony-stimulating factor (G-CSF) and/or cyclophosphamide (Cyc) to induce proliferation and release of bone marrow HSCs, which are harvested from peripheral blood by leukoapheresis. Cyc is used not only to ablate pathogenic clones and increase HSC yield, but provides a degree of immunosuppression by lymphocyte depletion. In MS, Cyc is believed to counter the risk of exacerbating CNS inflammation that has been associated with G-CSF use (15). While a direct correlation exists between $CD34^+$ cell dose and time to engraftment (120), *ex vivo* $CD34^+$ selection of the leukoapheresis product is not only costly but has not been shown to improve outcomes in a recent retrospective analysis of autoimmune patients in the European Bone Marrow Transplantation (EBMT) database (119). One randomized trial of $CD34^+$ selection in rheumatoid arthritis (121) and a recent retrospective study assessing $CD34^+$ selection in systemic sclerosis (122) did not show added benefit to overall or progression-free survival. A 2013 publication showed a reduced relapse incidence in systemic lupus erythematosus (SLE) patients undergoing AHSCT with $CD34^+$ selection (11 vs. 68%); however, a greater proportion of the non-graft selected patients had received a low-intensity conditioning regimen (123). Similarly, despite the complete arrest of MS disease activity post-AHSCT in the 2016 Atkins et al. trial (33), the use of a high-intensity myeloablative regimen makes it difficult to draw conclusions about the additional benefit of $CD34^+$ selection. Therefore, although $CD34^+$ selection is standard of care in some sites performing AHSCT for MS, compelling evidence to justify its use is lacking. Many centers opt instead for *in vivo* post-transplant T cell purging through the use of agents such as anti-thymocyte globulin (ATG) (119).

High-dose chemotherapy is used to ablate pathogenic lymphocytes, inducing pancytopenia and bone marrow aplasia prior to infusion of cryopreserved HSCs to variable intensity. Early clinical trials were designed on the back of animal studies (29, 30, 119, 124), suggesting a need for high-intensity immunoablation and employed myeloablative regimens using TBI and/or chemotherapeutics able to penetrate the blood–brain barrier. Higher intensity chemotherapy correlates with increasing adverse events, and a decreased tolerance for complications in the non-malignant setting has influenced treatment protocols in AIDs. Globally, the most widely used regimen continues to be BEAM—carmustine (BCNU), etoposide, cytarabine (AraC), and melphalan (15).

Treatment-related mortality (TRM) rates and MS disease progression following transplantation have limited the application of AHSCT in MS; however, historical results have been skewed by poor patient selection. An increase in the use of intermediate- and low-intensity regimens and better patient selection has resulted in a drop in the TRM reported by the EBMT. From 1995 to 2000, EBMT quoted a TRM rate of 7.3%, which fell to 1.3% in years 2001 to 2007 (125). However, in a 2017 meta-analysis of clinical trials for AHSCT in MS, TRM was 0.3% in the 349 patients who were transplanted after 2005 and no TRM was observed in those who underwent low-intensity conditioning (42). While determination of the optimal conditioning regimen in AHSCT for MS is of high research priority, a retrospective observational study from the EBMT autoimmune database (126) found that a transplant centers experience, and not intensity of conditioning had the strongest correlate with TRM.

The patient and treatment characteristics, results, and adverse events of pivotal trials of AHSCT in MS over the last 5 years have been summarized in **Table 2** (33–37, 39–41, 127, 128) and recently reviewed elsewhere (18, 39, 120). A degree of disease stabilization has been seen in all trials of AHSCT in MS, and a multitude of recent publications support sustained disease suppression in a subset of patients with highly active inflammatory disease. A more profound treatment effect is observed with higher intensity conditioning (39), although it should be noted that methodological variability limits comparability between trials. It is notable that all studies show a durable response in the majority of RRMS patients and consistent relapse-free survival rates within this cohort, exceeding results seen in Phase 3 trials of alternate IR therapies (12, 13); however in the absence of randomized controlled trials, one must be cautious in drawing comparisons across treatments.

IMMUNE MECHANISMS OF AHSCT IN MS

In general terms, IR therapies such as AHSCT are believed to exert their effects through (i) deletion of lymphocyte populations (129–133), (ii) induction of a lymphopenic state (129, 131, 134, 135), and (iii) subsequent development of a tolerant immune system which lacks the clonal expansion of functionally pathogenic T and B cells (131, 136) following recalibration of the TCR repertoire (**Figure 3**). This theoretical framework is supported by the observation that early clinical remission following AHSCT can be maintained for decades (33, 137). While the antigenic target of pathogenic lymphocytes is unique to each inflammatory disease, the sustained effects of AHSCT appear to relate less to MS-specific immune mechanisms, but more broadly to the principles of immune recalibration generated *via* the induction of lymphopenia and thymic reset. An exception to this is the ability of certain chemotherapeutics to cross the blood–brain barrier, enabling the elimination of MS-specific clones; however, this alone does not account for the durability of response observed in certain treatment trials. A recent meta-analysis and multi-center observational studies in MS have confirmed AHSCT to be a highly effective therapy, with event-free survival rates exceeding those seen best available pharmacotherapies (138). In order to optimize MS treatment in the future, it is vital to enhance our understanding of how IR therapies such as AHSCT adapt a patient's immune response to the antigenic target of disease.

TABLE 2 | Clinical and radiological outcomes in autologous hematopoietic stem cell transplantation (AHSCT) for MS.

Reference	Chen et al. (36)	Burman et al. (34)	Mancardi et al. (127)	Nash et al. (40)	Curro et al. (37)
Year	2012	2014	2015	2015—3 years/2017—5 years	2015
N# patients/gender	25/19 female	52/26 female (48 definite MS)	21 (9 AHSCT, 12 mitoxantrone)/14 female	24/17 female	7/3 female
Mean/median age (range)	37 (15–64)	31 (9–52)	36 (19–46)	37 (27–53)	28 (23–38)
Median baseline Expanded Disability Status Scale (EDSS) (range)	8.0 (3.0–9.5)	6.0 (1–8.5)	6.0 (5.5–6.5)	4.5 (3.0–5.5)	6 (5.0–7.0)
MS type	3 RR, 2 PR, 1 PP, 19 secondary progressive (SP)	40 RR, 1 PR, 2 PP, 5 SP	2 RR, 7 SP (AHSCT)	24 RR	7 RR
Median disease duration (months)	48 (7–147)	75 (4–300)	123 (24–276)	59 (7–144)	78 (48–144)
Mean/median follow-up (months)	59.6	47.4	48	46.5/62	range 36–60
Magnetic resonance imaging (MRI) activity at baseline	14 patients	66% MRI activity at baseline	ND	42%	100%
Conditioning regimen	Intermediate-intensity myeloablation (BEAM) + anti-thymocyte globulin (ATG)	Intermediate-intensity myeloablation (BEAM) + ATG, $n = 41$ Low-intensity lymphoablation Cyc + ATG, $n = 7$	Intermediate-intensity myeloablation (BEAM) + ATG	Intermediate-intensity myeloablation (BEAM) + ATG	Low-intensity lymphoablation Cyc + ATG
Outcome including relapse (event)-free survival (RFS)	PFS 74, 65, and 48% at 3, 6, and 9 years	4/48 relapses, ARR 0.03, all confirmed with new Gd^+ MRI lesion	79% reduction in lesions compared to MTX arm	Event-free survival was 78.4% at 3 years based either on clinical or MRI findings	Single relapse in 1 patient at 3 years
	40% EDSS improvement, 28% EDSS stabilization	Median change in EDSS −0.75 (−7 to 1.5), −1.5 in relapsing-remitting multiple sclerosis (RRMS)	100% absence of Gd^+ lesions during 4-year follow- up compared to 56% in MTX arm	Event-free survival was 69.2% at 5 years based either on clinical or MRI findings	6/7 had increase of EDSS at 3 years
	Follow-up MRI in 12 patients. 58% with Gd^+ at baseline had no MRI activity on follow-up	5/48 new MRI activity	Reduced ARR as compared to MTX arm	RFS was 86.3% and PFS was 90.0% at 3 years	Mean Gd^+/month decreased from 4.1 ± 4.1 pre-AHSCT to 0.3 ± 0.8 at 1 month and 0.2 ± 0.4 at 3 years
			No difference in EDSS or progression of disease	RFS was 86.9% and PFS was 91.3% at 5 years	6/7 developed Gd^+ lesions after 5–6 months
Mortality <100 days	0	0	0	0	0
Mortality >100 days	1 pneumonia 4.5 months, 1 VZV hepatitis 15 months	0	0	1 MS progression <2.5 years, 1 asthma <3.5 years, 1 cardiac arrest 4.5 years	0
Adverse events/others	13 patients with bacteremia	0 malignancy, 1 Crohn's Disease, 4 thyroid disease	3 severe AEs; 1 late engraftment, 1 systemic candidiasis/cytomegalovirus (CMV) reactivation, 1 ATG reaction		2/7 septicemia, 2/7 herpetic infections

(*Continued*)

TABLE 2 | Continued

Reference	Chen et al. (36)	Burman et al. (34)	Mancardi et al. (127)	Nash et al. (40)	Curro et al. (37)
	Burt et al. (35)	Shevchenko et al. (41)	Atkins et al. (33)	Cull et al. (128)	Muraro et al. (15, 39)
	2015	2015	2016	2017	2017
	151 (145 at end)/85 female	99/60 female	24 (21 at end)/14 female	13/11 female	281/164 female
	36 (18–60)	35 (18–55)	34 (24–45)	45 (22–60)	37 (15–65)
	4.0 (3.0–5.5)	3.5 (1.5–8.0)	5.0 (3.0–6.0)	7.0 (6.0–8.0)	6.0 (1.5–9.5)
	123 RR, 28 SP	43 RR, 3 PR, 18 PP, 35 SP	12 RR, 12 SP	3 PP, 10 SP	46 RR, 17 PR, 32 PP, 186 SP
	61 (9–264)	60 (6–288)	70 (16–134)	12.5 (36–360)	81 (<1–413)
	30	48.9	80.4	min. 36	79.2
	58%	40% MRI activity at baseline	87.5% 1 year pre-AHSCT	30.7% 1 year pre-AHSCT	N/A
	Low-intensity lymphoablation Cyc + ATG or alemtuzumab	Lesser than intermediate-intensity myeloablation (mini-BEAM like or carmustine + melphalan)	High-intensity myeloablation (busulfan + Cyc + ATG)	Low-intensity lymphoablation Cyc + ATG	Low intensity: Cyc ± ATG/ alemtuzumab/Fludarabine Intermediate intensity: BEAM + ATG, BEAM, Cyc, TLI + melphalan, carmustine + Cyc + ATG High Intensity: Cyc + total body irradiation + ATG, busulfan + Cyc + ATG, busulfan + ATG
	RFS was 80% at 4 years	80% had event-free survival at median follow-up of 49 months	Event-free survival was 69.6% at 3 years	PFS was 69.6% at 3 years	PFS RR was 82% at 3 years, 73% at 5 years. PFS SP was 33% at 5 years
	PFS was 87% at 4 years	47% improved EDSS score by at least 0.5 points and 45% were stable after long-term follow-up	70% had no EDSS progression with a median follow-up of 6.7 years	0% EDSS improvement. 69% had no EDSS progression	OS 93% at 5 years and 84% at 10 years
	Mean Gd^+ lesions 3.22 at 3–6 months pre-AHSCT to 0.08 at 5 years	MRI f/up in 55 patients. 15 patients had MRI activity at baseline, 3/15 developed Gd+ lesions on f/up	100% had absence of MRI activity after transplant	85% had absence of MRI activity after transplant	
	Decrease in T2 lesions volume from median of 8.57–5.74 cm^3	Improved QoL in all RRMS at 1 year	35% had sustained improvement in EDSS		
	0	0	1 *Klebsiella* sepsis, 60 days	0	8 (2.8%)
	1 cardiovascular disease at 30 months	0	0	0	29
	1 bacteraemia, 4 zoster reactivation. 14% ITP with alemtuzumab, 3% ITP with ATG		Two patients censored within 2 years after CCSVI treatment	Febrile neutropenia 96%. 1 JCV cystitis, 1 CMV reactivation. 1 case ITP 12/12 post-AHSCT	New onset malignancy 9, autoimmune disease (AID) 14

Induction of the Lymphopenic State: Effect on Clonal Populations and Cell Phenotype

Ablative chemotherapy induces an extreme reduction in the mature lymphocyte pool and triggers potent proliferative signals for cells that have either survived the conditioning regimen or have been reinfused with the leukoapheresis product and survived subsequent *in vivo* T cell depletion (typically ATG). Studies of AHSCT in MS have confirmed a pattern of lymphocyte reconstitution akin to that seen in alloHSCT and AHSCT for hematological indications (128, 129, 131–133, 135), when similar conditioning regimens are used. However, the use of T cell-depleting agents post stem cell infusion is rare in the hematology setting (139–142). B cells, NK cells, and monocytes repopulate within the first weeks to 6 months (128, 131–133, 135) while $CD3^+$ lymphocyte counts remain low in the initial post-transplant period, normalizing by 6–12 months (129, 133). Early proliferation of $CD8^+$ T_M and T_E lymphocytes (131, 135) results in an inversion of the $CD4^+/CD8^+$ ratio out to 24 months (128, 129, 131, 132, 134, 135). This expansion of CD8 memory cells is termed "lymphopenia induced proliferation" (LIP), a process of activation and proliferation of leukocytes in a lymphopenic environment which serves as a response to rapidly restore T cell

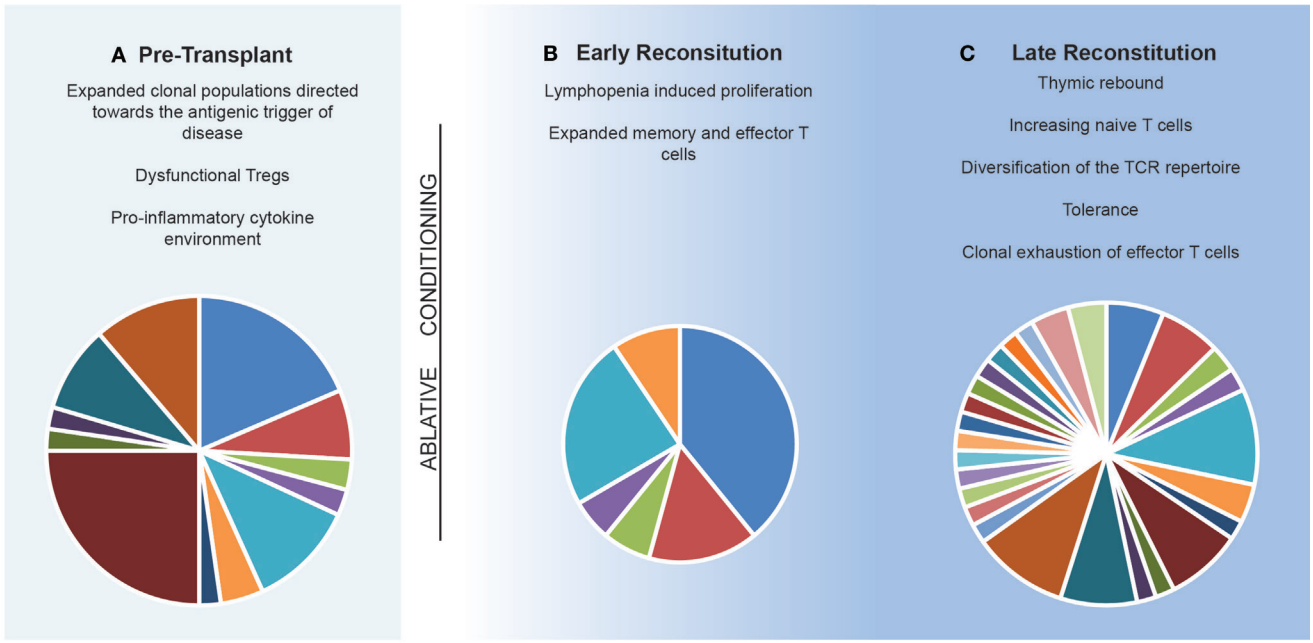

FIGURE 3 | Model of the immune reconstitution occurring following autologous hematopoietic stem cell transplantation. **(A)** The pre-transplant environment is defined by clonally expanded populations of lymphocytes driving central nervous system inflammation with dysfunctional immunosuppressive lymphocytes. **(B)** Early reconstitution is driven by lymphopenia-induced proliferation of predominately $CD8^+$ memory populations. Pie chart depicts T cell receptor (TCR) repertoire which is dominated by lymphopenia-induced proliferation induced expansions of clonal populations. **(C)** Late reconstitution involves a recovery of thymic output of naïve T cells. Pie chart depicts an increase in TCR diversity. Replicative senescence results in exhaustion of the previous expanded populations.

numbers. As in health, LIP promotes the expansion of CD8 more than CD4 lymphocytes (141). In theoretical terms, an "awareness" of available "space" (143) through alterations in the cytokine environment and availability of growth factors, induces lymphocytes to fill these voids. The mechanisms that result in LIP differ significantly from basal proliferation observed in the immune replete individual, whereby LIP is driven by a decreased threshold for TCR interactions with self-peptide MHC complexes and increased responsiveness to cytokines IL7, 15, and possibly 21 (144–146). This phenomenon has been observed in murine models in which rapid expansion of transferred lymphocytes occurs when injected to T cell deficient recipients, which is not seen on transfer to lymphoreplete mice (147).

Lymphopenia-induced proliferation results in lymphocyte phenotype switching, similar to those seen in the "progressive differentiation" model, with a rise in terminal effector populations. An early increase in the circulating $CD8^+CD28^-CD57^+$ cells has been seen across studies of AHSCT in MS (129, 133, 135), and it has been suggested that this population is a significant contributor to the rise seen in the aforementioned $CD8^+$ T_M population in the initial months post-AHSCT (133). These cells originate as an effector/cytotoxic population but may acquire an anergic, exhaustion-like, or regulatory phenotype (148–150), evidenced by weakened responses on TCR activation with lower cytokine release. Recent work in the field of systemic sclerosis demonstrated effector T cells undergo the greatest degree of expansion at 6 months post-AHSCT when compared with central or effector memory populations (151), and it is possible that the pace at which lymphocytes progress through cycles of differentiation and phenotype switching may be relevant to clinical outcomes. A study in type 1 diabetes mellitus demonstrated an early rise in T effector cells in the "short-response" population, opposed to the delayed rise in T central memory populations noted in the "prolonged-response" cohort (152). Recent work in MS has demonstrated that the inflammatory response of $CD8^+CD57^+$ cells correlates with relapse frequency, thereby confirming the relevance of this population to disease activity (153).

Molecular studies that have demonstrated a persistence of clonal populations following AHSCT despite patients remaining relapse free suggest that this early clonal expansion is dominated by lymphocytes reacting to common pathogens such as CMV or EBV (154, 155) and expansion of these terminal effector populations has been shown to predict viremia in the allogeneic transplant setting (156). However, the effect of these expansions on TCR diversity in the longer term remains unclear. Between 80 and 100% of MS patients are seropositive for EBV antibodies (157) and accumulating evidence demonstrates altered T-cell reactivity to EBV antigens (particularly EBNA) in MS (158). It has therefore been postulated that clonal expansions of EBV-specific T cells may shape the post-AHSCT TCR repertoire and limit the "space" available for diversification of the naïve T cell pool (133, 159–161). Notably, Muraro et al. identified an increase in the clonal frequency of EBV reactive T cells at 2 months in a subset of patients post-AHSCT and aforementioned investigations of $CD8^+CD57^+$ cells in MS demonstrates that a proportion of these cells are EBV specific (133). T cell competition ensures regulation of the size

and diversity of the circulating T cell pool, confirmed by murine experiments of monoclonal TCR-transgenic cells demonstrating dramatically longer survival rates when seeded at low vs. high frequencies (162). While clonal competition between infused and residual cells post-AHSCT is likely to favor the broadening of the T cell repertoire, early sizeable expansions of clonotypes in response to common viruses may limit the available space for diversification of the lymphocyte pool post-transplant (160). However, clonal expansion can also lead to replicative senescence which is one potential explanation for attrition of pathogenic clones. The proportion of the mature T cell repertoire that needs to be eliminated for clinical efficacy in MS is unknown and hindered by the absence of a defined antigen. Although observational data supports the concept that a higher intensity conditioning regimen results in improved patient outcomes (42), it is not known whether this relates to deletion of pathogenic clonal populations or the degree and duration of lymphopenia. A high-throughput sequencing study in MS patients undergoing HSCT (161) has confirmed that the $CD8^+$ lymphocyte pool is dominated by an expansion of pre-existing clones, while the $CD4^+$ pool is primarily comprised of lymphocytes not present pre-transplant and increased TCR diversification trended toward patient response.

Thymic Rebound and Diversification of the T Cell Pool

In contrast to the LIP of memory populations, a delayed repopulation of $CD4^+$ T_N cells (128, 129, 131, 133–135), presumably thymic derived, occurs after 12–24 months and correlates with a rise in TCR excision circles (TREC) and $CD31^+$ expression; a cell surface marker associated with recent thymic egress of $CD4^+$ cells (128, 131, 133, 135). Reconstitution of naïve, thymic-derived T cells is postulated as the mechanism for increasing TCR repertoire diversity. This is supported by publications demonstrating a "Gaussian" repertoire following HSCT, typically 2–3 years post-transplant (133, 134, 140, 142, 163) and is consistent with the timing of thymic reactivation post-AHSCT. TREC levels are higher in AHSCT with $CD34^+$ graft selection suggesting a correlation between $CD34^+$ load and thymocyte development (164); however, a correlation with degree and duration of lymphopenia in graft selected cases cannot be excluded. Although maturation of T cells within the thymus declines with age (164–166), reactivation of the thymus plays a fundamental role in lymphocyte reconstitution after chemotherapy (133, 140, 163). Residual thymic tissue appears able to support thymopoiesis and generate TREC-bearing lymphocytes following AHSCT. Given the significance of thymic output in the durable diversification of the T cell repertoire following AHSCT, it is perhaps unsurprising that younger age, independent of disease duration, correlates with improved clinical outcomes in MS post-AHSCT (42).

The Development of a Tolerant Environment

In ways still not fully understood, IR following AHSCT appears to favor the induction of tolerance. Both quantitative and qualitative abnormalities in Tregs have been observed in patients with inflammatory diseases including MS (167, 168) proposedly due to inadequate thymic production of naïve Tregs and impaired differentiation of memory Tregs (110, 111). Despite discrepancies in phenotypic definition, an early rise in Tregs post-AHSCT (129, 131, 132, 135) has been consistent across studies in MS. Early surges in Treg populations relative to the T cell pool are likely driven by LIP. Lymphopenia inducing therapies such as ATG and alemtuzumab have been independently associated with rapid expansions of $CD4^+CD25^+$ Tregs (169, 170), although the durability of these responses is unclear. Preliminary studies from our laboratory demonstrate an early spike in Tregs following AHSCT, which is not observed in lymphoma patients undergoing autografts with similar conditioning chemotherapy albeit without post-transplant ATG (171).

The sustained immune responses associated with AHSCT imply that a qualitative shift in the lymphocyte pool may relate to the context in which IR occurs. The significance of thymic reconstitution contributing to the restoration of Tregs has been highlighted by studies of recent thymic emigrants. Helios + Tregs, signaling thymic origin, have been shown to display greater proportions of RTE markers such as CD45RA and $CD31^+$ in SLE patients following AHSCT, compared to those with active disease (172). Furthermore, a Dutch study looking at AHSCT in juvenile idiopathic arthritis demonstrated an oligoclonal Treg repertoire prior to AHSCT, which diversified post-transplant in all patients bar one, whose disease relapsed, implying a correlation between a diverse, presumably thymic-derived Treg repertoire and disease response (136). Tregs fell post-transplant in a cohort of SPMS patients (128) treated with a conditioning cyclophosphamide/ATG regimen but without post-transplant T cell depletion; however, it is unclear whether this difference relates to chemotherapeutics, patients age, disease stage, or a combination of the three.

Treg suppressive capability was shown to be decreased at baseline in systemic sclerosis patients undergoing AHSCT compared with controls, and normalized post-transplant (173). Furthermore, Burman et al. showed that Tregs remain capable of recognizing myelin oligodentrocyte glycoprotein post-AHSCT and are more capable of suppressing the associated Th17 response compared with MS patients on natalizumab (130). While further functional Treg experiments are, to our knowledge, yet to be performed in MS patients undergoing AHSCT, this is a point of interest for our laboratory.

Akin to the progressive differentiation of T cells, B cell development is defined by maturation of B lymphocytes with a transition of surface marker proteins defining phenotype and splicing of Ig heavy-chain transcripts to induce class-switching of surface Ig. B cells are believed to contribute to MS pathogenesis by means of antigen presentation, auto-antibody production, and cytokine regulation (174). Not only may oligoclonal B cell populations traffic between the CNS and peripheral circulation, but the formation of ectopic lymphoid aggregates within the meninges are purported to act as a germinal center, enabling a compartmentalized CNS site for activation and differentiation of T and B cells. Mounting evidence suggests antigen-dependent B cell subsets may have both pro-inflammatory and regulatory characteristics, with B regulatory cells (Bregs) emerging in different disease settings to exert a suppressive function (175). It is plausible that in addition to Treg dysfunction, inadequacies of Bregs may play a

part in inflammatory disorders. Furthermore, a recent study of patients undergoing autografts for systemic sclerosis suggested that bone marrow "rebound" following AHSCT may induce proliferation of tolerant B cells (151). The reconstitution of these populations following IR therapy is yet to be studied in MS.

An Altered Inflammatory Response

Phenotypic studies have confirmed reduced function of pro-inflammatory Th17 (IL-17A$^+$IFN gamma$^-$) (131) and Th1.17 (IL-17A$^+$IFN gamma$^+$) cells post-transplant (131, 132), supporting a functional correlate to the changes in regulatory T-cell networks (159). Furthermore, Darlington et al. demonstrated a reduction in IL-17A post-AHSCT (131), while staining for IFN gamma, TGF beta, IL-23 and CD8$^+$ cytotoxic cytokine responses remained unchanged. By contrast, Sun et al. failed to find sustained difference in the cytokine profile post-AHSCT, despite persistent clinical responses (134). Despite an acknowledgment of the unidentified antigenic target(s) of MS, markers of autoreactivity have been frequently used a benchmark of the inflammatory state of disease. Studies analyzing changes in the populations of T cells targeting self-proteins such as myelin have persistently demonstrated spontaneous re-emergence of these populations following AHSCT (131, 134). While frequencies of autoreactive T cells do not appear effected by AHSCT, a reduced inflammatory Th17 response on MBP stimulation assays (131) supports the development of an altered antigenic response following AHSCT. A quantitative increase in circulating Tregs has associated with enhanced expression of immunoregulatory molecules such as CTLA-4 and glucocorticoid induced tumor necrosis factor-related protein, potentially modulating interactions between lymphocytes recruiting inflammatory cells to the CNS (176). Presumed pro-inflammatory mucosal-associated invariant T cells have been shown to be depleted post-AHSCT, to a greater degree than alternate IR therapies (135). Additionally, programmed cell death-1 protein (PD1), a cell surface marker known to downregulate inflammatory responses and induce tolerance following interaction with its ligand has been found to be more strongly expressed in lymphocytes following AHSCT, with degree of expression correlating with duration of disease remission (129, 132).

Normalization of Immune Gene Expression

The importance of the environment in modulating gene expression is now well established, and it is likely that epigenetic changes induced by the lymphopenic state post-AHSCT may underlie many of the immunological modifications which occur following transplantation. Downregulation of microRNAs (miR-155, miR-142-3p, and miR-16) previously demonstrated to be upregulated in MS (176) and a concurrent increase in the expression of their normally silenced target genes (FOXP3, FOXO1, and IRF2BP2) has been demonstrated out to 2 years following AHSCT, all of which associate with maintenance of a tolerant cytokine environment. The downregulation of miR-16 (which targets PDCD1, the gene encoding PD-1) following AHSCT has been postulated to account for increased secretion of IL-10 following AHSCT (159). Gene expression profiles by microarray DNA-chip technology have been used to study CD4$^+$ and CD8$^+$ cells pre- and post-AHSCT (177), showing a normalization of differentially expressed genes (DEGs) toward that of healthy controls. Interestingly, CD8$^+$ DEGs mirrored that of healthy controls at 2 years post-AHSCT, while CD4$^+$ gene expression remained distinct from pre-transplant expression and that of controls. Work in the field of malignancy has suggested that the HSCT induced transcriptional changes correlate with the degree of myeloablation and forced bone marrow expansion (178), implying that the intensity of conditioning influences the pathogenicity of lymphocytes. While it is acknowledged that more profound therapeutic effects can be achieved with higher intensity conditioning (119), what remains unclear is whether this is simply a reflection of lymphopenia induced by the transplant and the subsequent effects of LIP with presumed replicative senescence of pathogenic clones and creation of space for TCR diversification, or whether there are chemotoxic effects that influence the biology of resident lymphoid progenitors in the bone marrow and thymus, resulting in enhanced proliferation following engraftment.

Defects of central and peripheral tolerance implicated in MS pathogenesis (179, 180) may also normalize post-AHSCT. Intrinsic pro-apoptotic genes, abnormal in MS patients at baseline when compared with healthy controls, normalized post-AHSCT. Interestingly these genetic modifications appeared more pronounced in patients treated with a more aggressive conditioning regimen. Genes regulating extrinsic apoptotic processes (FAS, FASL, and c-FLIPL) also corrected post-AHSCT (181), supporting a re-establishment of activation induced cell death and clonal tolerance.

Unique Properties of AHSCT—Leukoapheresis Product

The potential for pathogenic clones within the graft product to be reinfused with CD34$^+$ HSCs is a subject of ongoing debate. Theoretically, if the conditioning regimen is sufficiently intense to abrogate all lymphocytes from the body, the graft serves as the sole source for IR. It is therefore conceivable that graft manipulation would result in improved rates of remission. Despite this hypothesis (33), a randomized trial assessing the role of graft manipulation in AHSCT for Rheumatoid Arthritis conducted by our unit did not show clinical benefit (121). We postulate that this relates not only to increased adverse event rates outlined earlier, but the importance of infusion of Tregs with the graft product. Furthermore, the significance of clonal populations within the graft product remains unclear. A single study assessing TCR BV-BJ gene usage in 4 MS patients undergoing AHSCT was unable to identify shared clonotypes between the apheresis product and post-AHSCT peripheral blood, despite the presence of shared clones between the pre- and post-AHSCT peripheral blood specimens (154). Cyclophosphamide (Cyc) is a potent alkylating agent that induces DNA fragmentation, exerting its toxicity on mature lymphocytes, while CD34$^+$ HSCs are relatively unaffected. While the leukoapheresis product is commonly evaluated pre-infusion with flow cytometry, the apoptotic potential of cells is not measured. Theoretically, cells entering the graft, which have been exposed to Cyc, would be less likely to engraft and proliferate, although recent work in the field of allogeneic transplant has suggested that a proportion of the engrafting cells are defined by

a Cyc-resistant phenotype (182). The influence of ATG on these populations remains unknown and is under investigation within our laboratory.

ALTERNATE IR THERAPIES—COMPARISONS WITH AHSCT

Current therapeutic approaches in MS can be dichotomized to chronic immunosuppression, or IR therapy where pulsed induction treatments are used to rebuild the immune system. While the goal of IR therapies is to induce long-term disease remission, practical experience suggests that retreatment may be required. Given the increasing application of alemtuzumab, a humanized IgG3 monoclonal antibody which targets the CD52 receptor of lymphocytes, monocytes, and dendritic cells (13, 183), and the recent EMA and pending FDA approval for cladribine, a 2-chlorodeoxyadenosine triphosphate prodrug inhibiting lymphocyte proliferation (12), there is an urgent need for improved understanding of the effects these treatments have on the immune system in comparison with AHSCT. This has been outlined in **Table 3** (12, 39, 42, 183–186) and explored further below.

TABLE 3 | Comparison of IR therapies.

	Autologous hematopoietic stem cell transplantation (AHSCT)	Alemtuzumab	Cladribine
Mechanism of action	Lympho/myeloablative chemotherapeutic conditioning followed by autologous stem cell rescue	IgG3 mediated cell lysis of CD52 lymphocytes. Dosed at 0 and 12 months	Purine anti-proliferative oral therapy mediating selective lymphocyte apoptosis. Dosed at 0, 1, 12, and 13 months
Clinical efficacy (for RRMS)	Phase 1/2 trials and observational studies estimate MRI and clinical disease-free survival 78–83% at 2 years	Phase 3 treatment-controlled trials report MRI and clinical disease-free survival 39% at 2 years	Phase 3 placebo-controlled trials report 57.6% reduction in relapse rate, 74.4% relative reduction in combined unique lesions on MRI
CD4 repopulation	Memory cell counts approach baseline by 18–24 months	70–80% baseline at 12 and 24 months	Fall by 40–60%. Naïve cells fall to greater degree than memory cells
CD8 repopulation	Early reconstitution 3–6 months dominated by CD8 memory cells	Fall by 80–90% post-dosing, reach 50% baseline at 12 and 24 months	Fall by 20–40% from baseline after dosing
B cell repopulation	Approach baseline levels at 6–9 months	$CD19^+$ cells return to baseline at 3–6 months, reach 120–130% prior to re-dosing	Fall by 90% after dosing, close to baseline prior to re-dosing at 12 months
Effect on thymic output	Multiple publications demonstrating increased $CD4^+CD31^+$ (RTE) and increased T cell receptor excision circle (TREC) post-AHSCT	Single study showing decrease in TREC post-alemtuzumab treatment	Unclear
Secondary AID	14/273 (5%) cases in largest longitudinal observational study	Estimated up to 50% at 7 years post treatment	None reported in Phase 3 trials

Alemtuzumab

Alemtuzumab has been used historically for treatment of hematological malignancy and in organ and BMT and has recently been approved for the treatment of RRMS. Alemtuzumab use in MS patients is associated with improvement in disability, and a sustained reduction in both relapse rate and MRI disease activity for up to 5 years in 70% of patients (13, 183). However, IR following alemtuzumab treatment results in secondary AID in up to 50% of patients at 7 years post treatment (185). As such, use of alemtuzumab is typically reserved for patients with aggressive disease, akin to patients referred for AHSCT. Both treatments deplete T and B lymphocytes resulting in profound lymphopenia (184) followed by IR.

Following alemtuzumab treatment, T cell repopulation is driven by early homeostatic proliferation of predominately $CD8^+$ memory lymphocytes (184) followed by slower reconstitution of $CD4^+$ cells with median recovery time to baseline of 61 months following a single infusion (187). 47% of patients reached pre-treatment total lymphocyte counts by 12 months (188) with $CD8^+$ populations approximating 50% of baseline and $CD4^+$ cells reaching 30% of baseline at this time (187–190). In contrast to AHSCT, T_N cells do not approach pre-transplant percentages until 24 months (184, 190). The depleted $CD4^+$ population is relatively enriched with $CD4^+CD25^+$ Tregs (188, 191), although absolute counts are significantly lower than baseline out to 24 months (184). Conversely, early B cell hyper-population is seen at 3–6 months post treatment correlating with a spike in B cell activating factor (BAFF) levels between 1 and 3 months (187, 189). The $CD19^+$ population is dominated by an arrested phenotype of immature ($CD19^+CD27^-CD38^+CD10^+$) cells (180% of baseline counts at 3 months) and to a lesser degree mature naïve ($CD19^+CD27^-CD38^+CD10^-$) cells, while $CD27^+$ memory B cell populations remain below 20% of baseline at 12 months (189). Thus, it is proposed that hyper-population of naïve B cells regenerating in the absence of Tregs may allow the development of autoreactive lymphocyte populations (184, 189). Elevated IL-21 levels, increased availability of antigen-presenting cells, and a lower threshold for cell proliferation in response to self-antigen skew the T cell repertoire following alemtuzumab therapy (192), further contributing to the development of secondary AID. Genetic studies have supported the role of IL-7 receptor polymorphisms in effecting $CD4^+$ reconstitution after anti-retroviral therapy in HIV and lower rates of GVHD following allogeneic HSCT (193). Although IL7 receptor expression does not appear to affect autoimmunity (188), it is possible that in the lymphopenic environment, the MS associated IL-7R SNPs (194) may support the proliferation of CD4 and 8 cells to variable degrees and influence rates of secondary AID. Circulating $CD34^+$ cells are present to a substantially lower extent in the post-alemtuzumab environment when compared to AHSCT, potentially explaining the prolonged lymphopenia

following anti-CD52 therapy. Interestingly, TREC levels do not appear to be increased in alemtuzumab therapy (192), supporting the theory that higher $CD34^+$ load more readily induces thymic T cell production. To determine whether these clear differences in mechanism of action between AHSCT and alemtuzumab correlate with differences in therapeutic efficacy will require a head-to-head trial.

Cladribine

Cladribine is a purine analog whose active metabolite, 2-chlorodeoxyadenosine (2-cDK), is selectively accumulated in lymphocytes as a result of intracellular enzyme characteristics. 2-dCK exerts a lymphoablative effect *via* disruption of cellular metabolism and impairment of DNA synthesis. A 2010 phase III, placebo-controlled trial demonstrated a significantly lower annualized relapse rate compared with placebo, a higher relapse-free rate (79.7 vs. 60.9%) and a significant reduction in new brain lesions on MRI (12). Although initial clinical application was limited by concerns regarding infection and malignancy risk, it is anticipated that cladribine use will increase in the near future following recent studies that have led to a revised risk assessment (195).

T and B cells undergo a dose dependent depletion following cladribine induction (186). B cells drop to roughly 10% of baseline, reaching ≈80% of baseline prior to re-dosing. Reconstitution kinetics of B cells were notably different to the early hyperpopulation seen within alemtuzumab cohorts (186). $CD4^+$ and $CD8^+$ T cells undergo a similar pattern of depletion; with more marked suppression seen in the $CD4^+$ population, reaching a nadir of 70% below baseline at 3 months in patients dosed at 5.25 mg/kg, and a 45% fall in the 3.5 mg/kg (EMA approved dose) cohort. $CD8^+$ populations fell by 30–50% (186). Consistent with other IR therapies; T_N cells fell to a greater extent than T_M populations and reconstituted more slowly. $CD4^+$ T_N cells fell by 80% 12 weeks after treatment cycle 1 and never rose above 70% of baseline. $CD4^+CD45RO^+$ T_M cells followed a similar pattern although recovered to 60–65%. $CD8^+$ T_N cells fell to a lesser extent; roughly 25% at 12 weeks and the $CD8^+$ T_M population only dropped by 10% (186). Although phase IV studies of cladribine are eagerly awaited, rates of secondary AID appear significantly lower than alemtuzumab (12). In regard to reconstitution profile, cladribine induction therapy mirrors the synchronized lymphocyte repopulation of AHSCT, reducing the risk of secondary autoimmunity. However, long-term clinical follow-up will be best placed to advise on the durability of this lympho-depleting therapy and future studies of deep immune phenotyping may offer further insights into the functional changes occurring in comparison with autologous transplantation.

AHSCT: A "FAST FORWARD" OF THE IMMUNE SYSTEM

It is clear that a sustained, qualitative change in the immune system occurs after AHSCT. As outlined above, the T cell population in "health" reflects a balance of space, attrition, and replicative senescence. Akin to longitudinal studies of aging which suggest that a senescent phenotype of memory lymphocytes accumulate through progressive differentiation and chronic antigen exposure, a similar process clonal exhaustion may explain the transition from relapsing remitting to secondary progressive (SP) MS, where the inflammatory response to the aforementioned driver of oligodendrocyte apoptosis is abrogated over time.

In that vein, AHSCT may provide a mechanism of "accelerating" the immune response to antigen, inducing senescence and possibly apoptosis of pathogenic T cell populations as well as boosting mediators of immune tolerance, in the absence of relapse-associated disability. We postulate that this centers around the induction of significant lymphopenia triggering fundamental shifts in the circulating lymphocyte pool. The early ablation of a proportion of clonally expanded, antigen-specific lymphocytes through the use of cyclophosphamide in the mobilization regimen, the conditioning chemotherapy and *in* or *ex vivo* T cell depletion not only significantly decreases the load of pathogenic cells surviving AHSCT, but induces LIP. LIP drives cells through progressive phenotype switching, resulting in an early expansion of $CD8^+CD28^-CD57^+$ terminally differentiated, effector lymphocytes. While the implication of this effector memory population remains debated, with some reviews suggesting an immunosuppressive effect on $CD8^+$ cells (135), we favor a role equivalent to that defined in studies of antigen experienced lymphocytes (93) where a senescent phenotype is dominant. It is proposed that a proportion of these cells may be implicated in MS pathogenesis and LIP drives them to clonal exhaustion, rendering them no longer pathogenic.

Attrition describes the contraction of clonal lymphocytes that occurs as a result of competition for space between memory cells (196). Despite controversies regarding the required purity of the $CD34^+$ leukoapheresis product, it is accepted that a proportion of circulating lymphocytes post-AHSCT have either survived the conditioning chemotherapy or post infusion serotherapy. Early LIP is likely to be dominated by surviving clones reacting to common pathogens such as CMV or EBV (156). Expansion of these populations may impact clonal space, and preliminary work in our laboratory has suggested that the degree of early $CD8^+$ oligoclonality post-AHSCT correlates with CMV status. Through immunoablation and subsequent clonal exhaustion, a proportion of the expanded T cell pool are sacrificed, creating "space" to ensure the survival of naive lymphocytes post-AHSCT. Moreover, evidence exists to support the concept that these cells are thymic derived (131, 133, 164), which appears critical in the maintenance of a diverse T cell repertoire. Whether signaling between the thymus and peripheral drivers of proliferation influences kinetics of lymphocyte expansion remains unclear, although it has been proposed that RTEs may suppress expansion of peripheral memory T cells (160). It is clear that lymphopenia induces thymic rebound beyond the levels expected for age (140). Whether this thymic rebound is triggered by the degree of lymphopenia induced by the conditioning regimen, enhanced by the presence of HSCs which may demonstrate a superior ability to track to and engraft the thymus, or a combination of the two mechanisms, remains uncertain. Comparative studies with alemtuzumab suggest the importance of HSCs and RTEs in supporting synchronized T and B cell maturation (192). Lymphopenia not only promotes development of Treg populations but results in a series of epigenetic changes that may ensure durability of the tolerant phenotype after recovery of lymphocyte counts (129, 181). Following AHSCT,

suppression of MS activity by clinical and radiologic measures appears to be accompanied by the reappearance of functional thymic T cell development, including Treg populations, enabling restoration of efficient antigen presentation.

Despite being highly efficacious in suppressing CNS inflammation and inducing disease stability IR therapies continue to be limited by morbidity and a small but significant mortality rate. There is evidence that IR therapies exert substantial changes in the quality of the immune system, indeed these changes are more likely to explain the induction of prolonged disease remission than the immunosuppression itself. Analysis of shifts in T cell populations over the period of IR allows the opportunity to gain significant insights into the pathogenesis of disease and treatment complications. Since the nucleotide sequence of the TCR can be used as a "fingerprint" of clonal populations, high-throughput sequencing analysis of individual T cell clones following IR therapy has the potential to monitor for pathogenic T cells. Development of targeted therapy in MS is however limited by our lack of identification of the antigenic target of disease. By identifying clonal T cell populations that correspond with disease (or an absence thereof) we envisage the potential for TCRs as a biomarker of MS, with utility in diagnosis, monitoring of treatment response or complications (AID) or for targeted cellular therapies in the future.

FUTURE TRIALS

Despite the increasing development of biologic therapies in MS, unmet needs exist for patients with aggressive and treatment-refractory MS. It is these patients who are at greatest risk of failing to respond to currently available disease-modifying therapy and in whom application of AHSCT is most appropriately targeted. More than 1,000 MS patients have been treated since 1997 and reported to the EBMT registry up until June 2017 (197). MS continues to be the most frequent indication for AHSCT in the non-malignant setting (197), with dominant countries of activity being Italy, Germany, Sweden, the United Kingdom, The Netherlands, Spain, France, and Australia. In the absence of a phase III, randomized controlled trial, autografts for MS should continue to be performed in the clinical trial setting and development of such trials is underway (18). In the United States, a National Institute of Health "BEAT-MS" trial will randomize patients to AHSCT or best available approved treatment including natalizumab, alemtuzumab, ocrelizumab, and potentially cladribine. The "NET-MS" study will follow a similar schema, while in Scandinavia the "RAM-MS" will compare AHSCT with alemtuzumab, with a similar "STAR-MS" study planned in the UK (18). It is hoped that these trials, along with the use of "real world" databases such as EBMT (197) and MSBase (198, 199) may better delineate which patients are most appropriately referred for AHSCT as opposed to alternate IR therapies, as this continues to be an undefined area of clinical practice influenced not only by MS severity but additional patient-specific factors.

Further investigation is also required to determine the optimal treatment protocol for AHSCT. Outstanding questions exist in regard to conditioning and mobilization regimens, which are unlikely to be answered in a head-to-head trial (15, 18). Attempts at modulating the "host environment" through the use of lymphodepletion or cytokine therapy has been trialed in the malignancy setting to improve engraftment and greater lymphocyte proliferation (200, 201). It has been postulated that establishing an anti-inflammatory environment prior to HSC infusion may benefit IR. Despite modest clinical benefit in Phase I–II clinical trials (202, 203), mesenchymal stem cells may possess unique immunomodulatory properties, inducing an anti-inflammatory milieu which may further enhance the benefits of AHSCT or other IR therapies (202–204).

CONCLUSION

In order to improve therapeutic advances in MS, it is necessary to understand (i) what factors initiate the dysfunctional state, (ii) what are the antigenic target(s) of clonal T and B cell populations, and (iii) how a tolerant immune system is restored. Here, we have outlined crucial components of our hypothesis addressing these questions and summarized the role of AHSCT as an IR therapy. While many outstanding questions remain, we are developing a clearer understanding of the immunological shifts that are induced with AHSCT. It is hypothesized that accelerating the demise of pathogenic clones through LIP, replicative senescence and clonal attrition, the creation of "space" and the capacity for thymic rebound, regenerating a tolerant immune system from $CD34^+$ HSCs are the core immunological shifts occurring in AHSCT. Future research identifying clonal T cell populations that correspond with disease and/or secondary autoimmunity and development of targeted T cell therapy remain the ultimate treatment goal. In the interim, exciting promise exists by closely examining these mechanisms in patients undergoing AHSCT.

AUTHOR CONTRIBUTIONS

JMassey conceptualized and designed the review, and drafted and edited the manuscript. IS conceptualized and designed the review and edited the manuscript. DM conceptualized the review and edited the manuscript. JMoore conceptualized the review and edited the manuscript.

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REVIEW
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Immune Reconstitution After Autologous Hematopoietic Stem Cell Transplantation in Crohn's Disease: Current Status and Future Directions. A Review on Behalf of the EBMT Autoimmune Diseases Working Party and the Autologous Stem Cell Transplantation In Refractory CD—Low Intensity Therapy Evaluation Study Investigators

Alan Graham Pockley[1], James O. Lindsay[2], Gemma A. Foulds[1], Sergio Rutella[1], John G. Gribben[3], Tobias Alexander[4,5] and John A. Snowden[6] On behalf of the EBMT Autoimmune Diseases Working Party (ADWP) and the ASTIClite Study Investigators*

[1] John van Geest Cancer Research Centre, School of Science and Technology, Nottingham Trent University, Nottingham, United Kingdom, [2] Centre for Immunobiology, Barts and the London School of Medicine and Dentistry, Blizard Institute, Queen Mary University of London, London, United Kingdom, [3] Centre for Haemato-Oncology, Barts Cancer Institute, Queen Mary University of London, London, United Kingdom, [4] Department of Rheumatology and Clinical Immunology, Charité – University Medicine, Berlin, Germany, [5] German Rheumatism Research Center Berlin (DRFZ) – a Leibniz Institute, Berlin, Germany, [6] Department of Haematology, Sheffield Teaching Hospitals NHS Foundation Trust, Sheffield, United Kingdom

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Edited by:
Maria Carolina Oliveira,
University of São Paulo, Brazil

Reviewed by:
Milton Artur Ruiz,
University of São Paulo, Brazil
Kristina Harris,
Immune Tolerance Network,
United States

***Correspondence:**
John A. Snowden
john.snowden@sth.nhs.uk

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Patients with treatment refractory Crohn's disease (CD) suffer debilitating symptoms, poor quality of life, and reduced work productivity. Surgery to resect inflamed and fibrotic intestine may mandate creation of a stoma and is often declined by patients. Such patients continue to be exposed to medical therapy that is ineffective, often expensive and still associated with a burden of adverse effects. Over the last two decades, autologous hematopoietic stem cell transplantation (auto-HSCT) has emerged as a promising treatment option for patients with severe autoimmune diseases (ADs). Mechanistic studies have provided proof of concept that auto-HSCT can restore immunological tolerance in chronic autoimmunity *via* the eradication of pathological immune responses and a profound reconfiguration of the immune system. Herein, we review current experience of auto-HSCT for the treatment of CD as well as approaches that have been used to monitor immune reconstitution following auto-HSCT in patients with ADs, including CD. We also detail immune reconstitution studies that have been integrated into the randomized controlled Autologous Stem cell Transplantation In refractory CD—Low Intensity Therapy Evaluation trial, which is designed to test the hypothesis that auto-HSCT using reduced intensity mobilization and conditioning regimens will be a safe and effective

means of inducing sustained control in refractory CD compared to standard of care. Immunological profiling will generate insight into the pathogenesis of the disease, restoration of responsiveness to anti-TNF therapy in patients with recurrence of endoscopic disease and immunological events that precede the onset of disease in patients that relapse after auto-HSCT.

Keywords: autologous stem cell transplantation, Crohn's disease, hematopoietic stem cell transplantation, immune reconstitution, inflammatory bowel diseases, T cell receptor repertoire

INTRODUCTION

Etiology, Epidemiology, and Management of Crohn's Disease (CD)

The intestinal inflammation associated with CD is caused by mucosal immune system reactivity to luminal antigen in genetically susceptible individuals. Active intestinal inflammation is associated with dysbiosis of the fecal and mucosal microbiota and increased intestinal permeability with alterations in innate lymphoid cell (ILC) populations. Defects in the innate immune pathway in CD are implicated by pathogenic mutations in the nucleotide-binding oligomerization domain 2 (*NOD2*) and autophagy-related protein 16-1 (*ATG16L1)* genes which suggest impairment of bacterial sensing and clearance. CD is associated with increased IL-12/IL-23 release from antigen-presenting cells and an imbalance in the differentiation of Th1/Th17 effector and regulatory lymphocytes (1, 2).

The incidence of CD is increasing in young adults who live with their disease for six decades (3). In addition, there has been a significant increase in CD incidence in newly industrialized countries of Africa, Asia, and South America (4). The prevalence of CD in the UK stands at approximately 145/100,000 (5) with 9.5 new cases per 100,000 annually (6). The highest prevalence in North America is reported in Canada at 319/100,000 (4). Traditional medical management focuses on controlling intestinal inflammation using conventional or biological therapy. Although many patients respond to first line biologic therapy, a recent prospective UK registry of 1,500 patients with CD commencing their first anti-TNF therapy reports primary non-response in between 16.9 and 23.7% of patients (7) and a secondary loss of response in a further 29% of patients over 2 years (7).

Recent single and multicenter cohort studies have reported a significant burden of adverse events with anti-TNF therapies including an increased risk of lymphoma independent of the use of concomitant thiopurine therapy (8, 9). The anti-integrin vedolizumab and the IL-12/23 antibody ustekinumab are both licensed as second-line biologic therapy for refractory CD. However, both therapies are less effective at inducing and maintaining remission in patients that have been exposed to anti-TNF therapy previously (10). Although novel biological therapies are in clinical development none have reported short- or long-term remission rates in more than 50% patients. Furthermore, biologic therapies currently constitute the largest proportion of the total treatment costs of patients with CD (11).

Patients refractory to medical therapy and those who develop stricturing or penetrating complications of disease progression require surgical resection of the affected intestine (12). However, disease recurrence after surgery is common and many patients face repeated or extensive surgeries that may require a stoma or result in short bowel syndrome and a requirement for parenteral nutrition support. Although the introduction of biologic therapies has been associated with a reduction in the rates of surgery over the recent decade, there has been no reduction in the requirement for repeat surgery (13). Patients with active disease refractory to currently licensed therapies in whom surgery is inappropriate or declined face ineffective biologic therapy and frequent courses of corticosteroids which are associated with a significant burden of treatment-related morbidity and mortality and high health-care resource utilization (11). There is a clear unmet need for an effective long-term therapy for this cohort of patients.

Clinical Studies Investigating Auto-HSCT for CD

Over the last two decades, autologous hematopoietic stem cell transplantation (auto-HSCT) has been identified as a promising therapeutic option for patients with severe autoimmune diseases (ADs). Mechanistic studies suggest that restoration of immunological tolerance in chronic autoimmunity occurs after auto-HSCT *via* eradication of immune memory and reconfiguration of the immune system. Although case reports suggested exceptional benefit for patients with refractory CD after auto-HSCT (14, 15), concerns about safety and a lack of understanding as to whether benefit relates to the chemotherapeutic agents administered during mobilization/pre-transplant conditioning regimen or the transplant itself, led to the Autologous Stem cell Transplantation International Crohn's disease (ASTIC) randomized controlled trial (NCT00297193) (16, 17). This was conducted at 11 accredited centers in 6 European countries (16, 17).

Autologous Stem cell Transplantation International Crohn's disease compared cyclophosphamide mobilization alone to mobilization, high-dose chemotherapy, and auto-HSCT in patients with refractory CD (16, 17). Eligible patients underwent peripheral blood stem cell mobilization with high-dose cyclophosphamide (4 g/m^2) and granulocyte colony-stimulating factor (G-CSF), after which they were randomized to immediate auto-HSCT or conventional care for 1 year (16, 17).

The primary endpoint for the trial was defined as clinical disease remission (CDAI < 150) for 3 months, with no medication for CD and no evidence of active disease on imaging and endoscopy at 1 year. Few patients randomized to immediate HSCT or who underwent mobilization and were then randomized to conventional care achieved the ambitious primary endpoint at 1 year.

However, ASTIC did demonstrate benefits of auto-HSCT over conventional care in more traditional endpoints for therapeutic trials in this area (18), such as steroid-free clinical remission (CR) and mucosal healing (16). In addition, after the primary endpoint had been assessed, patients who had undergone mobilization and then been randomized to conventional care were offered auto-HSCT and then followed for a further year with identical assessments as in the randomized trial. Subsequent analysis of the 38 patients who underwent auto-HSCT in the ASTIC program and had data at baseline and 1 year reported a significant reduction in clinical and endoscopic disease activity at 1 year, with 19 out of 38 (50%) patients showing regression of all endoscopic ulceration (17). There were also significant improvements in quality of life between baseline and 1 year after auto-HSCT (17, 19, 20). Importantly, disease recurrence after HSCT responded to the introduction of anti-TNF therapies (15, 17). The doses of cyclophosphamide used in both groups resulted in significant numbers of adverse events and one death (17, 21, 22). Subsequent expert review has suggested that the high dose cyclophosphamide regimen used at both mobilization and conditioning was a factor for many of the adverse events (17, 21, 22). This view is supported by the outcome of an uncontrolled series of 14 patients with refractory Crohn's disease (CD) who underwent auto-HSCT using a lower dose of cyclophosphamide during mobilization (2 g/m^2) and conditioning (50 mg/kg for 4 days). The median duration of anemia and neutropenia was shorter after both mobilization and conditioning than that seen in previous reports using higher cyclophosphamide dosing, and few episodes of febrile neutropenia were reported. The lower intensity regimen still resulted in marked reduction in clinical disease activity with 13 patients achieving disease remission (CDAI < 150) at 30 days (20).

In addition, favorable long-term outcome after auto-HSCT in a single-center cohort of 29 patients with CD (some of whom participated in the ASTIC trial) has been described (23). This includes 5-year follow-up data with scheduled clinical, endoscopic and radiological assessment. Drug-free clinical and endoscopic remission (CDAI < 150, SES-CD < 7) was seen in 61% at 1 year, 52% at 2 years, 47% at 3 years, 39% at 4 years, and 15% at 5 years. However, 80% of those patients who experienced a relapse responded to the re-introduction of anti-TNF therapy. Six out of the 29 underwent surgery after auto-HSCT, and 1 patient died of CMV infection.

Current Data on HSCT for CD From the EBMT Autoimmune Disease Working Party (ADWP) Registry

The long-term outcomes for all adult patients undergoing auto-HSCT for CD in Europe between 1997 and 2015 (outside the ASTIC study) have been evaluated using the EBMT registry. Clinical data were obtained for 82 patients from 19 centers in 7 countries, with clinical response being categorized as remission (no abdominal pain and normal stool frequency), significant improvement (improved pain and frequency), no change, or worsening of symptoms.

Median follow-up was 41 months (range 6–174). At 100 days post-HSCT, 64% of patients were in CR and a further 28% had experienced significant improvement. At 1 year after transplantation, data from 75 patients indicated that 43% were in CR, 20% improved, 17% unchanged and 20% worsened. 37% patients required surgery after auto-HSCT, and 73% re-started medical therapy. Of those requiring further treatment, 57% responded to therapies to which they had previously been refractory. Treatment-free survival, defined as survival without major surgery or medical therapy, was 27 and 22% at 3 and 5 years respectively.

In addition, the EBMT ADWP has produced a historical summary of the AD section of the registry, which has characterized outcomes following auto-HSCT in various indications. Compared with MS, relapse is more common in CD, which may reflect fundamental differences in disease processes (24).

As of November 2017, there have been a total of 166 transplant registrations for auto-HSCT of CD within the EBMT registry, with the majority 91.5% being in adults over 18 and 24% being treated as part of the ASTIC trial. Patients are predominantly being treated in Spain, UK, Italy, Belgium, The Netherlands, and France (**Figure 1**) (source, EBMT Office, Paris).

Taken together, current experience indicates that auto-HSCT can induce clinical and endoscopic remission, but does not result in sustained treatment-free disease remission in most patients. However, many patients become responsive to treatments to which they were previously refractory. Biomarkers that predict which patients will respond to anti-TNF therapy after auto-HSCT have not been identified and should be a focus of future trials in this area. In addition, auto-HSCT regimens including high dose cyclophosphamide are associated with significant adverse events, which may be avoided with lower intensity regiments. Hence further randomized controlled trials that assess the benefit of lower intensity regimens are warranted.

IMMUNE RECONSTITUTION FOLLOWING AUTO-HSCT FOR CD

Specific studies of immune reconstitution in CD patients after auto-HSCT are limited to a few observations (25, 26). Therefore, this review focuses on the general literature relating to immune reconstitution in ADs and highlights how it might be best investigated in patients with CD to identify mechanisms of action of auto-HSCT and gain insights into the pathogenesis of CD.

Rationale and Mechanistic Studies of Auto-HSCT for ADs

The original goal of auto-HSCT in ADs was to eradicate existing autoreactive immunological memory and regenerate a naïve, self-tolerant immune system (27, 28). Auto-HSCT has been shown to profoundly impact the immune system, as indicated by the regeneration of naïve B cells (29, 30), thymic reactivation (29, 31–33), the emergence of a polyclonal T cell receptor (TCR) repertoire (29, 31, 33), and restoration of Foxp3$^+$ regulatory T (Treg) (34, 35) and B regulatory cell levels (36). In addition, disease associated restriction of the peripheral blood Treg TCR repertoire is completely reversed by auto-HSCT, both in terms of number and uniqueness of Treg cell TCR sequences (37). Functional assays investigating the fate of autoreactive T cells post-transplantation

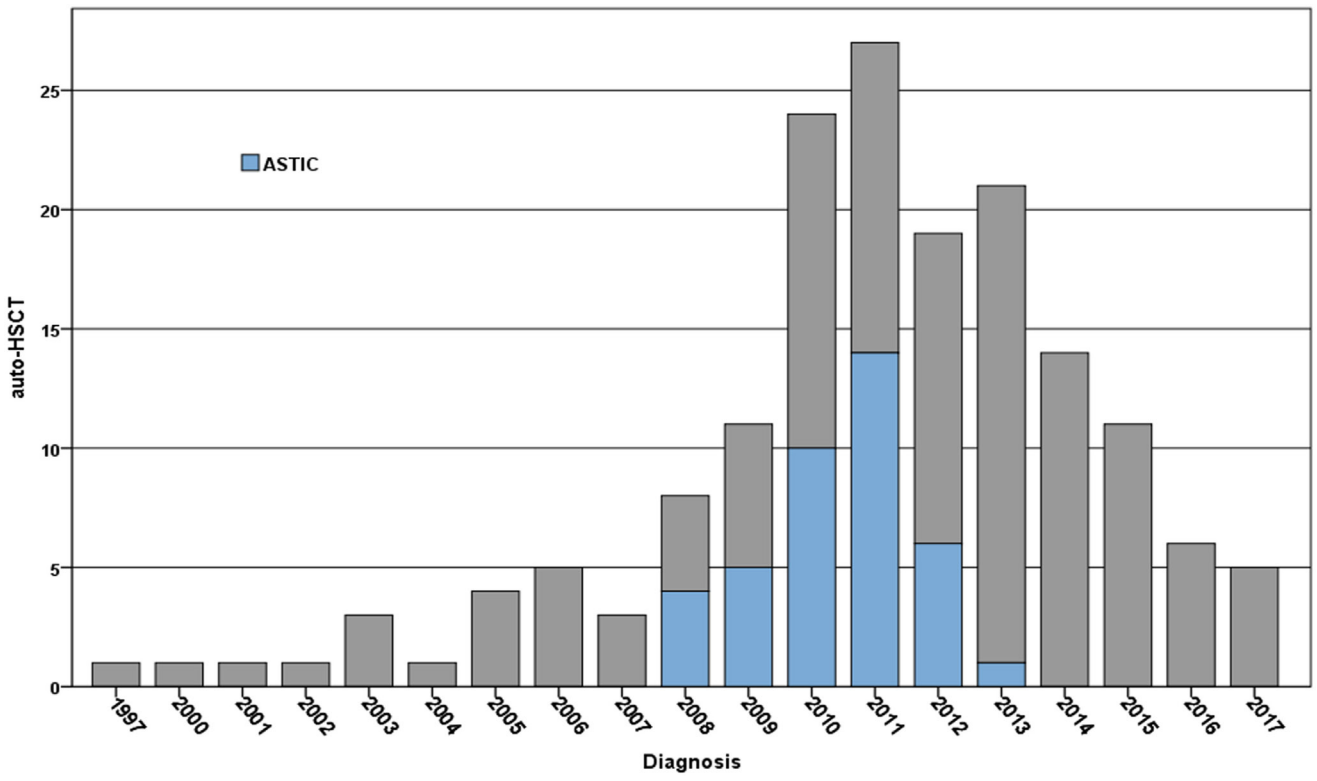

FIGURE 1 | Registrations per year for the treatment of Crohn's disease using autologous hematopoietic stem cell transplantation (auto-HSCT) within the EBMT registry 1994–2017 (note data for 2017 up to November 2017)—numbers treated on the Autologous Stem cell Transplantation International Crohn's disease (ASTIC) trial are indicated in blue. Patients have been predominantly treated in Spain, UK, Italy, Belgium, The Netherlands, and France in reducing order (source EBMT Office, Paris).

are limited due to restricted access to such cell clones in human disease compared to animal models. Nevertheless, *in vitro* stimulation assays in SLE have been able to demonstrate that T cell responses to antinuclear antigens were abolished, whereas virus-specific T cells were readily detectable in the first months after HSCT (29). Likewise, stimulation assays with myelin oligodendrocyte glycoprotein peptides in auto-HSCT-treated MS patients revealed reduced interleukin-17 responses and enhanced transforming growth factor-β1 production compared to natalizumab-treated patients, suggesting removal of autoreactive T cell clones as well as enhanced suppressive responses after HSCT (38). Collectively, these observations suggest that auto-HSCT has indeed the potential to restore self-tolerance by "resetting" the chronic autoreactive immune system into a naïve state.

Compared with MS and rheumatological diseases, there are relatively little data specifically relating to immune reconstitution following auto-HSCT in CD. The following sections will discuss how components of aberrant immunity may be investigated further in CD.

Impact of Auto-HSCT for CD on the Innate Immune System

The intestinal innate immune system not only protects against invading pathogens, it also regulates the interactions between the commensal intestinal microbiota and the host at multiple levels, many of which are altered in CD. A small study investigating the effect of auto-HSCT on the innate immune system in seven patients with moderate to severe CD has reported elevated numbers of peripheral blood TLR-4 expressing monocytes that also express TNF-alpha and IFN-gamma at baseline compared to healthy controls, which were normalized after transplantation (25). The impact of auto-HSCT on other intestinal innate immune populations has not been assessed. Studies on ILCs would be of particular relevance given their role in orchestrating immune defense and regulation at mucosal surfaces and their role in CD pathogenesis.

Impact of Auto-HSCT for CD on the Adaptive Immune System

The increased responses of $CD4^+$ Th17/Th1 cells toward bacterial-derived proteins that have been described in patients with CD suggest a potential mechanism for sustaining persistent disease (39). Elimination of such cells *in vivo* by immunoablative chemotherapy is presumed to ameliorate intestinal inflammation. Conversely, recurrence of regulatory $Foxp3^+$ Treg cells could restore self-regulation. The first investigation of the impact of auto-HSCT on peripheral blood lymphocytes in patients with CD demonstrated restoration of dysregulated T effector cell responses

with a normalization in the number of IFN-gamma-secreting $CD4^+$ T cells together with a significant increase in $Foxp3^+$ Treg cells (25). Interestingly, early restoration of circulating Treg numbers was predictive for treatment response at 3 months post-transplantation.

Impact of Auto-HSCT for CD on TCR Repertoire Profile

The extensive immune renewal that follows auto-HSCT is associated with a vast diversification of the TCR repertoire. For example, in MS, $CD4^+$ T cell dominant TCR clones that were present in peripheral blood before treatment were undetectable after immune reconstitution, with patients predominantly developing a new repertoire. More importantly, the T cell repertoire early during the reconstitution process were less diverse in patients who failed to respond to treatment (40). However, these data were exclusively obtained from peripheral blood and not from the site of inflammation. By contrast, next-generation sequencing (NGS) of the TCRβ locus in ileal and colonic biopsies collected at baseline (pre-mobilization) and after auto-HSCT (6 months and/or 1 year after transplantation) has been used to correlate TCR diversity with clinical and endoscopic outcome in 16 patients with CD (26). Quantifying repertoire similarities of T cell clones at different time points were quantified by the Morisita–Horn index (M–H; range 0–1) revealed monoclonal expansions in the mucosal T cell compartment at baseline prior to HSCT. The TCR repertoire was more polyclonal than expected (500 to 20,000 unique TCR sequences, clonality index 0.1 to 0.3), with no shared public TCR sequences being apparent in the mucosa of different patients. The clonality of the TCR in the mucosa was significantly increased after HSCT and the T cell repertoire appears reset, as the similarity index between baseline and after the procedure was low (26).

Impact of G-CSF on Immune Reconstitution Post Auto-HSCT in CD

Granulocyte colony-stimulating factor is given both to mobilize hematopoietic stem cells and to accelerate neutrophil recovery post auto-HSCT. However, it can profoundly affect innate and adaptive immunity by inducing the differentiation of type 1 Treg cells and tolerogenic dendritic cells (DCs) (41). Clinical benefit from G-CSF was reported in five patients with severe endoscopic postoperative recurrence of CD who received 300 μg of recombinant human G-CSF three times per week for 12 consecutive weeks (42). Administration of G-CSF was safe and associated with significant increases in neutrophil counts, and levels of IL-1 receptor antagonist and soluble TNF receptor p55 and p75.

A clinical trial in nine patients with CD at the USA National Institutes of Health confirmed the potential benefit of subcutaneous G-CSF given over a 4-week period (43). In this study, changes in immune cell phenotypes, including myeloid and plasmacytoid DCs, as well as cytokine production were measured in the peripheral blood and colonic mucosal tissues. Patients who responded to G-CSF or achieved CR had significantly more IL-10-producing $CD4^+$ memory T cells in the periphery, as well as a greatly enhanced $CD123^+$ plasmacytoid DC infiltration of the lamina propria. Interestingly, IFN-γ production was higher in non-responders to G-CSF compared with responders. Although a randomized controlled placebo trial has not yet been performed, these studies suggest that G-CSF *per se* might offer some therapeutic benefit for patients with CD. Whether stem cell mobilization with G-CSF affects the functional orientation of immune cell populations in patients with CD and whether G-CSF-mobilized immune cell subsets, such as Treg cells and/or plasmacytoid DCs, favor the re-establishment of immune tolerance after auto-HSCT remains to be evaluated.

Does Auto-HSCT Induce a Reset or Temporary Downregulation of Immunity in CD?

It is essential to determine whether there is a real "reset" of autoimmunity following auto-HSCT for CD, as has been observed for multiple sclerosis and systemic lupus erythematosus and which forms the basis for treatment-free remissions, or simply a temporary downregulation of innate and acquired immune responses. The relapse rate in CD after auto-HSCT is considerably higher compared to other ADs, which could be related to an insufficient eradication of the autoreactive immunologic memory by the conditioning regimens applied and/or a more prominent genetic contribution that favors disease recurrence. The latter fact is supported by epidemiologic studies demonstrating concordance rates among monozygotic twins of up to 50% (44). Alternatively, failure of achieving durable treatment-free remissions in CD post-transplantation could indicate that aberrant adaptive autoimmune responses and formation of a pathogenic immunologic memory are not the driving force in disease pathogenesis, as confirmed for other systemic ADs. In fact, perturbations in the innate immune pathway resulting in compromised mucosal barrier functions may have a stronger implication in driving chronic autoimmune responses in CD, which may not be corrected by "resetting" the immune system with auto-HSCT. Data supporting these considerations are not available yet, as studies investigating the influence of intestinal epithelial barrier changes on the dysfunction of mucosal innate and acquired immune responses after auto-HSCT in patients with CD are lacking.

Future Directions for Immune Monitoring After Auto-HSCT in CD

Guidelines and expert recommendations to develop and implement systematic approaches to monitor immune responses in patients with cancer have been recently published (45). Likewise, the EBMT Autoimmune Diseases and Immunobiology Working Parties have initiated a joint process to develop and implement guidelines for "good laboratory practice" to provide practical recommendations for biobanking and immune monitoring in patients with ADs undergoing HSCT (46). The analysis of biological specimens at the cellular, DNA, transcriptional, epigenetic, posttranscriptional, and protein levels, including peripheral blood and tissues, yields a massive amount of data, which need to be processed with novel bioinformatics methods.

For the analysis of TCR repertoire, several different approaches and methods exist. The approach to be used is dependent on the experimental questions being asked and methodological bias can make it difficult to compare results across different studies. The latest advances, available tools, the choice of starting material, and

the method for preparing samples have been reviewed in detail elsewhere (47). Most of the approaches involve the analysis of samples at the molecular level, commonly using deep and NGS (47).

Comprehensive protocols relating to multi-parameter flow cytometric analysis have been published previously (48, 49). In the future, such conventional flow cytometry may be combined with methods providing higher resolution, such as cytometry by time of flight mass spectrometry, as has already been used by Karnell and colleagues to investigate the kinetics of immune cell subset reconstitution in the periphery after HSCT and the impact of HSCT on the phenotype of circulating T cells in patients with MS (50). In addition, cytometric profiling may be complemented by an innovative flow cytometry approach which combines three monoclonal antibodies with two fluorophores to quantitate the TCR Vβ repertoire of human T lymphocytes (IOTest® Beta Mark TCR V-beta Repertoire Kit, Beckman Coulter).

Analysis of T Cell Reconstitution Based on T Cell Receptor Excision DNA Circles (TRECs)

T cell reconstitution after successful HSCT can occur *via* a thymic-independent pathway, which involves the expansion of graft-derived mature donor T cells, or a thymic-dependent pathway, the consequence of which is a regeneration of T cells with a more diverse TCR repertoire from graft-derived precursor cells (51). As thymic function is required for the *de novo* generation of T cells after transplantation, the potential function of T lymphopoiesis after auto-HSCT can be determined by quantifying TRECs (52). Signal joint TCR excision DNA circles (sjTRECs) result from the rearrangement of the TCR gene and the excision of circular DNA fragments from genomic DNA during thymocyte development. Measuring thymic function by quantifying sjTRECs in peripheral blood avoids disadvantages that are associated with the use of T cell surface molecules, such as CD45RA, as markers for recent thymic emigrants (RTEs). sjTRECs reflect developmental proximity to the thymus and the analysis of total sjTRECs levels and TCR beta variable region (TRBV) subfamily sjTRECs frequencies during immune reconstitution after HSCT is useful for more precisely determining thymic output function and T cell immune reconstitution (53). Although such analyses have not yet been undertaken in the context of CD, the increased precision of this approach has the potential to provide a more robust insight into the relationship(s) between immune status, disease status, and therapeutic resistance after auto-HSCT.

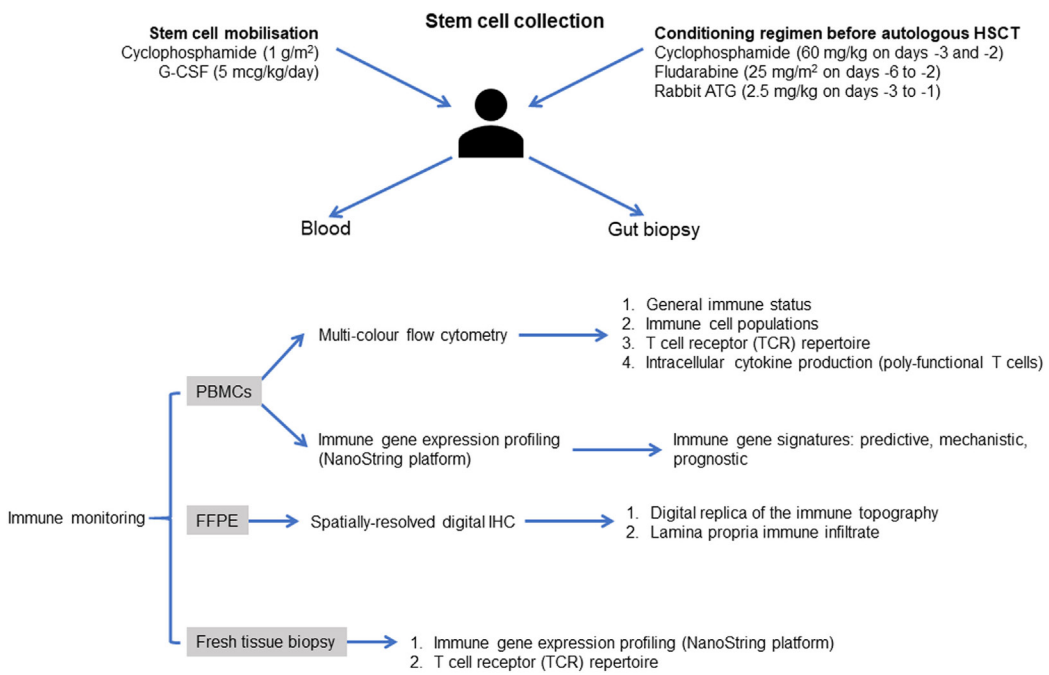

FIGURE 2 | Proposed approach to immune monitoring in the Autologous Stem cell Transplantation In refractory CD—Low Intensity Therapy Evaluation clinical trial. Immune reconstitution will be evaluated using patient-derived blood mononuclear cells at weeks 8 12, 24, 32, and 48 after infusion autologous hematopoietic stem cells. Multi-color flow cytometry and optimized multicolor immunofluorescence-like antibody panels will be used to assess the general immune status of the patients, as well as the ability to release immune regulatory cytokines at the single-cell level. Mucosal biopsies will be assessed at baseline, weeks 24 and 48. RNA will be extracted and will be analyzed on a NanoString FLEX™ gene expression profiling platform, as detailed in the main text. Additional studies could employ a multiplexed spatial protein profiling approach based on NanoString digital quantification of tissue proteins to generate a digital replica of the immune topography of intestinal biopsies and to gain mechanistic insights into the immune determinants of response after autologous HSCT, with emphasis on the reconstitution of plasmacytoid DCs, regulatory T cells, and TCR-Vβ repertoires. Abbreviations: G-CSF, granulocyte colony-stimulating factor; Cy, cyclophosphamide; Flu, fludarabine; PBMCs, peripheral blood mononuclear cells; FFPE, formalin-fixed paraffin-embedded; IHC, immunohistochemistry.

Amplification-Free Gene Expression Profiling of the Periphery and Tissue

Gene expression profiles reflect the immune *milieu* and are increasingly being used for immune monitoring purposes and identifying predictive biomarkers in patients with cancer. Microarrays have traditionally supported the high-throughput analysis of gene and miRNA expression, but they are limited by the requirement for relatively large quantities of high-quality RNA. Next Generation Sequencing (NGS) has become an important discovery tool, and the preferred choice for unbiased biomarker discovery of transcriptional signatures associated with disease activity, treatment outcomes, and mechanism of action studies of therapeutic agents. However, data analysis and interpretation require advanced bioinformatics approaches. Quantitative PCR (qPCR) provides a more accurate insight into gene expression than microarrays and requires lower amounts of RNA. However, the analysis of the expression of multiple genes with classical qPCR and digital PCR, which can also be used for high-throughput high-precision analysis, is more difficult.

NanoString-based molecular "bar coding" enables the high-throughput analysis of the expression of multiple genes using defined panels such as the nCounter™ Human Immunology, Human Inflammation, and Human Myeloid Innate Immunity Panels. The Human Immunology module could be particularly relevant to the post-HSCT immune monitoring of patients with CD, given that the ASTIC trial reported treatment-related infections to be the most frequent serious adverse events during the 100 days after conditioning and the subsequent follow-up (17). The NanoString nCounter™ analysis system detects the expression of up to 800 genes in a single reaction with high sensitivity and linearity across a broad range of expression levels (54). The platform utilizes digital detection and direct molecular barcoding of individual target molecules using fluorescently labeled capture and reporter probes incorporating 35- to 50-base target-specific sequences. This technology allows for direct, PCR amplification-free multiplexed measurements of gene expression from a low amount of mRNA (25–300 ng). Different sources of RNA, including total RNA, fragmented RNA and formalin-fixed paraffin-embedded (FFPE)-derived RNA, can be used. This approach is suitable for the discovery of gene expression signatures, their validation and diagnostic testing in large translational studies.

The Autologous Stem Cell Transplantation In Refractory CD—Low Intensity Therapy Evaluation (ASTIClite) TRIAL: An Opportunity to Investigate Immune Reconstitution Post Auto-HSCT in CD

Given that reduced intensity mobilization and conditioning regimens are associated with lower morbidity in malignant and AD (20, 55–58), the hypothesis that auto-HSCT using a reduced dose cyclophosphamide mobilization and low intensity conditioning (HSCTlite) will induce regression of ileocolonic ulceration in patients with refractory CD compared to standard of care will be tested *via* a soon-to-commence clinical trial (ASTIClite). This UK NIHR-funded national multicenter randomized controlled clinical trial aims to recruit 99 patients with a 2:1 randomization to compare HSCTlite with standard of care. For this, eligible outpatients with treatment refractory CD will be randomized to auto-HSCT with cyclophosphamide 1 g/m^2 + G-CSF for mobilization followed by transplant conditioning with fludarabine 125 mg/m^2,

TABLE 1 | Flow cytometry antibody panels for immune cell reconstitution in CD.

Cell type/subtype	Phenotype
CD4$^+$ and CD8$^+$ T cells[a]	
Naïve	CD3$^+$CD4$^{+/-}$CD8$^{-/+}$CD45RA$^+$CCR7$^+$
Effector	CD3$^+$CD4$^{+/-}$CD8$^{-/+}$CD45RA$^+$CCR7$^-$
Central memory	CD3$^+$CD4$^{+/-}$CD8$^{-/+}$CD45RA$^-$CCR7$^+$
Effector memory	CD3$^+$CD4$^{+/-}$CD8$^{-/+}$CD45RA$^-$CCR7$^-$
Gut-homing T cells	CD3$^+$CD4$^{+/-}$CD8$^{-/+}$CD49d$^+\alpha$4integrin$^+$CCR9$^+$
Recent thymic emigrants	CD3$^+$CD4$^{+/-}$CD8$^{-/+}$CD31$^+$
CD4$^+$ T helper (Th) subsets (option to include CD45RA$^{+/-}$)[a]	
Th1	CD3$^+$CD4$^+$CD8$^-$CXCR5$^-$CXCR3$^+$
Th2	CD3$^+$CD4$^+$CD8$^-$CXCR5$^-$CCR4$^+$CCR6$^-$
Th9	CD3$^+$CD4$^+$CD8$^-$CXCR5$^-$CCR4$^-$CCR6$^+$
Th17	CD3$^+$CD4$^+$CD8$^-$CXCR5$^-$CCR4$^+$CCR6$^+$CCR10$^-$
Th17.1	CD3$^+$CD4$^+$CD8$^-$CXCR5$^-$CXCR3$^+$CCR6$^+$CCR4
Th22	CD3$^+$CD4$^+$CD8$^-$CXCR5$^-$CCR4$^+$CCR6$^+$CCR10$^+$
Follicular T helper (Tfh)	CD3$^+$CD4$^+$CD8$^-$CXCR5$^+$PD-1$^+$ICOS$^+$
Gamma delta T cells	CD3$^+$TCR$\gamma\delta^+$
CD4$^+$ immunoregulatory T (Treg) cells	
	CD3$^+$CD4$^+$CD25^{high}CD127^{low}Foxp3$^+$CCR4$^{+/-}$CD45RO$^{+/-}$
Myeloid-derived suppressor cells (MDSCs)—Lineage (Lin) cocktail: CD3/CD19/CD20/CD56	
Monocytic	Lin$^-$HLA-DR$^{-/low}$CD11b$^+$CD14$^+$CD15$^-$CD124$^+$
Granulocytic	Lin$^-$HLA-DR$^{-/low}$CD11b$^+$CD14$^-$CD15$^+$CD124$^+$
Endothelial progenitor	Lin$^-$HLA-DR$^{-/low}$CD11b$^+$CD124$^-$
Dendritic cells (DCs)—lineage (Lin) cocktail: CD3/CD19/CD20/CD56	
Plasmacytoid	Lin$^-$CD14$^-$CD123$^+$CD11c$^-$
Conventional	Lin$^-$CD14$^-$CD123$^-$CD11c$^+$
Innate lymphoid cells (ILCs)—lineage (Lin) cocktail: CD3/CD14/CD19/CD20	
ILC1	Lin$^-$CD127$^+$CD161$^+$CD117$^-$CD294$^-$NKp44$^-$
ILC2	Lin$^-$CD127$^+$CD161$^+$CD294$^+$
ILC3	Lin$^-$CD127$^+$CD161$^+$CD117$^+$CD294$^-$NKp44$^-$
Mast cell progenitors (MCPs)—Lineage (Lin) cocktail: CD3/CD14/CD19/CD20	
	Lin$^-$FcεRIα$^+$CD203$^+$CD117$^+$
Natural killer (NK) cell subsets	
Cytotoxic	CD3$^-$CD56^{dim}CD16$^+$
Cytokine-producing	CD3$^-$CD56^{bright}CD16$^{+/-}$
Monocytes	
Classical	CD3$^-$CD14^{high}CD16$^-$
Intermediate	CD3$^-$CD14^{high}CD16$^+$
Non-classical	CD3$^-$CD14$^+$CD16^{high}
B cells/plasmablasts	
Naïve	CD3$^-$CD19$^+$CD27$^-$IgD$^+$
Switched memory	CD3$^-$CD19$^+$CD27$^+$IgD$^-$
Non-switched memory	CD3$^-$CD19$^+$CD27$^+$IgD$^+$
Plasmablasts	CD3$^-$CD19$^+$CD27^{high}IgD$^-$CD38^{high}
Regulatory B cells	CD3$^-$CD19$^+$CD1d^{high}CD5$^+$CD21$^+$CD24^{high}
T cell responsiveness to stimulation	
	CD3$^+$CD4$^+$CD8$^-$IL2$^{-/+}$IL4$^{-/+}$IL17$^{-/+}$TNFα$^{-/+}$IL10$^{-/+}$IFNγ$^{-/+}$
	CD3$^+$CD4$^-$CD8$^+$IL2$^{-/+}$IL4$^{-/+}$IL17$^{-/+}$TNFα$^{-/+}$IL10$^{-/+}$IFNγ$^{-/+}$

[a]*CD4 differentiation and helper subsets can be gated upon after Foxp3$^+$CD25^{hi}CD127^{low} Treg cells have been excluded from the CD4$^+$ T cell parent population.*

cyclophosphamide 120 mg/kg, and rabbit anti-thymocyte globulin 7.5 mg/kg (total doses) versus standard of care and followed for 48 weeks. The reduced doses of cyclophosphamide in the mobilization and conditioning regimens are based on the concerns raised following the single case of TRM in the original ASTIC trial, which may have been related to cumulative toxicity from higher mobilization dose of cyclophosphamide (4 g/m^2) followed by the transplant conditioning (21, 22). It is hoped that this measure will improve overall safety and reduce the likelihood of neutropenic sepsis during the mobilization phase, which may be performed as an outpatient procedure.

The standard care group will be able to receive any licensed biologic, immunosuppressive or nutritional therapy for CD at the discretion of the treating physician. Pre-specified interim analyses will be undertaken to confirm that the mobilization regimen is effective for stem cell harvest with no negative impact on disease activity. Colonoscopy and MRI at week 24 will assess the requirement for re-initiation of maintenance of anti-TNF therapy in those with evidence of disease activity post auto-HSCT. Immunological profiling of blood and mucosa before and after HSCTlite will identify its mechanism of action. It is expected that HSCTlite will have an acceptable side-effect profile.

Additional secondary clinical endpoints will be the impact of HSCTlite on clinical disease activity, steroid requirements, quality of life, and the presence of adverse/serious adverse events, as compared to standard of care [and historical HSCT, as observed in ASTIC (16, 17)]. Exploratory endpoints will be the safety and efficacy of maintenance anti-TNF therapy in those patients with the recurrence of endoscopic disease after HSCTlite.

At the core of this study is comprehensive and informative immunological profiling of the periphery and mucosa before and after treatment (**Figure 2**). Profiling will be focused on generating insight into the pathogenesis of CD, the responsiveness, and restoration of responsiveness to anti-TNF therapy in patients with endoscopic disease recurrence and the immunological events that precede recurrence of disease and occurrence of resistance to anti-TNF therapy in patients that relapse after auto-HSCT.

Reconstitution of immune potential is being monitored by determining the re-appearance of monocyte subsets, T cell subsets ("helper," "cytotoxic," naïve, central memory, effector memory, regulatory, RTEs), B cell subsets, and NK cell subsets (cytokine-secreting $CD3^-CD56^{bright}CD16^{+/-}$ and cytotoxic; $CD3^-CD56^{dim}CD16^+$) using multi-parameter flow cytometry. Representative panels that can be used for the monitoring of immune reconstitution in patients with CD following auto-HSCT are summarized in **Table 1**, the ability to deliver which will be dependent on the instrumentation available in the analytical laboratory. Comprehensive protocols relating to flow cytometric analysis have been published previously (48, 49).

Combined with post-transplant vaccination, the immune reconstitution of "ASTIClite" will therefore aim to establish whether the effect of AHSCT is merely temporary downregulation of immunity or whether there is a significant component of immune reset.

CONCLUSION

Based on clinical trials and EBMT registry data, auto-HSCT represents a promising therapy for patients with severe resistant CD. Prolonged responses have been achieved in some patients that have otherwise been resistant to conventional treatments and biological therapies. In the patients who relapse, there appears to be a re-sensitization to previous agents, consistent with a "setting back of the immunological clock." Previous clinical trials and case series report a relatively high relapse rate and high frequency of serious adverse events. Future trials will assess the efficacy and safety of lower intensity mobilization and conditioning regimens and the benefit of protocoled introduction of maintenance therapy in patients who relapse after auto-HSCT.

In addition, clinical trials of auto-HSCT provide a unique opportunity to characterize the nature of immune reconstitution as well as the interaction between the peripheral and mucosal immune system in CD. This will allow deep interrogation and characterization of the localized mucosal immune environment in patients with disease before and after auto-HSCT during ASTIClite, as well as the immunome of the periphery (by profiling peripheral blood mononuclear cells, PBMCs) before and after auto-HSCT and during the progression toward disease relapse.

Recently, the EBMT and European Crohn's and Colitis Organisation have published a review to encourage and guide inter-specialty collaboration in both clinical and scientific development of this auto-HSCT in CD (59). Thus, in addition to therapeutic benefits, destroying and re-building the dysfunctional immune system and mucosal environment, a program of modern scientific investigation carefully scheduled around auto-HSCT may yield valuable insights into the etiology, pathogenesis, and mechanisms of treatment resistance in CD.

AUTHOR CONTRIBUTIONS

AP drafted the initial version of the manuscript, to which all other authors made significant content and editorial contributions, with JS and TA leading the contribution from the EBMT Autoimmune Diseases Working Party. All authors are integrally involved in the clinical and/or scientific aspects of the ASTIClite trial (Chief Investigator, JL).

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Disclaimer: Although drug doses have been checked against primary sources, this review should not be used as a means of assuring prescription of chemotherapy and other drugs in clinical practice.

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OPINION
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A Future for Autologous Hematopoietic Stem Cell Transplantation in Type 1 Diabetes

Kayleigh M. van Megen[1†], Ernst-Jan T. van 't Wout[1†], Stephen J. Forman[2] and Bart O. Roep[1,3]*

[1] Department of Diabetes Immunology, Diabetes & Metabolism Research Institute, Beckman Research Institute at the City of Hope, Duarte, CA, United States, [2] Department of Hematology & Hematopoietic Cell Transplantation, T Cell Therapeutics Research Laboratory, City of Hope Beckman Research Institute and Medical Center, Duarte, CA, United States, [3] Department of Immunohaematology and Blood Transfusion, Leiden University Medical Center, Leiden, Netherlands

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INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease caused by destruction of insulin producing β-cells in the pancreas. Standard of care therapy consists of life long symptomatic insulin treatment and in rare and severe cases patients undergo islet transplantation (1). Until today, autologous hematopoietic stem cell transplantation (aHSCT) proved to be the only intervention therapy for T1D reaching complete and sometimes even lasting remission (2–7). In spite of many other immunotherapies assessed around the globe, none matched the clinical efficacy of aHSCT (8, 9). Indeed, aHSCT had insulin-independency as primary end-point, rather than delayed loss of insulin production or decreased insulin needs. aHSCT is already widely and successfully used as a treatment for hematological malignancies (10, 11). Interestingly, one diabetic patient, when treated with aHSCT for multiple myeloma, became insulin independent (12). aHSCT was evaluated as a treatment for several autoimmune disorders as well, such as rheumatoid arthritis (13), systemic sclerosis (14, 15), multiple sclerosis (16), and juvenile idiopathic arthritis (17). By 2012, up to 3,000 aHSCT had been performed for autoimmune diseases (18). Yet, in the case of T1D, aHSCT remains controversial (19–21).

Indeed, the use of aHSCT as a strategy to cure T1D has been received with mixed enthusiasm. Concerns were raised about the short follow-up, the possibility that a positive effect of aHSCT may be attributable to a honeymoon phase and the absence of a placebo-treated trial arm for comparison (19, 21). Furthermore, the ethics of including minors in the trial was being questioned (19). Although valid at the time, these concerns have all since been addressed, as will become evident in the following paragraphs.

aHSCT IN T1D

The rationale behind using aHSCT in autoimmune diseases is to halt autoimmune destruction of the targeted tissue and reestablish tolerance. While the mechanism by which this is achieved remains incompletely resolved, the importance of a diverse T-cell receptor repertoire (22), thymus reactivation (23), and the number of regulatory T-cells (Treg) has been established (24).

The first evidence to demonstrate that aHSCT can reestablish tolerance in new-onset T1D patients comes from Voltarelli et al. (25, 26). Recent-onset (<6 weeks) T1D patients were included to undergo aHSCT with mobilized [cyclophosphamide (2.0 g/m^2) and granulocyte colony-stimulating factor (10 μg/kg/day)] peripheral blood-derived hematopoietic stem cells after an intermediate-intensity conditioning regimen consisting of cyclophosphamide (200 mg/kg total) and rabbit antithymocyte globulin (4.5 mg/kg total). Similar mobilization and conditioning regimes were used in other discussed studies, unless mentioned otherwise. In total, 25 patients were included, of which 21 were

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****Correspondence:***
Bart O. Roep
broep@coh.org

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

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treated according to protocol and became insulin independent, for a median of 43 months (2); a result unmatched by any intervention therapy up until this point. These results were substantiated independently around the world, accomplishing insulin independence in all studies, with maximum insulin independence ranging from 38 to 56 months and increasing with further follow-up (3–7). These studies prove that aHSCT is a promising therapy for T1D, while providing crucial and unique metabolic and immunological data of T1D patients in remission (27, 28).

BALANCING THE RISK OF aHSCT WITH THE RISK OF DIABETES-ASSOCIATED COMPLICATIONS

Depending on the intensity of the conditioning regime, aHSCT can cause a wide range of complications. In the T1D trials (2–7), these ranged from relatively mild symptoms such as febrile neutropenia, nausea, and alopecia to more severe complications such as *de novo* autoimmunity and systemic infections, which in one case resulted in an unfortunate death (7). Temporal oligospermia was witnessed in some of the studies, but not all. Of note, multiple children have been conceived after aHSCT. Apart from these complications, there is also a concern of increased risk of malignancies after aHSCT, particularly myelodysplasia. With allotransplantation, this risk is well established and can be attributed to the heavy conditioning regime, while this regime is much milder in the autologous setting for autoimmune diseases. Furthermore, in contrast to aHSCT as a treatment for malignancies, stem cells of T1D patients have not sustained any damage from previous chemotherapy. Consequently, the incidence of malignancies was reported to be lower, although further prospective studies with longer follow-up and proper control groups are warranted to asses if these malignancies are aHSCT related (29).

Containment of adverse events from aHSCT is constantly improving as illustrated by decreased morbidity and mortality to <1% (30). Furthermore, in the setting of T1D, it will be performed in relatively young and otherwise fit subsets of patients with a low to intermediate conditioning regimen (2, 31), associated with reduced risk (29) without compromising treatment efficacy. This was attested by a recent trial exploring the possibility of a simplified method of aHSCT in an outpatient setting, with a conditioning regime consisting of cyclophosphamide (2.0 g/m^2 total) and fludarabine (120 mg/m^2 total), still reaching 44% prolonged insulin independence for up to 56 months and beyond, without significant adverse effects (4).

To make a compelling and fair case of aHSCT in T1D, the complications of aHSCT need to be juxtaposed with the short- and long-term complications of T1D. It is important to realize that acute and possibly life threatening events related to T1D and insulin treatment such as a hypoglycemic coma (32) and diabetic ketoacidosis (DKA) (33) are not uncommon. Indeed, T1D remains a deadly disease, where insulin therapy merely provides palliative care. In addition to a significantly reduced life expectancy, T1D also imposes severe and often lifelong negative impact on the quality of life of T1D patients. The major burden of the disease is caused by long-term micro- and macrovascular complications, with T1D still being a main cause of end stage renal disease and non-inherited blindness (34, 35). Even with optimal education and state-of-the-art treatment options, good glycemic control is not achieved in the vast majority of patients (36). This is of particular importance, since good glycemic control early in the course of the disease reduces long-term complications and preserves endogenous insulin production (37). Interestingly, patients that experienced a honeymoon phase showed significantly less macrovascular complications after 7 years of follow-up (38, 39). This could imply that a similar effect can be expected from an aHSCT induced prolonged period of insulin independence.

Importantly, side effects are inherent to immunotherapy. The adverse events of, for instance, DMARD, TNF blockers, sirolimus, cyclosporine, azathioprine, prednisone, thymoglobulin, alemtuzumab, or imatinib, all considered in the context of T1D, are certainly not negligible.

CLINICAL OUTCOME OF aHSCT CORRESPONDS WITH THE DEGREE OF ISLET AUTOREACTIVITY BEFORE THERAPY

Currently, after almost 15 years of experience in the application of aHSCT for the treatment of T1D, much knowledge has been gained about the mechanism of action of aHSCT and, concomitantly, about which patient population benefits most (2, 3, 5–7, 27, 28, 40–42).

Earlier this year, the first aHSCT in T1D trial reported its *ad hoc* analysis with a mean follow-up of 67.5 months (some patients remain insulin-independent beyond 106 months) and included 25 patients (2). HLA-A2 positive patients were divided into low and high cytotoxic T lymphocytes (CTL) autoreactivity groups according to the cumulative frequencies of islet-specific CTLs at baseline. Low CTL autoreactivity associated with higher c-peptide levels after aHSCT compared with high CTL autoreactivity. Furthermore, while 83% of patients in the high CTL group had resumed insulin therapy at 24 months after aHSCT, all patients with low frequencies of islet-autoreactive CTLs at baseline remained insulin independent. In addition, patients were divided into those with "short-remission" and "prolonged remission" depending on whether they were insulin-free for less or more than 3.5 years after aHSCT, respectively. A trend was seen of persistently lower cumulative frequencies of islet-specific CTLs in the prolonged remission group compared with the short-remission group. This outcome may point that the conditioning regimen with thymoglobulin was insufficient to deplete autoreactive T-cells. Diabetes relapse could then result from clonal expansion of autoreactive CTLs that escaped the conditioning procedure. In any case, these immunological parameters associated with superior or inferior clinical outcome of aHSCT before therapy point to patient and disease heterogeneity and present a good case for personalized and precision medicine in which tailoring the conditioning therapy might lead to more effective reversal of islet autoimmunity.

Additional evidence in favor of an immunogenic heterogeneity that relates to the outcome of aHSCT came from a study of

13 patients that was conducted in China with a mean follow-up of 42 months (5). Expressing more than one preexisting autoantibody negatively correlated with the preservation of beta-cell function as quantified by c-peptide levels. Yet, a larger study including 123 patients with a mean follow-up of 16 months found no difference in baseline presence of any of the autoantibodies between responding and non-responding patients (27). Serum levels of interleukin-10, interleukin-4, transforming growth factor-β, and fasting c-peptide after aHSCT correlated with the number of infused CD34+ cells, whereas tumor-necrosis factor-α (TNF-α) and insulin doses showed an inverse relation. Furthermore, prolonged insulin-free survival was negatively correlated with baseline TNF-α levels, which may provide another suitable negative predictor of prolonged remission (3).

In summary, current clinical evidence points to heterogeneity between patients and in disease, as well as provides immune correlates of disease remission or relapse that may offer opportunity for patient selection, precision medicine, and guidance for tailored immunotherapy following aHSCT.

THE SUCCESS OF aHSCT IN RELATION TO PREEXISTING FUNCTIONAL BETA-CELL MASS

Besides a baseline immune signature, *post hoc* analyses have revealed the importance of preexisting beta-cell mass for the outcome of aHSCT (27). One small study (5) found that the baseline c-peptide level was a positive predictor of post-aHSCT c-peptide levels, which was corroborated by other, larger studies (3). The largest study including 123 patients stratified subjects into a responder group and a non-responder group according to the presence of a post-aHSCT clinical response assessed by a β-score (27). The β-score is mainly used in the islet transplantation setting and consists of four components: fasting plasma glucose, HbA1c, c-peptide, daily insulin use or usage of oral hypoglycemic agents. The β-score was already significantly higher at baseline in responders compared with non-responders. Moreover, baseline fasting c-peptide levels proved to be an effective positive predictor of prolonged remission and the age of onset of diabetes a negative predictor. Obviously, baseline c-peptide levels are an indication of functional β-cell mass (27), although increasing evidence points to a disconnect between beta-cell mass and function in the case of diabetes (43, 44). β-Cell regeneration may occur until adolescence, after which regenerative capacity appears to stagnate (45). Indeed, early intervention within 6 weeks after diagnosis of T1D led to remission in the vast majority of cases, whereas later intervention achieved remission in less than half of the cases (42), suggesting that timely therapy matters.

The influence of DKA before aHSCT on clinical outcome could be substantial (6). Indeed, DKA at diagnosis has been associated with lower c-peptide levels, higher insulin needs and HbA1c levels, suggesting lower remaining β-cell function (46). Yet, another trial including 24 patients with 52 months as a mean follow-up found no relation between duration of insulin independence and the time from diagnosis to AHSCT, baseline c-peptide levels, nor number of CD34+ cells (7).

To summarize, patients with sufficient beta-cell function at baseline, no DKA at diagnosis, and treated early after diagnosis appear to benefit most. These characteristics all point toward the

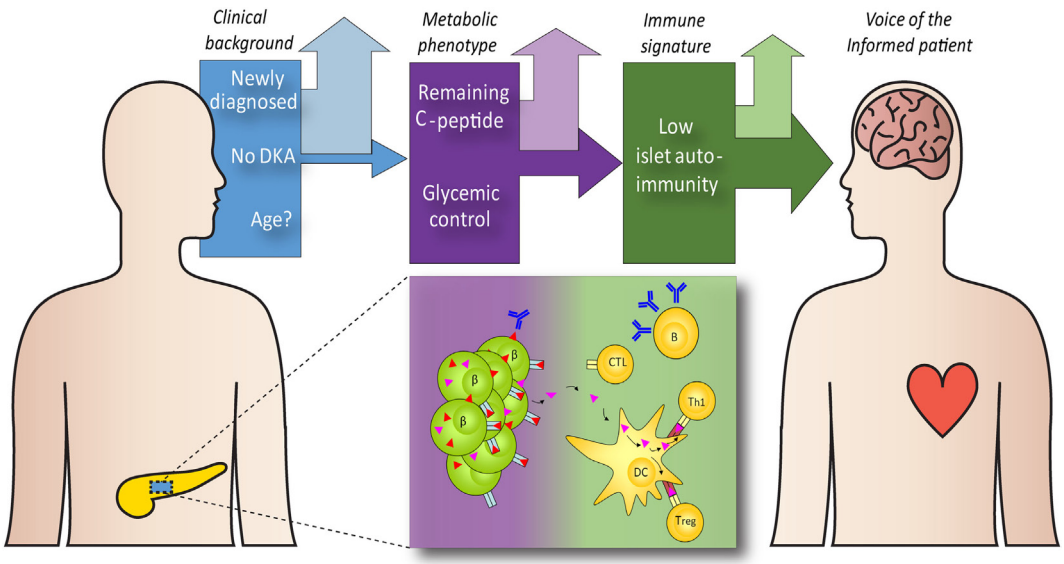

FIGURE 1 | Guidance on the selection of type 1 diabetes (T1D) patients for autologous hematopoietic stem cell transplantation (aHSCT). aHSCT is unlikely to benefit all T1D patients. Factors that may help selecting the preferred candidates include the clinical background [disease duration, age, and diabetic complications, such as diabetic ketoacidosis (DKA)], metabolic features [remaining functional beta-cell mass (β), glycemic control, HbA1c] and immunopathogenic features [the number and type of islet autoantibodies, the frequency and specificity of islet-autoreactive cytotoxic T lymphocytes (CTL), and other effector (Th1) and regulatory (Treg) immune cells, and cytokine profiles]. With the opportunity to identify patient subgroups with particularly great or smaller chances for clinical benefit, we propose that we engage the patient community to guide shared decision-making.

pivotal role of remaining functional beta-cell mass for success of aHSCT in T1D (27). To verify whether the age of onset matters (3), inclusion of minors in trials of aHSCT in T1D would be required. The potential capacity to regenerate their beta cells would further support considering young patients to offer this intervention therapy. Teenagers are a particularly challenging population to treat as diabetes-related distress, which is present in one-third of adolescents with T1D, is linked to poor glycemic control (47–49). Consequently, 84% of teens do not reach target HbA1c levels (36), which jeopardizes their future health with regards to increased long-term complications, but also their career perspectives (50).

SELECTING ELIGIBLE PATIENTS FOR aHSCT IN T1D

Understanding which patient groups respond better to aHSCT and why, enables us to transform aHSCT from a general therapy to personalized medicine, thus envisioning a future of aHSCT in T1D. Yet, we contend that the choice for aHSCT as therapeutic option is not confined to the care providers. The voice of the patient is equally relevant, both in terms of refusing the risk for treatment related adverse events or accepting these in favor of temporal disease remission, preservation of beta-cell function, and reduced risk of diabetic complications. In case of minors, parents face the difficult task of weighing the best therapy for the patient in consultation with the care provider, which makes careful information provision even more important. We envision a future in which care providers, in dialog with the patient and caregivers, use a framework of evidence-based risk assessment to assess whether aHSCT is a viable option (see **Figure 1**).

CONCLUSION

While aHSCT will not be the magic bullet universally curing T1D, there is a promising future for its implementation in a distinct group of patients (20). Indeed, none of the alternative intervention strategies match, or even get close to, the clinical outcome achieved in a considerable number of patients treated with aHSCT. We propose that this patient group should be identified, diligently informed and offered the possible benefits of an extended period of insulin-free and burden-free survival, while medical science continues their pursuit of developing alternative intervention strategies for those less eligible, or declining, aHSCT. T1D enters the era of personalized medicine.

AUTHOR CONTRIBUTIONS

KM and ET performed a literature review, contributed to the design of the work, analyzed the data, and wrote and edited the manuscript. KM designed the figure. BR conceived the idea of this work. BR and SF analyzed the data, revised and edited the manuscript, and contributed to the discussion. All the authors provided approval for publication and are accountable for all aspects of the work.

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Natural Killer Cells Regulate Th17 Cells After Autologous Hematopoietic Stem Cell Transplantation for Relapsing Remitting Multiple Sclerosis

Peter J. Darlington[1], Brandon Stopnicki[1], Tarik Touil[2], Jean-Sebastien Doucet[2], Lama Fawaz[2], Morgan E. Roberts[2], Marie-Noëlle Boivin[2,3], Nathalie Arbour[4], Mark S. Freedman[5], Harold L. Atkins[6] and Amit Bar-Or[2,7] for the Canadian MS/BMT Study Group*

[1] Departments of Exercise Science and Biology, PERFORM Centre, Concordia University, Montreal, QC, Canada, [2] Neuroimmunology Unit, McGill University and Montreal Neurological Institute, Montreal, QC, Canada, [3] Clinical Biological Imaging and Genetic Repository, McGill University, Montreal, QC, Canada, [4] Department of Neurosciences, Université de Montréal, Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM), Montréal, QC, Canada, [5] Department of Medicine, Ottawa Hospital Research Institute, University of Ottawa, Ottawa, ON, Canada, [6] Blood and Marrow Transplant Program, Ottawa General Hospital, Ottawa, ON, Canada, [7] Center for Neuroinflammation and Experimental Therapeutics and Department of Neurology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, United States

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Antoine Toubert, Paris Diderot University, France

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***Correspondence:**
Amit Bar-Or
amitbar@upenn.edu

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In autoimmunity, the balance of different helper T (Th) cell subsets can influence the tissue damage caused by autoreactive T cells. Pro-inflammatory Th1 and Th17 T cells are implicated as mediators of several human autoimmune conditions such as multiple sclerosis (MS). Autologous hematopoietic stem cell transplantation (aHSCT) has been tested in phase 2 clinical trials for MS patients with aggressive disease. Abrogation of new clinical relapses and brain lesions can be seen after ablative aHSCT, accompanied by significant reductions in Th17, but not Th1, cell populations and activity. The cause of this selective decrease in Th17 cell responses following ablative aHSCT is not completely understood. We identified an increase in the kinetics of natural killer (NK) cell reconstitution, relative to $CD4^+$ T cells, in MS patients post-aHSCT, resulting in an increased NK cell:$CD4^+$ T cell ratio that correlated with the degree of decrease in Th17 responses. *Ex vivo* removal of NK cells from post-aHSCT peripheral blood mononuclear cells resulted in higher Th17 cell responses, indicating that NK cells can regulate Th17 activity. NK cells were also found to be cytotoxic to memory Th17 cells, and this toxicity is mediated through NKG2D-dependent necrosis. Surprisingly, NK cells induced memory T cells to secrete more IL-17A. This was preceded by an early rise in T cell expression of *RORC* and *IL17A* mRNA, and could be blocked with neutralizing antibodies against CD58, a costimulatory receptor expressed on NK cells. Thus, NK cells provide initial co-stimulation that supports the induction of a Th17 response, followed by NKG2D-dependent cytotoxicity that limits these cells. Together these data suggest that rapid reconstitution of NK cells following aHSCT contribute to the suppression of the re-emergence of Th17 cells. This highlights the importance of NK cells in shaping the reconstituting immune system following aHSCT in MS patients.

Keywords: multiple sclerosis, natural killer cells, aHSCT, Th17 cells, NKG2D, CD58

INTRODUCTION

Pro-inflammatory helper T (Th) cells contribute to disease activity in several autoimmune inflammatory conditions including multiple sclerosis (MS). In MS, Th1 and Th17 subsets have been considered pathogenic, while Th2 and regulatory T cell subsets may limit disease activity (1). Th17 cells express IL-17A and the transcription factor RORγt. These cells, including those co-expressing IL-17 and IFN-γ, have the capacity to disrupt the blood–brain barrier and directly injure neurons, and therefore have been of particular interest in the study of MS (2, 3).

Natural killer (NK) cells are part of the innate immune system, specialized in killing virus-infected cells and tumors through several mechanisms and receptors including NKG2D-dependent cytotoxicity. Infected or stressed cells express ligands for NKG2D including MICA, MICB, and the ULBP ligands. In addition, NK cells can regulate adaptive immunity (4, 5). There are at least two major subtypes of NK cells in humans referred to as $CD56^{bright}$ and $CD56^{dim}$ NK cells, circulating in blood and located within tissues (6, 7). They are discernable by flow cytometry based on their relative intensity of CD56 expression. In blood, about 90% of NK cells are expected to be $CD56^{dim}$ NK cells, this proportion varies for NK cells within tissues (6, 7). The traditional view was that $CD56^{dim}$ NK cells are cytotoxic while $CD56^{bright}$ NK cells played more of a regulatory role, however, $CD56^{bright}$ NK cells can acquire cytotoxic properties, and both types can produce cytokines (8). NK cells can also be subdivided based on CD16, a low affinity Fc receptor. $CD56^{dim}$ NK cells are predominantly $CD16^{+}$ with only a minor proportion that are $CD56^{dim}CD16^{-}$, and $CD56^{bright}$ NK cells are mostly $CD56^{bright}CD16^{-}$ with a minor population expressing intermediate levels of CD16, referred to as $CD56^{bright}CD16^{dim}$ (7).

Several lines of evidence indicate that NK cells play a protective role in autoimmunity. Depletion or removal of NK cells exacerbates collagen-induced arthritis (9, 10), a transfer model of colitis (11), and experimental autoimmune encephalomyelitis (12–15). Furthermore, dysregulation or impairment of NK cells has been linked to MS relapses and MRI lesions (16–24). The mechanism by which NK cell dysfunction contributes to MS is still unclear, but may include loss of cytotoxic capacity against autologous T cells (25). Following activation, effector T cells are sensitized to NKG2D-dependent cytotoxicity by upregulating NKG2D ligands (26–29). Given their regulatory capacity, NK cells are potential therapeutic targets in autoimmune diseases including MS. Several MS therapeutics limit MS disease activity and concomitantly expand NK $CD56^{bright}$ cells, including daclizumab (30), alemtuzumab (31), dimethylfumarate (32), and IFN-β (33). NK cells are also capable of injuring astrocytes and neurons (34) and promoting demyelination (35). Their overall contribution to autoimmunity is thus a balance of immune-regulatory and pro-inflammatory actions.

Autologous hematopoietic stem cell transplantation (aHSCT) as a treatment for relapsing remitting MS, has been tested in several clinical trials and has been found to be effective at inducing prolonged disease stabilization (36). In a Canadian multi-center phase 2 clinical trial, relapsing remitting MS patients with aggressive disease received immunoablative chemotherapy followed by $CD34^{+}$ aHSCT. In this open label study, treatment resulted in complete abrogation of all clinical relapses and there were no new brain MRI lesions with follow-up now exceeding 13 years (37–39). The concept behind aHSCT therapy in MS is to ablate the existing pathogenic immune cells with the induction chemotherapy regimen and then allow reconstitution of a newly derived, non-pathogenic, autologous immune repertoire by the transplanted stem cells. Studies carried out following aHSCT demonstrated that central nervous system-reactive Th1 cells re-emerged to levels that were indistinguishable from those documented before the treatment. By contrast, Th17 responses, including IL-17 secretion, were much reduced in the reconstituted immune system following aHSCT (40).

Given the importance of NK cells in MS as well as their capacity to limit effector T cell responses, we questioned whether the decrease in Th17 responses after aHSCT, occurs as a result of NK cell-mediated restriction of the generation and/or maintenance of Th17 cells in the early reconstituting immune repertoire. NK cells are known to reconstitute rapidly following allogeneic stem cell transplantation for leukemia (41). Furthermore, a recent study on immunoablative and stem cell reconstitution in MS found a transient increase in NK cells and lower Th17 cell responses post stem cell transplantation (42). In this study, we demonstrated that NK cells were elevated in aHSCT samples.

MATERIALS AND METHODS

Collection of aHSCT Blood Samples From MS Patients

Multiple sclerosis patients were serially enrolled in the immune monitoring sub study as part of the Canadian Collaborative MS/BMT Study (registered at ClinicalTrials.gov, NCT01099930), following informed consent as approved by the institutional ethics review boards. aHSCT therapy was performed on a cohort of MS patients as previously described (39). In brief, patients with aggressive disease who were refractory to drug treatments underwent autologous hematopoietic stem cell mobilization using cyclophosphamide and filgrastim followed by stem cell graft collection by peripheral vein leukopheresis. Immune cells were depleted from the graft using CD34 immunomagnetic selection and the graft was cryopreserved. High dose busulfan, cyclophosphamide, and antithymocyte globulin were administered for immune ablation followed by infusion of the thawed CD34 autologous hematopoietic stem cell graft. The detailed protocol, patient characteristics, and clinical results are published elsewhere (39). Venous blood was sampled from MS patients within 2 months before aHSCT, then at 3 weeks, 3 months, 12 months, and 21 months following aHSCT. A portion of the blood sample was analyzed by flow cytometry with antibodies for CD3 (UCHT-1), CD56 (B159), CD4 (RPA-T4), CD8 (RPA-T8) (from BD Bioscience; Mississauga, ON, Canada), and CD58 (TS2/9; eBioscience). The remaining blood sample was processed into peripheral blood mononuclear cell (PBMC) using Ficoll, aliquoted, and cryopreserved in fetal bovine serum with 10% dimethyl sulfoxide in a cryovial stored in liquid nitrogen.

Analysis of aHSCT Blood Samples

After completion of the clinical trial, selected aliquots were thawed in batches and used for NK cell bright dim analysis, the Th17 assay, and the CD56-depletion experiment. NK bright and dim were assessed by staining samples for CD3-APC (UCHT-1), CD56-PE (B159), and CD16-FITC (CLB/FcGran1) all from BD Bioscience, then by flow cytometry determining the two different populations, which were visible on the CD3 and CD56 plots as the $CD3^{-}CD56^{bright}$ or $CD3^{-}CD56^{dim}$ populations, and gated using Flow Jo software (Tree Star Inc., Ashland, OR, USA). The Th17 analysis was done according to previously published protocol (40), in brief, PBMC was incubated for at least 1 h after thawing in media containing RPMI1640, 10% FBS, 1% pen strep, 1% L-glutamine. Samples were activated with soluble function grade anti-CD3 (OKT3 1.0 μg/ml; eBioscience, San Diego, CA, USA), soluble anti-CD28 (CD28.2; 1.0 μg/ml; eBioscience), neutralizing anti-IFN-γ and anti-IL-4 antibodies (5 μg/ml, R&D systems, Minneapolis, MN, USA), and 10 ng/ml of recombinant human IL-23 (R&D systems). The Th17 proportion was determined with CD3 and CD4 surface staining, followed by intracellular cytokine staining: samples were incubated for 4 h with ionomycin, phorbol myristate acetate, and brefeldin A, stained for CD3 (UCHT-1), and CD4 (RPA-T4), then fixed and permeabilized with a BD Cytofix/Cytoperm kit (BD Biosciences), followed by staining for IFN-γ (B27) and IL-17A (SCPL1362) antibodies from BD Biosciences. The proportion of Th17 cells was the percentage of cells with the phenotype $CD3^{+}CD4^{-}IL\text{-}17A^{+}IFN\text{-}\gamma^{-}$. The Th17 change was calculated by dividing the proportion after therapy by the proportion before therapy and multiplying by 100%. For the CD56-depletion, samples were thawed and incubated in media for at least 1 h. The samples were divided, half kept complete, the other half depleted with CD56 positive selection by magnetic activated cell sorting according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA, USA). Depletion was confirmed with flow cytometry using antibodies for CD3 (UCHT-1) and CD56 (B159). Complete or depleted samples were then incubated with functional grade anti-CD3 (OKT3 1.0 μg/ml; eBioscience), anti-CD28 (CD28.2; 1.0 μg/ml; eBioscience), and neutralizing anti-IFN-γ and anti-IL-4 antibodies (5 μg/ml, R&D systems) for 4 days, then intracellular cytokine stained for IL-17A and IFN-γ, and supernatants analyzed for IL-17A using uncoated enzyme-linked immunosorbent assay (ELISA) kit for human IL-17A (eBioscience) and human IFN-γ (eBioscience).

Mechanism of Action of Cytotoxicity Between NK Cells and Th17 Cell in Healthy Human Samples

PBMCs were isolated from venous blood of healthy adult participants following informed consent per approved institutional protocol (McGill University). CD4 memory T cells were obtained by magnetic activated cell sorting with negative selection, which depletes CD45RA, CD8, CD14, CD16, CD19, CD56, CD36, CD123, anti-TCRγδ, and CD235a (glycophorin A) according to manufacturer's instructions (Miltenyi Biotec, Auburn, CA, USA). The resulting phenotype was $CD3^{+}CD4^{+}CD45RO^{+}CD45RA^{-}$ as determined by flow cytometry with antibodies for CD3-PerCP (UCHT-1), CD4-FITC (RPA-T4), CD45RO-APC (UCHL1), and CD45RA-PE (HI100) from BD Biosciences. Purified autologous NK cells were obtained with $CD56^{+}$ selection magnetic activated cell sorting (Miltenyi Biotec). The resulting phenotype was $CD3^{-}CD56^{+}$ determined by flow cytometry using antibodies for CD3-FITC (UCHT-1), NKG2D-APC (1D11), and CD56-PE (B159). The purified CD4 T cells and NK cells were incubated in media containing RPMI1640, 10% FBS, 1% pen strep, 1% L-glutamine, at 37°C with 5% CO_2, in 96-well U-bottom plates at 0.2×10^6 cells per well. Activation was with soluble anti-CD3 antibodies (OKT3; 1.0 μg/ml; eBioscience) and anti-CD28 antibodies (CD28.2; 1.0 μg/ml; eBioscience). Th17 polarization was with neutralizing anti-IFN-γ and anti-IL-4 antibodies (5 μg/ml, R&D systems) and 10 ng/ml of recombinant human IL-23 (R&D systems) for 4 days. ELISA and intracellular cytokine staining were performed as previously described to measure IL-17A and IFN-γ (40). Detection of MICA was achieved with MICA polyclonal antibody and Zenon labeling kit according to manufacturer's instructions (Thermo Fisher Scientific, Carlsbad, CA, USA). For proliferation assay, memory CD4 T cells were pre-labeled with CFSE as previously described (40). For cell death studies, annexin V and 7AAD were performed on live cells, this was done by washing cells, adding annexin V PE in annexin V binding buffer (BD Bioscience) at 1:10 ratio in 100 μl for 20 min, and then 7AAD (BD Bioscience) at a 1:20 ratio for additional 10 min at room temperature before reading on flow cytometer within 1 h. Before annexin

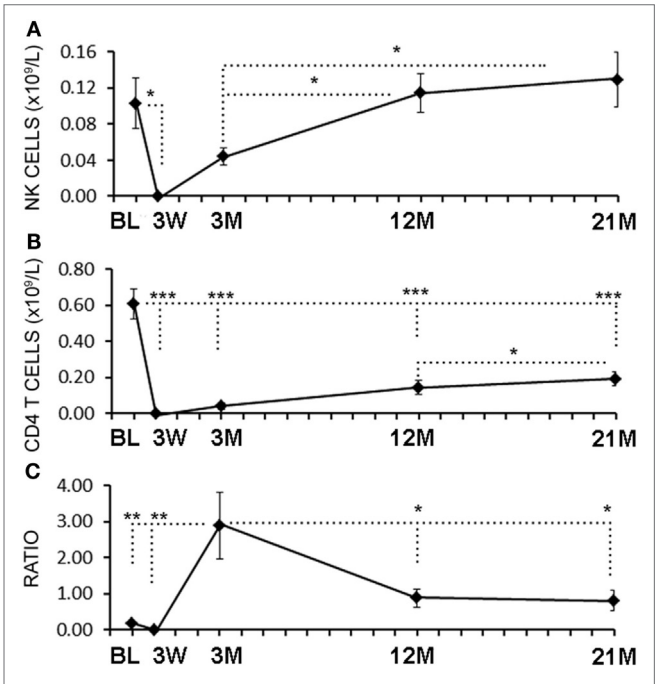

FIGURE 1 | Rapid reconstitution kinetics of natural killer (NK) cell counts compared with CD4 T cells in aHSCT-treated multiple sclerosis (MS) patient blood. From freshly drawn blood samples, absolute number of NK cell counts **(A)** and $CD4^{+}$ T cell counts **(B)** were determined at baseline (BL), and at 3 weeks (3 W), 3 months (3 M), 12 months (12 M), and 21 months (21 M) post-aHSCT. The ratio of NK cells to $CD4^{+}$ T cells was calculated **(C)**. *N* = 7 patients.

V and 7AAD labeling, surface staining with CD4 and CD56 was performed to identify gates using FlowJo software (Tree Star Inc.). The NKG2D neutralizing antibody (Amgen, Inc., Seattle, WA, USA) was used at 40 µg/ml, an amount shown previously to significantly attenuate cytotoxicity of NK cells toward glial cells as described in a previous publication (34). CD58 neutralizing antibodies and respective isotype control were used at 10 µg/ml (R&D systems). These neutralizing experiments were done by combining purified CD4 memory T cells and purified NK cells at equal ratio and incubating for 4 days under activation (CD3 and CD28) and polarization conditions (anti-IFN-γ and anti-IL-4), then measuring IL-17A by ELISA from the supernatants.

RNA Extraction and PCR

Quantitative PCR was performed as previously described (43, 44). In brief, total RNA was extracted using RNeasy® Mini kit (QIAGEN, Toronto, ON, Canada) and then transcribed into complementary DNA using QuantiTect® Reverse Transcription kit (QIAGEN) according to the manufacturer's instructions. Relative gene expression levels were determined using primers and TaqMan® FAM™-labeled MGB probes for ROR-γ, IL17, and ribosomal 18 S (VIC®-labeled probe) (Applied Biosystems, Foster City, CA, USA). Quantitative PCR cycling was performed for 40 cycles in a 7900HT fast-real-time PCR system (Applied Biosystems) as recommended by the manufacturer instructions. Gene-specific messenger RNA was normalized compared with 18S, the endogenous control.

Statistics

Statistical comparison with multiple comparisons was made using one-way ANOVA ($p < 0.05$), followed by *post hoc* Tukey's honest significant difference test. For statistical comparison of

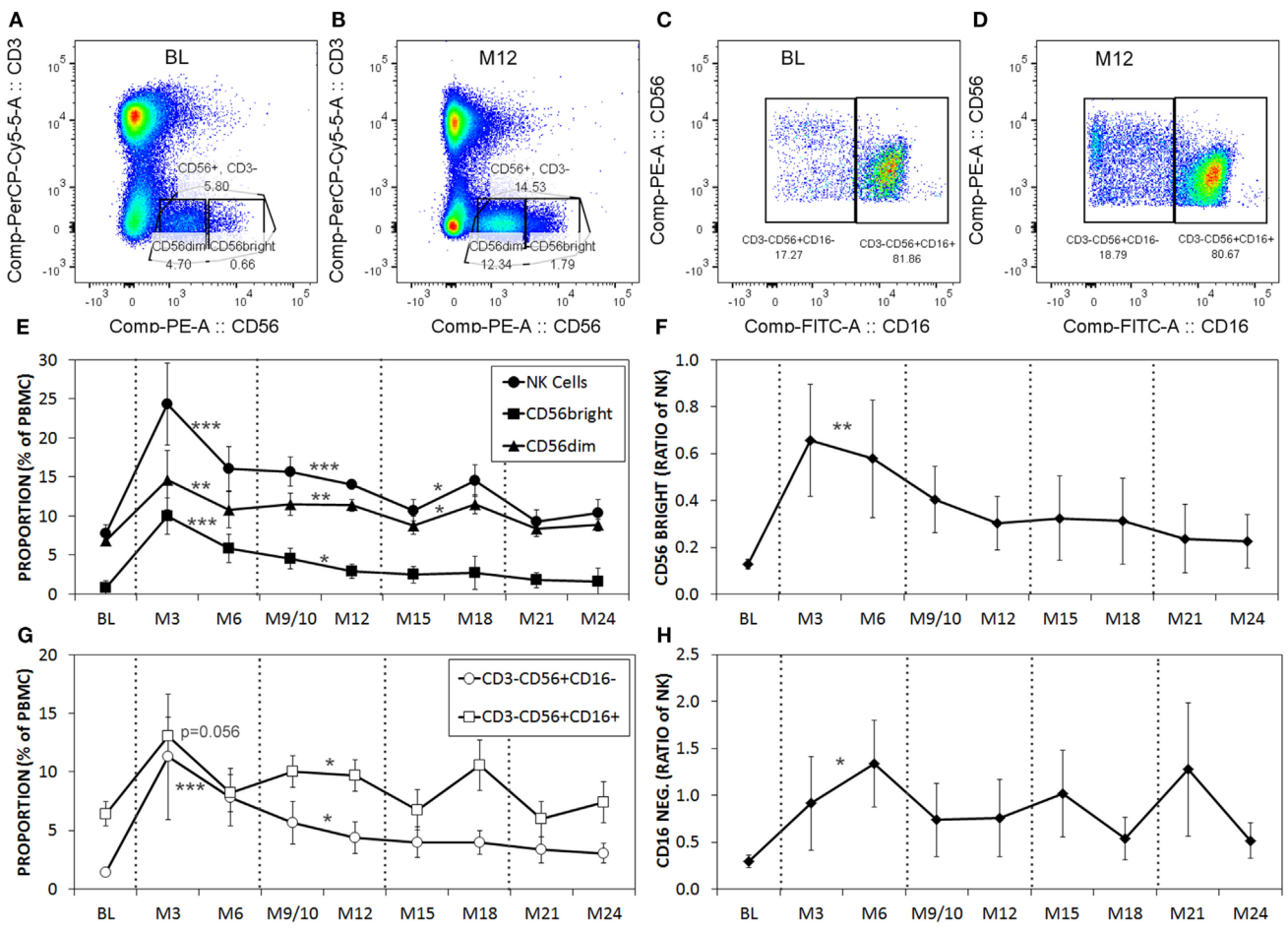

FIGURE 2 | The proportions of CD56^{dim} and CD56^{bright} natural killer (NK) cells are higher post-aHSCT compared witho baseline (BL). Cryopreserved peripheral blood mononuclear cell (PBMC) from aHSCT-treated MS patients was analyzed by flow cytometry. Representative plots for BL **(A,C)**, and month 12 [M12; **(B,D)**] stained for CD3 and CD56 **(A,B)**, gated on CD3$^-$CD56$^+$, then shown as CD56 with CD16 **(C,D)**. Example gates for CD56^{bright} and CD56^{dim} are shown. Additional time points include BL, and serial samples approximately every 3 months for up to 24 months post-aHSCT. The average proportions of total NK cells as well as CD56^{dim} and CD56^{bright} NK cell subsets expressed as a proportion of total PBMC **(E)**. The ratio of NK cells with the CD56^{bright} phenotype calculated as CD56^{bright}/CD56^{dim} NK cells **(F)**. The proportions of NK cells with CD16$^+$ or CD16$^-$ phenotype expressed as a proportion of total PBMC **(G)**. The ratio of gated NK cells with the CD16$^-$ phenotype calculated as CD16$^-$/CD16$^+$ **(H)**. *N* = 7 patients. For statistical analysis, the time points were grouped in M3–M6, M9–M12, M15–M18, and M21–M24, followed by univariate one-way ANOVA with pairwise comparisons with the BL values.

two groups before and after aHSCT a paired Student's *t*-test was used. The level of significance is indicated by $*p < 0.05$ was considered to be significantly different, where indicated, $**p < 0.01$, and $***p < 0.001$. Unless otherwise indicated, the error bars represent SEM.

RESULTS

NK Cells Regulate Th Cell Responses After aHSCT in MS

Serial blood samples were collected before aHSCT, 3 weeks after treatment and then at approximately 3-month intervals thereafter. The number of NK cells began returning toward baseline (BL) levels by 3 months post-ablation, whereas $CD4^+$ T cell counts did not reach BL levels even at 21 months (**Figures 1A,B**). The relatively rapid return of NK cells during immune-reconstitution may have been due to insensitivity to the chemotherapy or that NK cells or NK cell precursors were present in the graft. The ratio of circulating NK cells to $CD4^+$ T cells increased from less than 0.2:1 before aHSCT, to a high of 3:1 at 3 months post-aHSCT, and then a steady-state ratio of 1:1 from 12 months post-aHSCT onward (**Figure 1C**). A more detailed analysis of immune cell subsets revealed that frequencies of both the $CD56^{dim}$ and the $CD56^{bright}$ NK cell subsets rose sharply between month 3 and month 6 post-aHSCT, and remained elevated until month 18 and were still significantly higher at 12–18 months post-aHSCT samples (**Figures 2A,B,E**). The ratio of NK bright cells ($CD3^-CD56^{bright}$/$CD3^-CD56^{dim}$) was approximately 0.1 at BL, significantly increased to a ratio of 0.6 from month 3 to 6, and dropped to BL values by 24 months (**Figure 2F**). We also used CD16 as a defining marker for NK bright and dim cells (**Figures 2C,D**). The $CD3^-CD56^+CD16^-$ NK cells were significantly higher at month 3 until month 12 compared with BL, as were the $CD3\text{-}CD56^+CD16^+$ NK cells although they only has a trend to increase from month 3 to month 6 (**Figure 2G**). The ratio of $CD3^-CD56^+CD16^-$ cells ($CD3^-CD56^+CD16^-$/$CD3^-CD56^+CD16^+$) was approximately 0.3 at BL, and this ratio rose significantly to about 1.0 from month 3 to month 6, and then declined to BL levels by month 24 (**Figure 2H**). Thus, we found similar conclusions whether the CD56 intensity or the CD16 expression was used to define NK bright and dim cells.

We next evaluated the relationship between the level of change of NK cells and Th17 responses in the aHSCT samples. The proportional change in Th17 cells was inversely correlated to the change in NK cells in PBMC samples. Patients with a greater increase in NK cells exhibited the greatest reductions in Th17 responses ($r^2 = 0.5679$; **Figure 3A**). Within the Th17-polarized cultures, there was a substantial proportion of $CD3^+CD4^+$IL-17A$^-$ IFN^+ cells consistent with Th1 cells. The proportion of Th1 cells was variable, with no overall reduction following aHSCT, and the change in Th1 cells correlated to changes in NK cells, but to a lesser degree than Th17 cells (**Figure 3B**). We next sought to determine the effect of NK cells on the induction of T cell responses *ex vivo*. To do this, $CD56^+$ cells were depleted from post-aHSCT PBMC samples, which successfully removed NK cells (**Figures 4A,C**). This procedure also removed $CD3^+CD56^+$ cells, which was a minor population in the post-aHSCT samples (**Figure 2B**). The proportion of Th17 cells and Th1 cells was significantly lower in activated PBMC that contained $CD56^+$ cells, compared with CD56-depleted cultures (**Figure 4**). On average, Th17 and Th1 cells were 2.6 and 1.8-fold higher in the absence of $CD56^+$ cells, respectively, suggesting that Th17 cells were more sensitive to the presence of NK cells. Thus, NK cells may contribute to the regulation of T cell subset responses following aHSCT therapy in MS patients.

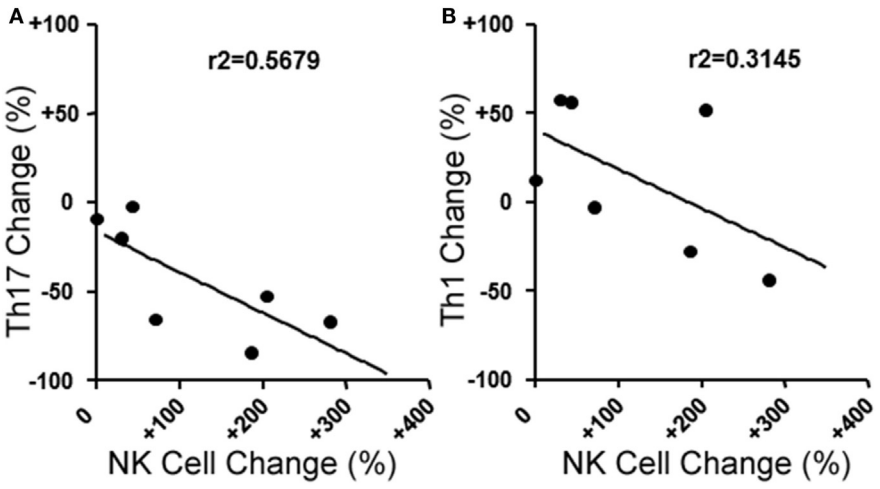

FIGURE 3 | Changes in blood natural killer (NK) cell populations correlate with changes in helper T (Th) cells populations following aHSCT in MS patients. MS patient peripheral blood mononuclear cell (PBMC) samples from baseline (BL) and 12 month post-aHSCT were activated *in vitro* with anti-CD3, anti-CD28, and Th17 polarizing factors for 4 days. Th17 and Th1 cells were assessed by analysis of cytokine production by intracellular flow cytometry ($CD3^+CD4^+$IL-17A$^+$IFN-γ^- or $CD3^+CD4^+$IL-17A$^-$IFN-γ^+, respectively). The change in frequency of Th17 cells **(A)** or Th1 cells **(B)** was plotted against the change in NK cell frequency, and linear regression was performed on the data points. *N* = 7 patients.

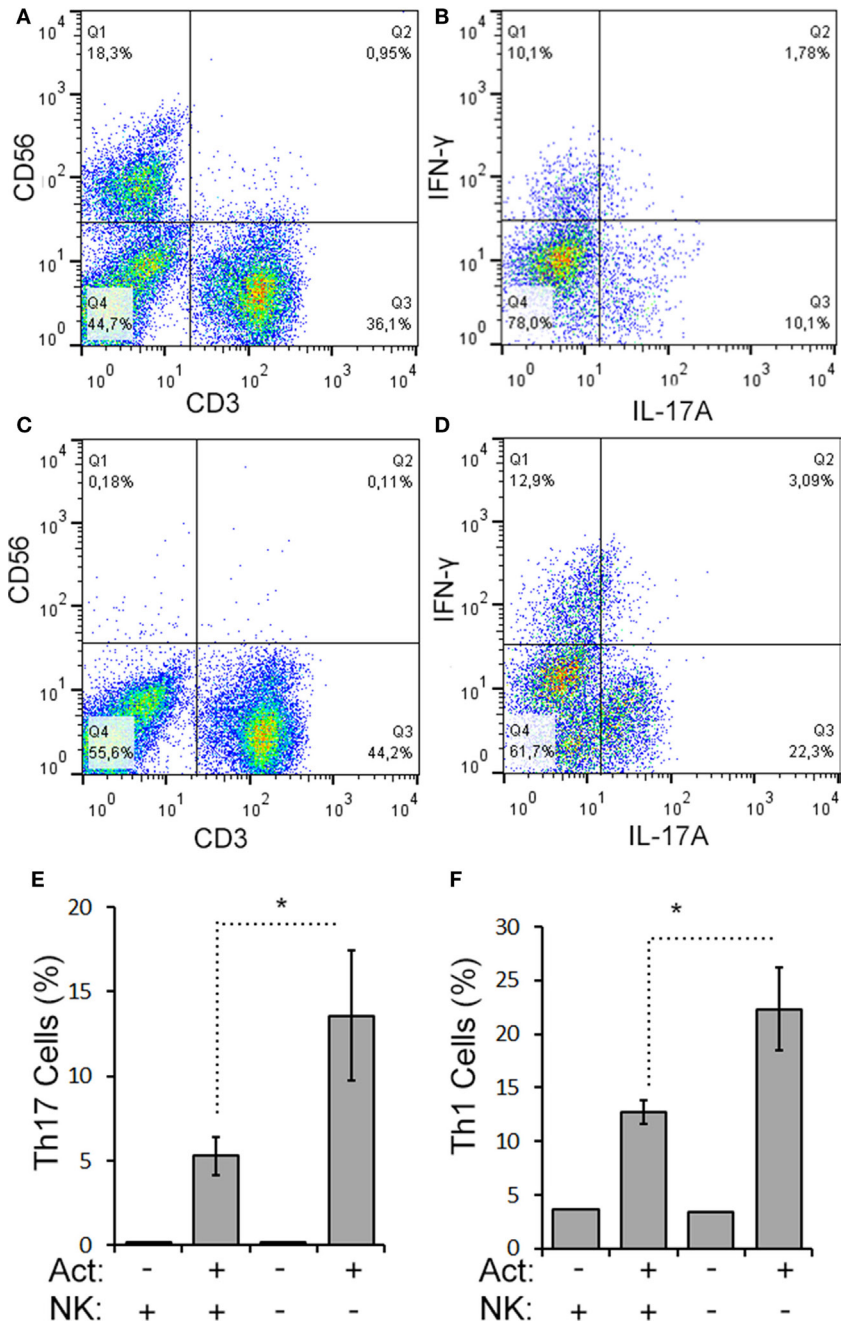

FIGURE 4 | CD56$^+$ cells suppress Th17 and Th1 cell responses in aHSCT. CD56$^+$ cells were depleted from MS patient PBMC samples collected at 12 months post-aHSCT. Representative plots are shown for complete **(A,B)** and CD56-depleted samples **(C,D)**. Cells were activated *in vitro* with anti-CD3, anti-CD28, and Th17 polarizing factors (Act) for 4 days. The proportions of Th17 and Th1 cells were assessed by analysis of cytokine production by intracellular flow cytometry. Representative plots are shown for complete samples **(B)** and CD56-depleted samples **(D)**. The average proportion of Th17 **(E)** or Th1 cells **(F)** is shown. $N = 3$ patients.

NK Cells Kill Th17 Cells by NKG2D-Mediated Necrosis

Given the limited cell numbers available from aHSCT PBMC samples, we continued to explore the mechanism in a series of cell culture experiments with healthy subjects PBMCs. Healthy PBMCs were fractioned into memory (RO$^+$RA$^-$) CD4$^+$ T cells and CD3$^-$CD56$^+$ NK cells (representative purity confirmation in **Figures 5A–C**). Th cells and NK cells were then activated in co-culture at a 1:1 ratio to approximate the ratio observed in the post-aHSCT clinical trial samples. NK cells caused a 50% decrease in response of Th17 cells (defined as CD3$^+$CD4$^+$IL-17A$^+$ IFN-γ$^-$) when proportion and number were measured using intracellular

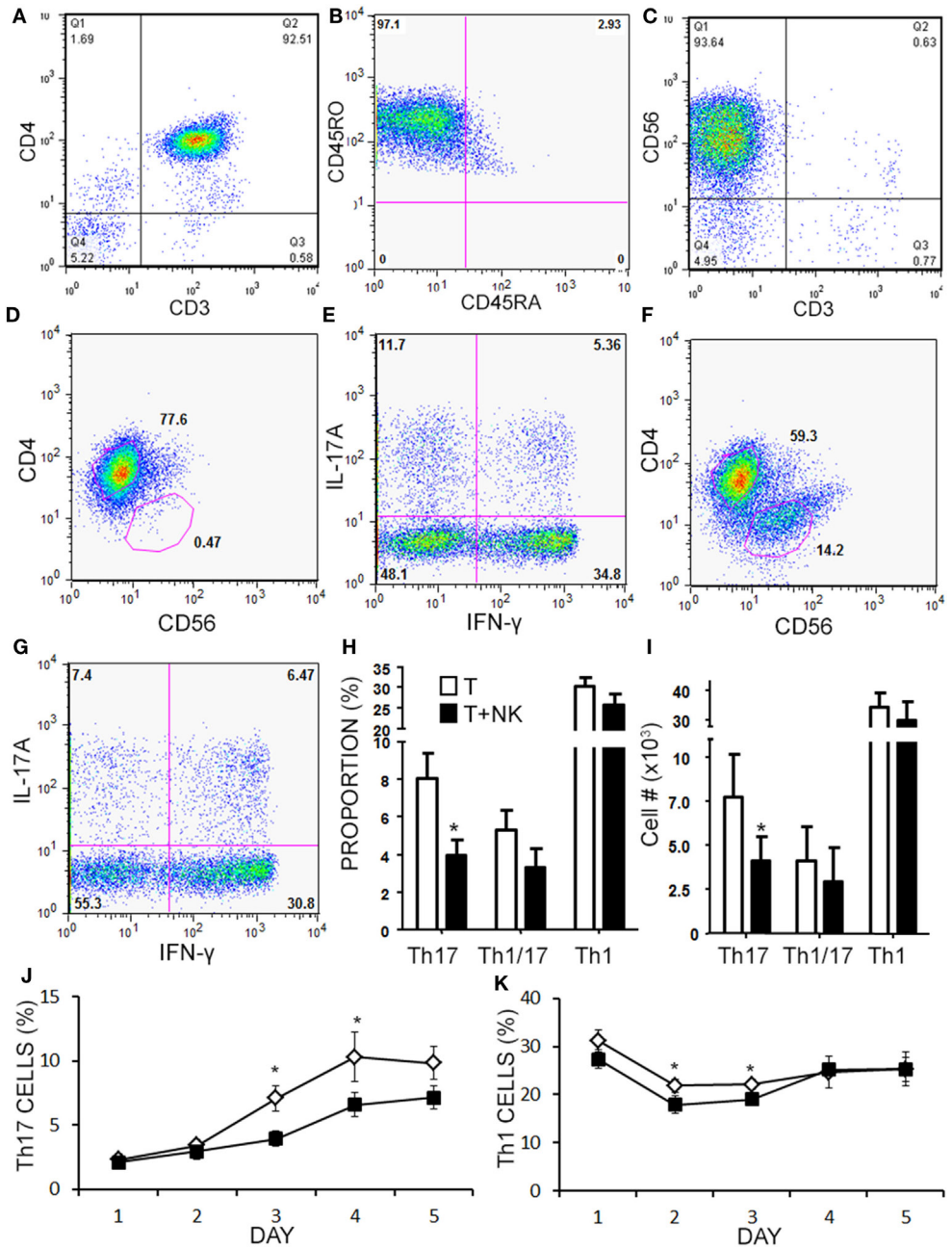

FIGURE 5 | Natural killer (NK) cells reduce the proportion of Th17 and Th1 cells *in vitro*. Memory CD4+ T cells and NK cells were isolated from healthy subject PBMC. Memory CD4+ T cells were $CD3^+CD4^+CD45RO^+CD45RA^-$ as shown in representative dot plots **(A,B)**. NK cells were $CD3^-CD56^+$ as shown in a representative dot plot **(C)**. Memory CD4+ T cells were cultured without **(D,E)**, or with NK cells at a 1:1 ratio **(F,G)** for 4 days with anti-CD3, anti-CD28, and Th17 polarizing factors for 4 days. Plots are shown for CD4 × CD56, which was used to gate CD4+ helper T (Th) cells. Expression of IL-17 and IFN-γ by CD4+ T cells was assessed by intracellular flow cytometry (Th17 = $CD3^+CD4^+IL\text{-}17A^+IFN\text{-}\gamma^-$, Th1 = $CD3^+CD4^+IL\text{-}17A^-IFN\text{-}\gamma^+$, and Th1/17 = $CD3^+CD4^+IL\text{-}17A^+IFN\text{-}\gamma^+$). Data pooled from 12 experiments showing the proportion **(H)** and absolute number of Th cells **(I)**. A time-course analysis for Th17 cells **(J)** and Th1 cells **(K)** was performed for 5 days using intracellular cytokine staining. Open diamond = T cells and closed square = T cells + NK cells.

flow cytometry (**Figures 5D–I**). In the same samples, the Th1/17 cells ($CD3^+CD4^+IL\text{-}17A^+$ $IFN\text{-}\gamma^+$) or Th1 cells ($CD3^+CD4^+IL\text{-}17A^-$ $IFN\text{-}\gamma^+$) were not altered by NK cells, although a trend toward decrease was noted for each (**Figures 5H,I**). A more detailed time-course analysis showed that NK cells began to decrease the proportion of Th17 cells on day 3, while the proportion of Th1 cells was slightly reduced on days between 2 and 3 (**Figures 5J,K**). Despite the fact that NK cells reduced the proportion and number of Th17 cells by about 50%, the mean fluorescent intensity of IL-17A inside of Th cells was significantly elevated

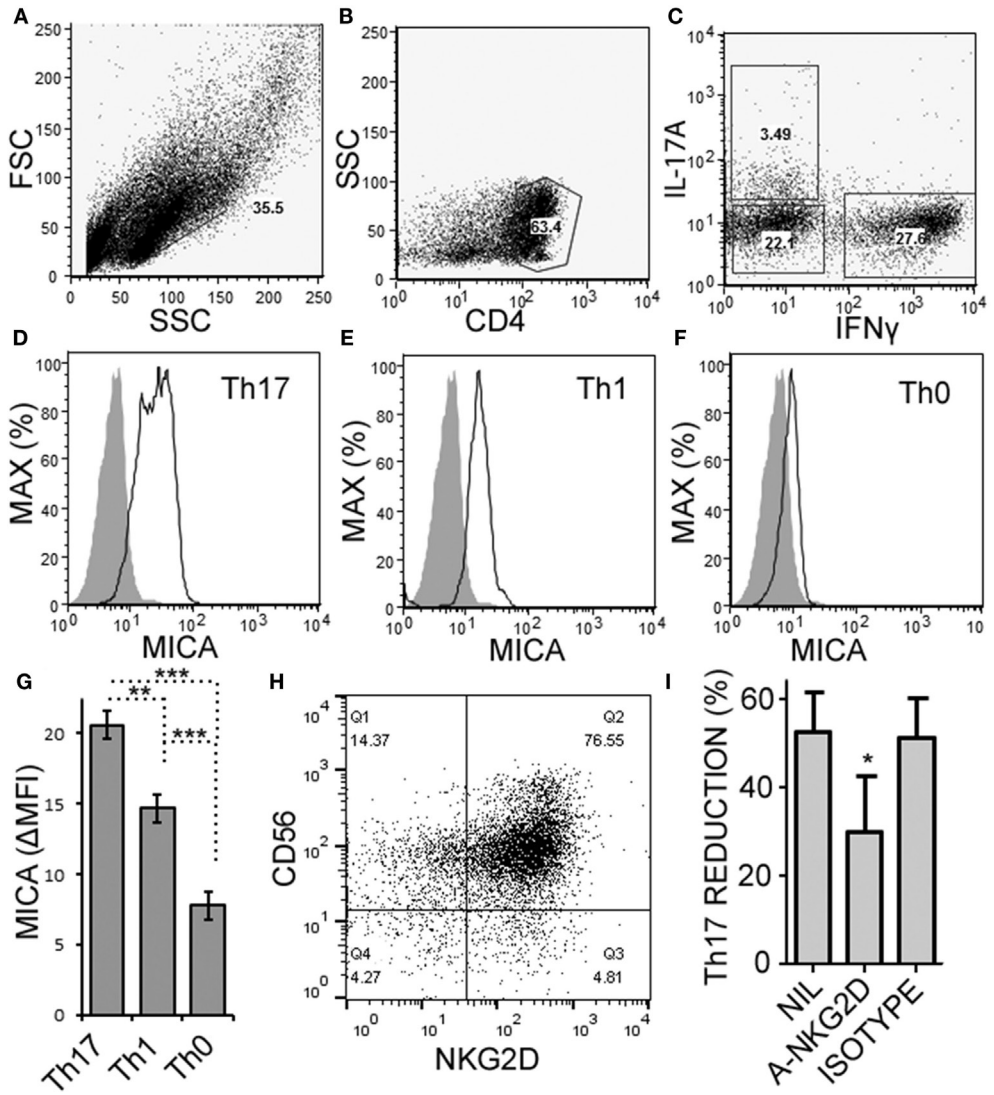

FIGURE 6 | Activated memory CD4+ T cells express MICA and are sensitive to NKG2D-mediated natural killer (NK) cell cytotoxicity. Memory CD4+ T cells were obtained from healthy subject PBMC, as described in **Figure 5**, and activated with anti-CD3, anti-CD28, and Th17 polarizing factors for 4 days. Expression of CD4, MICA (Zenon labeled), IL-17A, and IFN-γ were assessed by intracellular cytokine staining and flow cytometry. Representative plots of FSC × SSC **(A)**, CD4 × SSC **(B)**, and IL-17A × IFN-γ **(C)** are shown. MICA expression on Th17 **(D)**, Th1 **(E)**, and the Th0 cells **(F)** is shown. Open histogram indicate MICA stained cells and closed histograms indicate an isotype control. The average mean fluorescent intensity of MICA minus the isotype control is shown [ΔMFI; **(G)**]. Expression of CD56 and NKG2D by NK cells was assessed by flow cytometry and a representative plot is shown **(H)**. NK cells were cultured with memory CD4+ T cells and activated with anti-CD3, anti-CD28, and Th17 polarizing factors, at the same time treated without antibody, (NIL), anti-NKG2D neutralizing antibody, or isotype control antibody **(I)**. *N* = 7 samples.

from 200 MFI (SE 35) up to 283 MFI (SE 74) in the presence of NK cells ($p = 0.042$). The fluorescent intensity of IFN-γ in Th cells was not significantly altered by the presence of NK cells although a trend toward a reduction was noted, from 682 MFI (SE 85) without NK cells, to 634 MFI (SE 97) with NK cells.

Natural killer cells have previously been reported to inhibit cell cycle of T cells (45). Given the decrease in Th17 cells in the cultures over time, we questioned whether NK cells were limiting the proliferation of the T cells in our cultures. NK cells did not alter the proliferation of Th17, Th1/17, or Th1 cell as indicated by dilution of a proliferation-tracking dye combined with intracellular staining for cytokines (Figure S1 in Supplementary Material). Next, we evaluated the possibility of NKG2D-dependent cytotoxicity by NK cells. Memory CD4+ T cells obtained from healthy volunteer PBMCs were activated under Th17 polarizing conditions (**Figures 6A–C**). MICA, an NKG2D ligand, was found on Th17, Th1, and Th0 subsets (the latter defined here as negative for IL-17A and IFN-γ; **Figures 6D–F**), and Th17 cells had the highest MICA expression (**Figure 6G**). We chose to focus on MICA because the other ligands (MICB and ULBP1,2,3) were below detection thresholds on PBMC samples (data not shown). As expected, NKG2D was expressed on the NK cells and expression

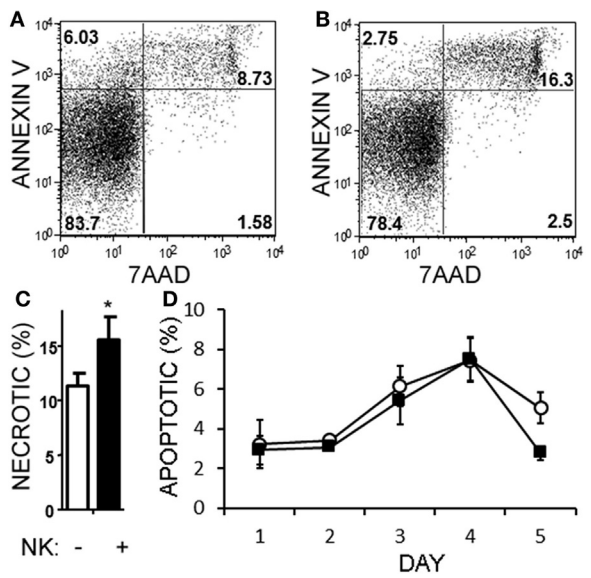

FIGURE 7 | Natural killer (NK) cells are cytotoxic toward helper T (Th) cells through necrosis but not apoptosis. Memory CD4+ T cells from healthy subject PBMC were activated with anti-CD3, anti-CD28, and Th17 polarizing factors, either in the absence or presence of NK cells for 4 days. Samples were stained with CD3, CD56, 7AAD, and annexin V. Representative plots of T cells (CD4+CD56−) cultured without **(A)** or with NK cells **(B)** are shown. The average proportion of necrotic (7-AAD+annexinV+) T cells is shown **(C)**. Open bars = memory CD4 T cells, closed bars = memory CD4+ T cells plus NK cells. *N* = 8 samples. A time-course analysis of apoptotic (7-AAD−annexin V+) T cells is shown **(D)**. Open circles indicate memory CD4 T cells alone and filled squares indicate T cells plus NK cells.

was identified on both CD56^{dim} and CD56^{bright} (**Figure 6H**). When memory CD4+ T cells were cultured with NK cells, neutralization of NKG2D significantly attenuated the NK cell-mediated reduction in Th17 responses (**Figure 6I**). To determine the mode of cell death, samples were stained with annexin V and 7-AAD dyes. NK cells caused memory CD4+ T cells to become annexin V+ and 7-AAD+, which is a profile consistent with late apoptosis or necrosis (**Figures 7A,B**). The proportion of necrotic (annexin V+ 7-AAD+) Th cells was significantly elevated after NK cells were added (**Figure 7C**). Furthermore, a time-course analysis revealed no evidence that NK cells were inducing apoptotic (annexin V+ 7AAD−) T cells even at earlier time points (**Figure 7D**). Together, these data demonstrate that NK cells can limit Th cell expansion by inducing necrotic cell death.

CD58 Expression on NK Cells Contributes to a Transient Increase in IL-17A Production from CD4+ T cells

When we analyzed the levels of cytokines in cell culture supernatants of activated Th cells exposed to NK cells, we observed significantly higher levels of IL-17A, and IFN-γ (**Figures 8A,B**). This seemed at odds with a reduced proportion of Th17 cells, however, we noted an increased MFI of IL-17A in the flow cytometry-based analysis suggesting that surviving cells were making more IL-17A. Upon activation, Th cells expressed *RORC* (encodes RORγt) mRNA on day 1 of the experiment, which increased (**Figure 8C**), and *IL17A* mRNA levels were detected at day 2 and day 3 of the experiment (**Figure 8D**). With NK cells added, there was more *RORC* on day 1, and more *IL17A* on day

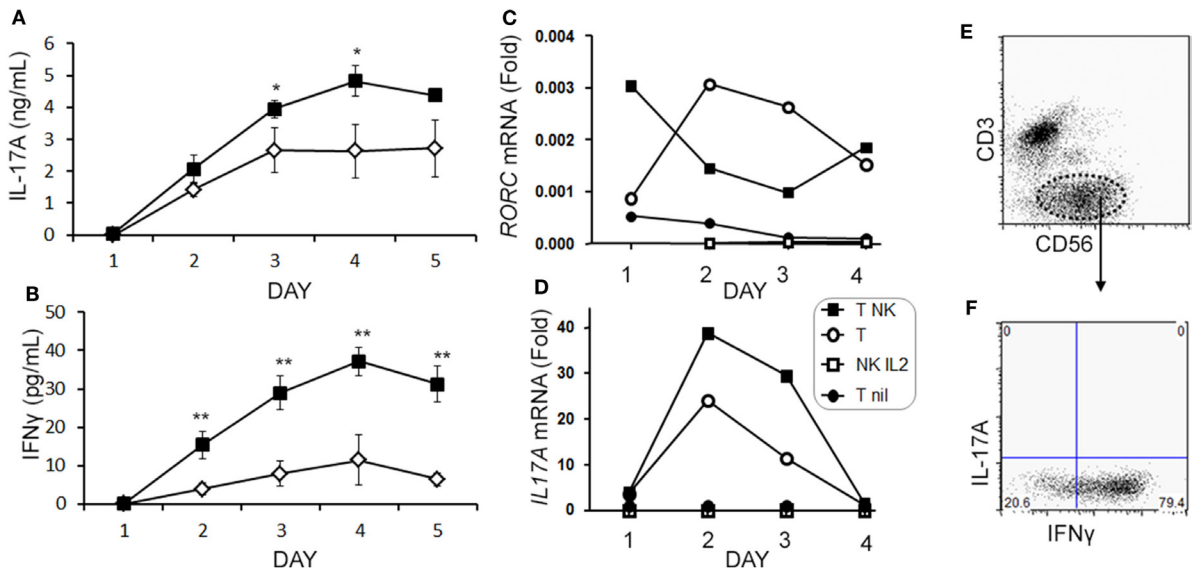

FIGURE 8 | Natural killer (NK) cells augment IL-17A and *RORC* levels in memory CD4 T cells. Purified memory CD4+ T cells from healthy subject PBMCs were activated with anti-CD3, anti-CD28, and Th17 polarizing factors without (open diamond) or with NK cells (closed square). Cytokines IL-17A **(A)** and IFN-γ **(B)** were measured in the supernatant by enzyme-linked immunosorbent assay. Expression of *RORC* **(C)** and *IL17A* **(D)** mRNA was measured by qPCR at the indicated time points. Data are representative of three samples. Groups included non-activated memory CD4+ T cells (T nil; closed circles), activated memory CD4+ T cells (T; open circle), activated memory CD4+ T cells with NK cells (T NK; closed squares), and NK cells cultured alone with IL-2 (NK IL-2; open squares). Representative plots of IL-17A and IFN-γ expression in NK cells (CD3−CD56+) are shown **(E,F)**.

2 and day 3. NK cells cultured on their own with IL-2 (a potent activator of NK cells) exhibited no detectable mRNA for either *RORC* or *IL17A*, indicating that T cells were the likely source of IL-17 in the co-cultures. Intracellular flow cytometry confirmed that the NK cells did not express IL-17A, although they did highly express IFN-γ (**Figures 8E,F**). One issue regarding measurement of IFN-γ is the presence of IFN-γ neutralizing antibodies used in the Th17 conditions. This lowers the amount of IFN-γ detected by ELISA when compared with cultures without the neutralizing IFN-γ (data not shown). However, the amount of IFN-γ even in the presence of the neutralizing IFN-γ was significantly increased in the presence of NK cells. To investigate the mechanism by which NK cells induced more IL-17A, selected adhesion molecules were studied. We first considered CD54 (ICAM-1), an adhesion molecule found on antigen presenting cells and NK cells. Neutralizing CD54 did not alter the NK cell-mediated transient boost in IL-17A (data not shown). CD58, another adhesion molecule, was expressed by both $CD56^{bright}$ and $CD56^{dim}$ NK cells (**Figure 9A**). The presence of neutralizing antibodies against CD58 in the co-cultures partially attenuated the NK cell-mediated increase in IL-17A (**Figure 9B**). To determine the expression of CD58 on NK cells from the MS cohort, we stained samples from BL until M24 and analyzed by flow cytometry (**Figure 10**). There was a very low percentage of $CD58^+$ NK cells in these samples (less than 1% of total PBMC), this proportion was not changed at M12, and significantly higher by M15–M24 compared with BL although remaining below 1% of PBMC.

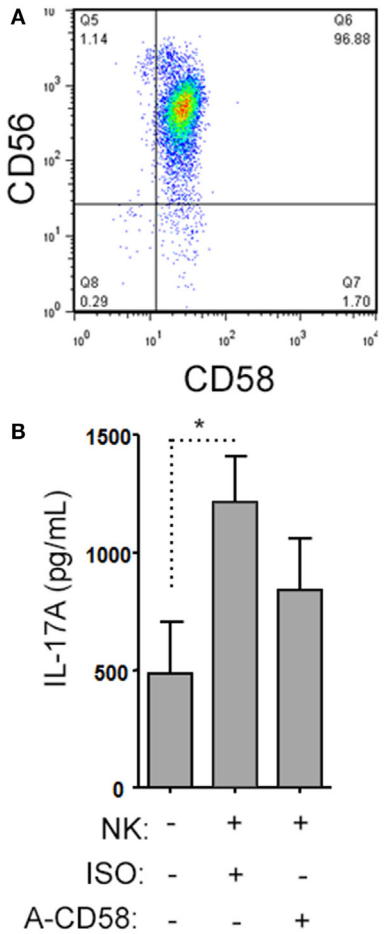

FIGURE 9 | Natural killer (NK) cells support IL-17A expression by helper T (Th) cells by CD58 co-stimulation. A representative plot of CD58 expression by $CD3^-CD56^+$ NK cells is shown from healthy subject peripheral blood mononuclear cell (PBMC) **(A)**. Memory $CD4^+$ T cells from healthy subjects PBMC were activated with anti-CD3, anti-CD28, and Th17 polarizing factors with NK cells in the presence or absence of CD58 neutralizing or isotype control antibody for 4 days. The cytokine IL-17A was assessed by enzyme-linked immunosorbent assay from cell culture supernatants **(B)**. Graph indicates mean IL-17A concentration for $N = 3$ samples.

DISCUSSION

Autologous-HSCT is a promising new therapy for aggressive MS, which can abrogate clinical relapses and stabilize brain MRI lesions. The reconstituting immune system has a lesser neuroinflammatory capacity in post-aHSCT samples. This suggests that changes had occurred following treatment, which decrease disease progression. The data presented here demonstrate that NK cells reconstitute rapidly following aHSCT, while $CD4^+$ T cells remained below BL for up to 21 months. One explanation for functional suppression of CD4 T cells could be that conventional regulatory T cells ($CD3^+$ $CD4^+$ $FoxP3^+$ $CD25^+$ $CD127^-$) that were shown to rapidly reconstitute following a non-ablative aHSCT in MS were suppressing CD4 T cells (46). However, in the Canadian ablative cohort, regulatory T cells were only transiently increased in MS patients after aHSCT, and returned to BL by 9 months post-treatment (40). Another possibility is that thymic atrophy accounts for the low Th cell counts. However, the re-emergence of recent thymic emigrant naive T cells, and the broad clonal diversity that developed in MS patients who underwent aHSCT, suggests that the thymus (which exhibits decreased function early after chemotherapy) is still sufficient to facilitate T cell maturation and selection (40, 47). In this study, we tested the hypothesis that NK cells were playing a regulatory role in the reconstituting immune system. NK cells correlated inversely with the change in Th17 cells, and depleting NK cells from aHSCT samples resulted in a higher proportion of Th17 cells. Th1 cells were also inhibited by NK cells, albeit to a lesser extent than Th17 cells. These results indicate that NK cells reconstitute rapidly, possibly due to incomplete ablation or presence of NK cells in the graft, following aHSCT and suppress the re-emergence of Th17 cells.

Natural killer cells are known to suppress T cells, but the relative effect on Th cell subsets has not been widely explored. In cell culture experiments, we found that NK cells reduced the proportion of Th17 cells by necrosis in an NKG2D-dependent fashion. Previous studies have reported that NK cell–T cell interactions may result in apoptosis (48), or limit proliferation of T cells (45). Here, we only observed necrotic cell death of T cells with no differences in apoptosis or proliferation of Th cells. Th17 cells expressed more MICA than Th1 cells that may account for the higher sensitivity of Th17 cells to NK cell-mediated cytotoxicity. Despite the clear necrotic effects, NK cells caused an early rise in expression of IL-17A and RORγt. Moreover, the IL-17A increase was, in part, mediated by CD58. Thus, NK cells provide early co-stimulation to Th17 cells followed by NKG2D-dependent

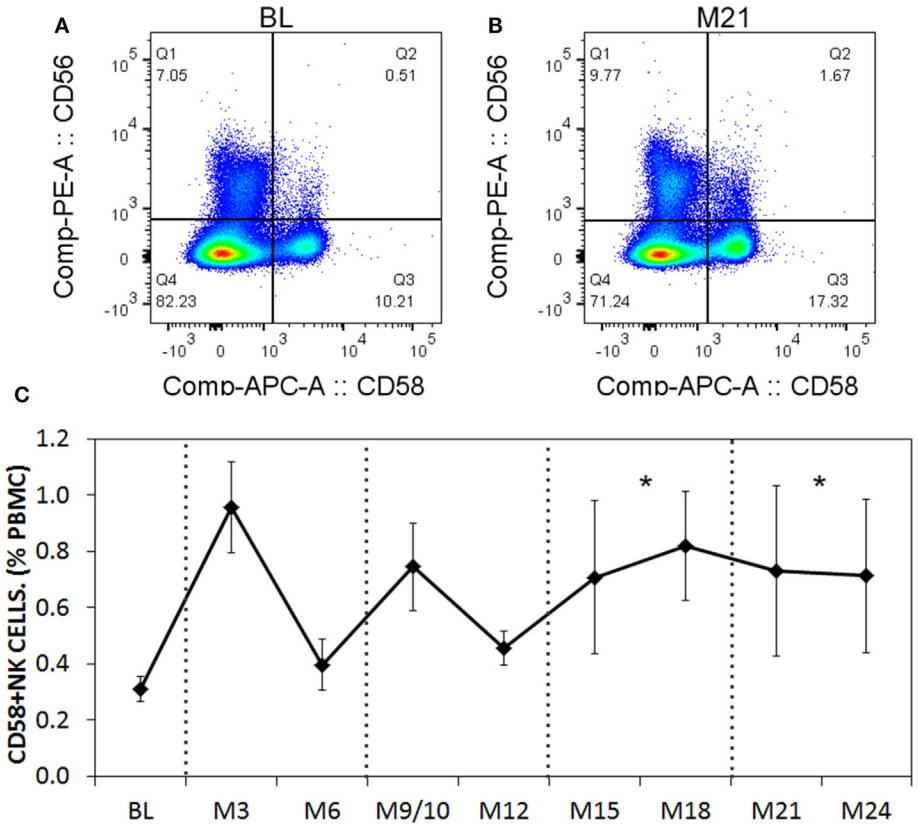

FIGURE 10 | CD58 expression levels on natural killer (NK) cells before and after aHSCT treatment of multiple sclerosis (MS) patients. Cryopreserved peripheral blood mononuclear cell (PBMC) from the aHSCT cohort of MS patients was stained for CD3, CD56, and CD58. Representative plots for CD56 and CD58 are shown for baseline (BL) **(A)** and month 21 [M21; **(B)**]. A time series of samples from BL until month 24 (M24) is presented **(C)**. For statistical analysis, the time points were grouped in M3–M6, M9–M12, M15–M18, and M21–M24, followed by univariate one-way ANOVA with pairwise comparisons with the BL values. *N* = 7 patients.

cytotoxicity. We previously identified a decrease in IL-17A in culture supernatants of PBMC (activated with anti-CD3, anti-CD28, and Th17 polarizing factors) at 12 months post-aHSCT (40). While it is possible that NK cells transiently boosted Th17 responses, the longer-term outcome is that Th17 responses, whether assessed by flow cytometry or ELISA, were reduced in post-aHSCT samples. Further examination of the aHSCT MS samples showed a very low level (<1%) of $CD58^{+}$ NK cells, and this value increased slightly by month 15 post therapy. It is not clear that this has a biologically significant impact on the patients.

Our observations add to the growing understanding of how NK cells regulate T cell responses. NK cells can provide co-stimulation and even antigen presentation that activates T cells (49, 50). Upon activation, T cells upregulate NKG2D ligands and are then reduced in number through cytotoxic action of NK cells (26–28). We did not perform kinetics of expression of MICA, however, a previous study showed that T cells only express MICA and related ligands after 3 days of activation, leading to their sensitization to NK cell-mediated killing (27). This timing is consistent with our observations where NK cells were initially costimulatory, and then began to kill by necrosis by the third day of the cell culture experiment. Thus, there is a brief window of time for NK cells to boost Th17 cell responses. In our experiments, the proportion of Th17 cells remaining after NK cell exposure was around 50% which suggests that the purpose of NK cells is to attenuate, but perhaps not fully eliminate, T cell expansion, to preserve immunological memory while preventing uncontrolled T cell activation. This may allow NK cells to accelerate the normal life cycle of Th17 cells by transiently boosting IL-17A expression and secretion, and then accelerating down-regulation of the effector response through cytotoxicity.

We identified an increase in both $CD56^{bright}$ and $CD56^{dim}$ NK cells at 12 months post-aHSCT. Similar conclusions were obtained whether the CD56 intensity or the CD16 expression was used to define NK cell subsets. The ratio of $CD56^{bright}$ NK cells was substantially increased from month 3 to month 6 after the therapy suggesting that they replenished faster than the $CD56^{dim}$ NK cells. From our phenotype data it is not possible to draw conclusions about the functional properties of NK cell subsets. In an elegant study by Laroni et al., the two subsets were sorted from healthy donors and their cytotoxic capacity was compared in the presence of inflammatory cytokines (25). The authors found that $CD56^{bright}$ NK cells were more cytotoxic, and they required pro-inflammatory cytokines to be cytotoxic to human autologous CD4 T cells. However, other studies found comparable cytotoxicity of $CD56^{bright}$ and $CD56^{dim}$ NK cells toward

human autologous CD4 T cells (51). Thus, both subsets can have cytotoxic capacity depending on the protocols used. The ability for NK cells to potentially up- or down-regulate inflammatory T cell responses warrants further detailed investigation into the molecular mechanisms of NK cell-T cell interactions, and into their clinical relevance for novel therapeutic strategies.

CANADIAN MS/BMT STUDY GROUP

The Canadian MS/BMT Study Group includes **Harold Atkins**, **Lothar Huebsch**, **Lisa Walker**, **Marjorie Bowman**, **Mark S. Freedman**, Ottawa Hospital Research Institute (OHRI), Ottawa, ON, Canada; **Amit Bar-Or**, **David Haegert**, **Douglas Arnold**, **Farzaneh Jalili**, **Hyunwoo Lee**, **Jack Antel**, **Jacqueline Chen**, **Peter Darlington**, **Pierre Laneuville**, **Robert Brown**, **Yves Lapierre**, Montreal Neurological Institute/McGill University, Montreal, QC, Canada; **Rafick Pierre Sekaly**, **Remi Cheynier**, Université de Montreal, Montreal, QC, Canada; **Paul O'Connor**, University of Toronto, Toronto, ON, Canada.

ETHICS STATEMENT

Samples were collected, as specified in the immune monitoring sub study, from patients enrolled in the trial "Autologous Stem Cell Transplant for Multiple Sclerosis (MS/BMT)" (NCT01099930) following informed consent as approved by the institutional review boards. PBMCs were isolated from healthy donors following informed consent per approved institutional protocol (McGill University).

AUTHOR CONTRIBUTIONS

PD designed, performed, analyzed experiments as well as made figures, wrote and edited the manuscript. BS made figures, wrote and edited the manuscript. TT conceived of project, designed performed, analyzed, experiments. J-SD performed and analyzed experiments. LF conceived project and designed experiments. MR designed performed and analyzed experiments. M-NB designed, performed and analyzed experiments as well as made figures. NA designed performed and analyzed experiments as well as edited the manuscript. MF designed implemented transplant procedure, analyzed blood samples and edited the manuscript. HA designed implemented transplant procedure, analyzed blood samples, and edited the manuscript. AB-O conceived project, designed and analyzed experiments, analyzed clinical data, wrote and edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00834/full#supplementary-material.

FIGURE S1 | Natural killer (NK) cells do not inhibit helper T (Th) cell proliferation. Memory T cells from healthy subjects were labeled with CFSE and then activated with anti-CD3, anti-CD28, and Th17 polarizing factors with NK cells for 4 days. T cell proliferation was determined by CFSE dilution as assessed by flow cytometry. Th cell subsets were identified based on cytokine production by intracellular flow cytometry. Representative histograms indicating CFSE dilution are shown for Th17 **(A)**, Th1/17 **(B)**, and Th1 **(C)** cells. Non-activated T cells (gray filled), activated T cells (thick line), and activated T cells cultured with NK cells (thin line) are shown. The average proportion of T cells that have undergone division are shown **(D)**. Open bars = T cells, closed bars = T cells plus NK cells. N = 7 samples.

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New Horizons in the Treatment of Type 1 Diabetes: More Intense Immunosuppression and Beta Cell Replacement

Carlos E. B. Couri[1,2]*, *Kelen C. R. Malmegrim*[1,3] *and Maria C. Oliveira*[1,2]

[1] Center of Cell-Based Therapy, Regional Blood Center of Ribeirao Preto, Ribeirao Preto Medical School, University of Sao Paulo, Ribeirao Preto, Brazil, [2] Department of Internal Medicine, Ribeirao Preto Medical School, University of Sao Paulo, Ribeirao Preto, Brazil, [3] Department of Clinical, Toxicological and Bromotological Analysis, School of Pharmaceutical Sciences of Ribeirao Preto, University of Sao Paulo, Ribeirao Preto, Brazil

Since the discovery of autoimmunity as the main pathophysiologic process involved in type 1 diabetes, many attempts have tried to delay or stop beta cell destruction. Most research protocols in humans have investigated the effects of therapeutic agents targeting specific steps of the autoimmune response. In spite of safety and some degree of beta cell preservation, the clinical impact of such approaches was similar to placebo. Recently, research groups have analyzed the effects of a more intense and wider immunologic approach in newly diagnosed type 1 diabetic individuals with the "immunologic reset," i.e., high-dose immunosuppression followed by autologous hematopoietic stem cell transplantation. This more aggressive approach has enabled the majority of patients to experience periods of insulin independence in parallel with relevant increments in C-peptide levels during mixed meal tolerance test. However, on long-term follow-up, almost all patients resumed exogenous insulin use, with subsequent decrease in C-peptide levels. This has been at least in part explained by persistence of islet-specific T-cell auto-reactivity. Here, we discuss future steps to induce immune tolerance in individuals with type 1 diabetes, with emphasis on risks and possible benefits of a more intense transplant immunosuppressive regimen, as well as strategies of beta cell replacement not requiring immunomodulation.

Keywords: type 1 diabetes, immunotherapy, autologous hematopoietic stem cell transplantation, immunologic reset, autoimmunity, beta cell preservation

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Anne Cooke,
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Lund University, Sweden
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The University of Queensland,
Australia

***Correspondence:**
Carlos E. B. Couri
ce.couri@yahoo.com.br

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THE COMPLEX AUTOIMMUNE REPERTOIRE OF TYPE 1 DIABETES (T1D)

Type 1 diabetes is an autoimmune disease characterized by a silent phase of progressive beta cell destruction, followed by a symptomatic phase of hyperglycemia, when great amount (not well defined) of beta cell mass and function has been reduced. This phenomenon occurs in individuals with genetic background exposed to still undefined immunologic triggers (1). The rate of beta cell destruction is not absolutely linear as previously described. The pattern of temporal beta cell loss is typical of relapsing-remitting diseases, as periods of exacerbated beta cell destruction alternate with those of inactivity (1). In addition, subjects who are older at diagnosis present a slower process of autoimmune destruction, and, as a consequence, larger residual beta cell mass (2, 3).

Recent data from the TEDDY (The Environmental Determinants of Diabetes in the Young) trial showed that autoantibodies against islet antigens may occur early in life of a subset of patients. In fact, the age of patients at diagnosis of overt diabetes and the order of antibody positivity depend

both on HLA and non-HLA genotypes (4, 5). The exact triggers of autoimmunity against pancreatic islet beta cells have not yet been identified, and much of the immunologic mechanisms involved in this process are still under study. There is a pathological cross talk involving B and T lymphocytes, regulatory cells (B and T reg cells), autoantibodies, monocytes, natural killer cells, cytokines, and the beta cells themselves (1, 6–8). Although there is evidence that CD8+ effector T cells may be directly involved in beta cell death, other cell types most certainly participate in this autoimmune response (8, 9). In this scenario, which are the effects of immune interventions in humans with T1D?

THE ROLE OF IMMUNOINTERVENTIONS IN THE PRESERVATION OF BETA CELL MASS

Since the discovery of autoimmunity as the key phenomenon leading to T1D, many immunologic interventions have been investigated as strategies to stop beta cell destruction. The Diabetes Control and Complication Trial showed that higher C-peptide levels are associated with lower incidence of diabetic nephropathy, retinopathy, and less episodes of hypoglycemia (10). Preservation of beta cell mass is also associated with less exogenous insulin requirement (3). Autoimmunity in T1D is complex and involves different pathways, connections, organs, and cells. Nevertheless, most research protocols have attempted to hinder beta cell destruction targeting specific molecules or pathways, instead of wider immunosuppressive approaches. The argument is that systemic immunosuppression may expose the patient to undesirable adverse effects. **Table 1** summarizes the main outcomes of recent clinical trials on beta cell preservation in patients with T1D (secondary prevention trials).

Effector T cells are directly related to beta cell death, and secondary prevention trials with teplizumab (anti-CD3 monoclonal antibody) (11) and otelixizumab (anti-CD3 monoclonal antibody) (12–15) have used these cells as targets to preserve pancreatic function. In these studies, treated patients presented a less accelerated rate of decline in C-peptide levels and also some reduction in daily insulin doses, when compared to non-treated patients, indicating a beneficial effect of these immunomodulatory agents on beta cell preservation. However, less than 5% of individuals experienced periods free from insulin.

The combination of antithymocyte globulin plus granulocyte colony-stimulating factor (G-CSF) has also been investigated, as another attempt to stop T cell auto-reactivity. Individuals up to 2 years after diagnosis of T1D (different from other secondary prevention trials) were included. Along 1 year, the decline in C-peptide levels was slower in treated patients, when compared to the control group, but there was no difference in insulin requirements (16). In 2014, Peskovitz and colleagues turned the focus on B cells, treating newly diagnosed T1D individuals with rituximab (anti-CD20 monoclonal antibody). The treatment was considered safe and able to induce slower decline in C-peptide levels; however, no significant difference in insulin requirements was detected between treated and placebo groups (17).

Later, T cells were again targeted by immunomodulatory approaches with alefacept (LFA3-IgG1 fusion protein that binds CD2) (18) and abatacept (CTLA-4-IgG1 fusion protein that binds CD80/CD86) (19) that induce apoptosis and inhibit activation of

TABLE 1 | Recent secondary prevention trials in individuals with type 1 diabetes and their effect on beta cell preservation.

Immunomodulatory approach	Main target of medication	Follow-up	Effect on C-peptide on time	Comments
Teplizumab (11)	T cell (CD3+)	2 years	Slower decline compared with placebo	At 1 year, 5.3% (11/207) of patients in the full-dose group were free from insulin. In year 2, three of these 11 were still insulin-free
Otelixizumab (12–15)	T cell (CD3+)	4 years	Slower decline in C-peptide with higher doses of otelizumab. No effect with lower doses	The higher the dose of otelizumab, the better the clinical response, but the more the risk of side effects. No patient became insulin-free
ATG + granulokine (16)	T cell (CD4+, CD8+), B cell, T reg	1 year	Slower decline compared with placebo	No differences in insulin dose compared with placebo. No patient insulin-free. Time of disease at randomization: 4 and 24 months
Rituximab (17)	B cell	2 years	Slower decline compared with placebo	Lower insulin requirements in the treated group. No patient insulin-free
Alefacept (18)	T cell (CD4+, CD8+)	2 years	Slower decline compared with placebo	Reduced insulin dose in treated group. No patient insulin-free
Abatacept (19)	T cell (CD80+, CD 86+, CD28+)	3 years	Slower decline compared with placebo	No differences in insulin dose compared with placebo
Autologous mesenchymal stem cell (21)	T cell, T reg	1 year	No change compared with placebo	No differences in insulin dose compared with placebo. Cells were harvested from bone marrow
Autologous T regs (23)	T reg	2 years	No changes in C-peptide along the time compared to baseline	No differences in insulin dose along the time
Chemotherapy followed by autologous hematopoietic stem cell transplantation (26–35)	"Immunologic reset"	Up to 7 years	Increase in C-peptide > 3 y post-transplantation and then returned to baseline levels after 6 years and (compared to baseline)	Around 80% of patients became insulin-free for variable periods. Lack of randomized, parallel, double-blind, placebo-controlled trials. One death occurred in the Polish group. Potential risk of severe side effects

T-cells, respectively. Both drugs promoted beta cell preservation, with slower rate of reduction of C-peptide levels and were considered safe. Nevertheless, clinical effects were disappointing, as alefacept promoted only slight reduction of insulin requirements, while abatacept did not change them at all.

Cell-based therapies were also investigated in secondary prevention trials. Multipotent mesenchymal stromal cells (MSCs) are considered safe promising tools to change the natural history of T1D and other immune-mediated diseases. They exhibit immunomodulatory potential, migratory capacity to injured and inflamed areas and can contribute to tissue regeneration [directly or by the secretion of bioactive factors (20)]. In 2015, Carlsson and colleagues analyzed the effect of autologous MSCs in adults with recent-onset T1D. In contrast with non-treated T1D patients, who presented a significant decline in serum C-peptide levels, beta cells were still preserved in the MSC-treated group at 1-year follow-up, with stable levels of C-peptide. The beneficial effect on pancreatic function, however, did not reflect on insulin requirements of the MSC-treated group. No relevant side effects were observed (21).

The pathophysiology of T1D also involves defective function of regulatory T cells (Tregs), and perhaps T effector cells that may be refractory to Treg suppression (22). In 2015, Bluestone and colleagues evaluated the effects of intravenous infusions of expanded autologous polyclonal Tregs in recent-onset T1D patients (22). In this phase I open label study, autologous Treg infusions were safe, but did not change the temporal secretion of C-peptide and exogenous insulin use along 2 years of follow-up. Phase 1/2 trials have also investigated the effects of low-dose exogenous IL-2 on Treg function (23, 24). However, although the studies have detected increments in Treg numbers, the effects on glycemic control have yet to be established.

Most of the research protocols in humans used approaches that aimed to target only some steps of autoimmunity repertoire. Despite safety and some degrees of beta cell preservation, the clinical impact of such agents was similar to the placebo groups. Moreover, most of these immunomodulatory agents were originally developed and/or tested to prevent progression of other autoimmune diseases, and they may not necessarily share similar pathophysiological mechanisms of T1D.

Under the rationale of having a wider approach to the complex immune dysfunctions linked to T1D, in 2007 Voltarelli and colleagues published the first trial analyzing the effect of the "immunologic reset" in newly diagnosed T1D patients. The strategy included ablation of the autorreactive immune system, followed by generation of a new and tolerant system through infusion of autologous stem cells. Hematopoietic stem cells were mobilized with cyclophosphamide (2.0 g/m^2) and G-CSF (10 μg/kg per day) and then were harvested from the peripheral blood by leukapheresis and cryopreserved. Subsequently, hematopoietic stem cells were injected intravenously after conditioning with cyclophosphamide (200 mg/kg) and rabbit antithymocyte globulin (4.5 mg/kg). In this prospective non-randomized trial, most patients (21 out of 25) became insulin-free after transplant. At 4 years posttransplantation, C-peptide levels were significantly higher than pretransplant levels (25–30). Furthermore, at 7 years, most patients had already resumed exogenous insulin use, but C-peptide levels were still similar to those pretransplantation.

Independent research groups have reproduced the Brazilian transplant protocol with slight modifications to increase efficacy of the procedure (31–34). In the Polish protocol, patients underwent 2 or 3 plasmapheresis sessions before transplant and acarbose was used as maintenance drug (31, 32). In the Mexican protocol, the transplant conditioning regimen included cyclophosphamide plus fludarabine (34). Nevertheless, results regarding duration of insulin independence and transient increase in C-peptide levels were similar to those shown by Voltarelli and colleagues.

Recently, to compare long-term effects of the "immunologic reset" with the real world scenario, a cross-sectional analysis was made with BrazDiab1 (the largest multicenter observational study in T1D in Brazil) data. During the long-term follow-up of 8 years, none of the transplantated patients had developed microvascular complications, while 21.5% of the non-transplanted BrazDiab1 patients had presented at least one microvascular complication (30). Despite limitations, this study suggests that hematopoietic stem cell transplantation may promote long-term beneficial metabolic effects beyond insulin freedom.

Ethical and safety issues are key points of research protocols that involve high-dose systemic immunosuppression. Since there are potential short-term risks of infection, acute organ dysfunction and death, and theoretical long-term risks of malignancies and secondary autoimmune diseases, the inclusion of young children with T1D has been restricted in these trials. The majority of patients included in the studies presented only nausea, vomiting, alopecia, and fever as transplant-related adverse events. In the Polish study, however, there was one death due to *Pseudomonas aeruginosa* sepsis (32). To date, no severe long-term side effects have been described.

In 2017, Malmegrim and colleagues analyzed the effects of autologous hematopoietic stem cell transplantation on the immune system (27). Although $CD8^+$ T-cells reconstituted early after transplant, $CD4^+$ T-cell remained lower than baseline for several months, resulting in a prolonged inversion of the CD4/CD8 ratio. B cells reconstituted to baseline levels at 2–3 months posttransplantation and regulatory T cell ($CD4^+CD25^{hi}FoxP3^+$ and $CD8^+CD28^-CD57^+$) counts increased. In the overall population, memory cells comprised most of T cells detected on follow-up of patients after transplantation; however, in patients that remained insulin-free for longer periods after transplant, there was slower reconstitution of effector memory cells. When analyzed separately, islet-specific autoreactive $CD8^+$ T cells were still present after high-dose immunosuppression, indicating insufficient ablation of these cells.

The lack of knowledge of the exact mechanisms of disease, genetics, and environmental triggers may be one of the reasons for not restoring immunological balance in secondary prevention trials. On the other hand, the organ-specific autoreactivity may be too intense and persistent to be controlled, even by systemic ablation of the immune system.

THE FUTURE: BETA CELL REPLACEMENT OR MORE INTENSE IMMUNOSUPPRESSION?

As secondary prevention trials did not achieve complete restoration of immune balance, development of new strategies to preserve

and/or increase beta cell mass are still required. If even the most intense immune-based approach of "immunologic reset" with autologous hematopoietic stem cell transplantation was not able to change the natural history of T1D, it is less probable that low intensity or target-specific immunomodulatory approaches would achieve clinical success in this field.

Figure 1 presents some of the strategies that can be investigated in the near future to manage T1D autoreactivity.

Increasing the Intensity of the Conditioning Regimen for Autologous Hematopoietic Stem Cell Transplantation

As previously shown, clinical trials with autologous hematopoietic stem cell transplantation for T1D included similar drugs as conditioning regimen. The Brazilian protocol used high dose cyclophosphamide plus rabbit ATG, while the Polish protocol added plasmapheresis to the procedure and the Mexican protocol used fludarabine plus cyclophosphamide. All provided similar outcomes, indicating insufficient control of islet-specific autoreactivity. Therefore, new protocols of autologous hematopoietic stem cell transplantation should be developed, aiming to increase effectiveness of the immunosuppressive approach. One possibility is to increase the intensity of transplant conditioning regimens. In this context, a three-drug immunosuppressive regimen (cyclophosphamide + fludarabine + rabbit antithymocyte globulin) regimen has been proposed, aiming to more efficiently destroy the memory T and B cell compartment and possibly improve treatment outcomes (28).

Graft manipulation with $CD34^+$ may also be investigated as a strategy to be added to future transplant protocols. Although there is no consensus and still a matter of debate in transplantation for other autoimmune diseases, $CD34^+$ selection has not been investigated in T1D. The rationale for this approach is that during unselected infusions, memory T cells are reinfused, perpetuating the autoimmune process after transplant. Importantly, graft manipulation is associated with higher incidence of post-transplant viral infections.

For every newly proposed intervention, safety and long-term toxicity must be considered, especially when higher immunosuppression is expected. Despite lifetime insulin-dependence and poor quality of life of patients, T1D is a non-malignant disease, and new strategies to improve glycemic control are constantly under investigation.

In addition to the immune-based interventions, other ongoing research protocols investigate means to restore insulin secretion. Most of the current effort involves development of technologies for beta cell or stem cell encapsulation.

Intra-Abdominal Endocrine Pancreas Bioengineering

One of the greatest challenges in islet transplantation is the need for chronic immunosuppression (with or without corticosteroids) to avoid rejection of allogeneic cells. This problem may be circumvented by encapsulation of the islet cells to physically prevent host immune cells from reaching the graft.

Recently, Baidal and colleagues (35) reported the case of a woman with longstanding T1D who received pancreatic islets from a deceased donor and became insulin-free 17 days after the procedure. Islets were combined with host autologous plasma and were laparoscopically layered onto the omentum. A degradable biologic scaffold was created, but immunosuppression regimen still had to be used in this case.

The next step will be the improvement of the technique of islet encapsulation so that immunosuppression will be no longer

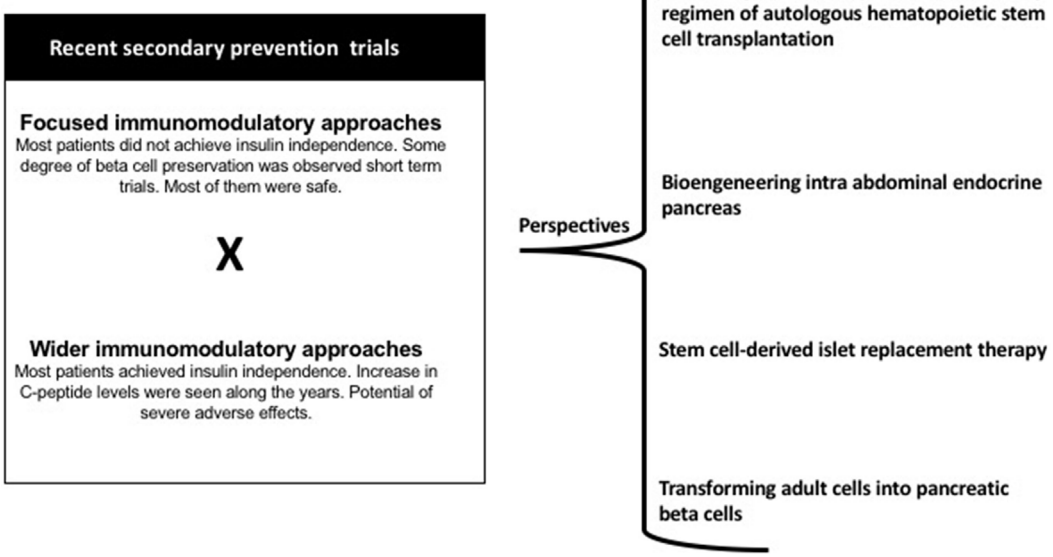

FIGURE 1 | Main results of recent secondary prevention trials and perspectives in the immunologic approaches for individuals with T1D.

needed. In the future, embryonic stem cells or even induced pluripotent stem cells (iPS) may be used as alternative sources for insulin-producing cells.

Stem Cell-Derived Islet Replacement Therapy

As a further step in the use of allogeneic insulin-producing cells, an ongoing study investigates transplantation of pancreatic endoderm cells derived from human embryonic stem cells to restore insulin and glucose homeostasis (NCT02239354). Immune-mediated rejection is prevented by a surrounding semi-permeable and protective membrane, enabling pancreatic endoderm cells to further differentiate into mature and functional pancreatic cells, and not requiring use of immunosuppressive drugs.

Transforming Adult Cells Into Pancreatic Beta Cells

The use of autologous sources for beta cell replacement is another strategy to avoid rejection. *Doiron* and colleagues designed a lentiviral vector construct expressing the glucokinase gene under control of the cytomegalovirus promoter (36). In this study, insulin-secreting cells could be generated, *in vivo,* from adult pancreatic tissue of a mouse model of partial pancreatectomy. Treated animals presented long-term normalization of glucose tolerance and insulin secretion. Despite technical difficulties still to be circumvented, mainly the use of non-viral vectors, this is an attractive approach to restore organ function on humans. As beta cells would be generated from the patient's own tissue, no immunosuppression would be necessary.

CONCLUSION

Many attempts have been made to modulate or even to reset the immune system in type 1 diabetic individuals, aiming to avoid pancreatic beta cell destruction. However, even high-dose immunosuppression followed by infusion of autologous hematopoietic stem cells was not able to sustainedly restore immune tolerance. Given these observations, new approaches need to be developed. These would include the use of more intense immunosuppressive protocols to preserve beta cell mass, perhaps coupled with replacement of beta cells protected against immune destruction.

AUTHOR CONTRIBUTIONS

CEBC, KCRM, and MCO researched data and wrote the manuscript.

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REVIEW
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The Role of Endothelial Progenitors in the Repair of Vascular Damage in Systemic Sclerosis

Nicoletta Del Papa and Francesca Pignataro*

Department of Rheumatology, ASST G. Pini-CTO, Milano, Italy

Systemic sclerosis (SSc) is a connective tissue disease characterized by a complex pathological process where the main scenario is represented by progressive loss of microvascular bed, with the consequent progressive fibrotic changes in involved organ and tissues. Although most aspects of vascular injury in scleroderma are poorly understood, recent data suggest that the scleroderma impairment of neovascularization could be related to both angiogenesis and vasculogenesis failure. Particularly, compensatory angiogenesis does not occur normally in spite of an important increase in many angiogenic factors either in SSc skin or serum. Besides insufficient angiogenesis, the contribution of defective vasculogenesis to SSc vasculopathy has been extensively studied. Over the last decades, our understanding of the processes responsible for the formation of new vessels after tissue ischemia has increased. In the past, adult neovascularization was thought to depend mainly on angiogenesis (a process by which new vessels are formed by the proliferation and migration of mature endothelial cells). More recently, increased evidence suggests that stem cells mobilize from the bone marrow into the peripheral blood (PB), differentiate in circulating endothelial progenitors (EPCs), and home to site of ischemia to contribute to *de novo* vessel formation. Significant advances have been made in understanding the biology of EPCs, and molecular mechanisms regulating EPC function. Autologous EPCs now are becoming a novel treatment option for therapeutic vascularization and vascular repair, mainly in ischemic diseases. However, different diseases, such as cardiovascular diseases, diabetes, and peripheral artery ischemia are related to EPC dysfunction. Several studies have shown that EPCs can be detected in the PB of patients with SSc and are impaired in their function. Based on an online literature search (PubMed, EMBASE, and Web of Science, last updated December 2017) using keywords related to "endothelial progenitor cells" and "Systemic Sclerosis," "scleroderma vasculopathy," "angiogenesis," "vasculogenesis," this review gives an overview on the large body of data of current research in this issue, including controversies over the identity and functions of EPCs, their meaning as biomarker of SSc microangiopathy and their clinical potency.

Keywords: systemic sclerosis, endothelial progenitors, stem cells, vasculogenesis, angiogenesis

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Mirko Manetti, Università degli Studi di Firenze, Italy
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****Correspondence:***
Nicoletta Del Papa
nicoletta.delpapa@asst-pini-cto.it

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INTRODUCTION

Systemic sclerosis (SSc) is a chronic connective tissue disease of unknown etiology characterized by immunologic abnormalities, microangiopathy, and excessive deposition of collagen in the skin and different internal organs (1). Clinical and pathologic findings of vascular damage and endothelial activation strongly support the hypothesis that the vascular involvement could be the most important and the primary process in the pathogenesis of scleroderma (2). Morphological changes in the vessels of patients with SSc range from considerable initial derangement with capillary thrombosis, to often abortive reparative neoangiogenesis with abnormal capillary proliferation, and to almost complete loss of vessels with persistent ischemic injury in target tissues (3, 4). Although tissue hypoxia is a strong inducer of neovascularization, new microvessel formation appears to be defective in SSc patients and no evidence exists of an effective replacement of damaged capillaries (5). Recent data suggest that the scleroderma impairment of neovascularization could be related to both angiogenesis and vasculogenesis failure (6). Angiogenesis is defined as the creation of new vessels sprouting out of pre-existing ones, whereas vasculogenesis refers to the *in situ* formation of blood vessels from hemangioblasts or vascular stem/progenitor cells (7, 8). In SSc, serum levels of both pro-angiogenic mediators and powerful inhibitors of angiogenesis are largely alterated, especially in the active phases of the disease. In addition, abnormalities in pro-angiogenic signal transduction pathways have been reported, suggesting an intrinsic impaired response of SSc endothelial cells to the mechanisms of vascular angiogenic repair (9). In this scenario, the endothelial cell apoptosis could be recognized as an additional feature of disturbed angiogenesis (10, 11).

In contrast to angiogenesis, during vasculogenesis the formation of new blood vessels can occur in the absence of pre-existing blood vessels through the recruitment and differentiation of endothelial progenitor cells (EPCs). Interest in EPC biology has been growing continuously since their discovery (12) and now EPCs are regarded as biomarkers in cardiovascular diseases and also potential sources of cell for revascularization strategies, which may include direct cellular transplantation and tissue engineering. Significant advances have been made in understanding the biology of EPCs, and preclinical studies using transplanted EPCs provided promising results in the treatment of ischemic diseases (13). Altogether, in the last decade, these data have given rise to several studies regarding the role of EPCs in SSc vasculopathy. The purpose of this review is to evaluate the relevant scientific literature to determine the current state of knowledge on EPCs in the context of the scleroderma vasculopathy. We conducted an online literature search (PubMed, EMBASE, and Web of Science, last updated December 2017) using keywords related to "endothelial progenitor cells" and "Systemic Sclerosis," "scleroderma vasculopathy," "angiogenesis," "vasculogenesis." Eligible papers were evaluated on four pertinent criteria: (1) SSc study populations and appropriate controls, (2) markers used to define EPC phenotype, (3) methods used for assessing EPC function, and (4) evaluation of the possible correlation between EPC detection and angiogenesis/vasculogenesis processes in SSc.

Herein, we summarize the pertinent findings of these studies and discuss the potential role of EPCs as biomarker of scleroderma microangiopathy, the controversies over the identity and functions of EPCs, and their potential clinical potency.

CHARACTERIZATION AND BIOLOGY OF EPCs

The term "Endothelial Progenitor Cells" (EPCs) should be basically used to refer to populations of cells that are capable of differentiation into mature endothelial cells in vasculogenesis (*de novo* formation of vascular networks) (14). Many studies have attempted to identify cell surface markers that are unique to EPCs and distinguish them from mature endothelial cells. EPCs were identified for the first time in 1997 by Asahara et al. in human peripheral blood (PB) as a subset of hematopoietic cells with vasculogenic properties *in vivo* and *in vitro* (12). They identified these cells as $CD34^+$ (a protein with unknown function expressed in early hematopoietic cells) and KDR^+ [kinase-insert domain containing receptor that encodes for vascular endothelial growth factor receptor 2 (VEGFR2)]. Since $CD34^+$/$VEGFR^+$ cells may also identify circulating mature endothelial cells shed from damaged vessel, subsequent works have included CD133 as the stemness marker of EPCs (15). However, the use of CD133 remains controversial. Case et al. showed that mobilized adult PB $CD34^+/VEGFR2^+/CD133^+$ cells represent an enriched population of $CD45^+$ hematopoietic precursors, which do not differentiate into endothelial cells *in vitro* (16). Other authors suggested VE-cadherin and E-selectin as additional surface markers to identify progenitor cells in a more advanced stage of endothelial maturation (17). Due to this controversial scenario, it is evident that the family of EPCs is characterized by lineage and functional heterogeneities, with a spectrum of phenotypes not yet fully defined.

Another approach to EPC isolation and characterization is represented by the use of defined culturing assays to culture unselected PB mononuclears cells (MNCs). For EPCs isolated by cell culture assays, there is now agreement that two different populations can be identified. Hur et al. (18) plated MNCs on a fibronectin- and/or gelatin-coated plate with VEGF containing medium to obtain two types of EPCs, early and late EPCs. Early EPCs with spindle-shaped morphology showed peak growth at 2–3 weeks, whereas late EPCs, cobblestone-shaped, showed exponential growth at 4–8 weeks. The definition of early and late EPCs, based on their time of appearance in culture, reflects a very different phenotype, one being hematopoietic and the other endothelial, respectively (19). Recently, Medina et al. recommended identifying in early EPCs myeloid angiogenic cells (MACs) to clarify their lineage and function. MACs are defined as cultured cells derived from PB mononuclear cells which are grown under endothelial cell culture conditions. These cells share multiple surface antigens of monocytes (CD45, CD14, CD31) and are negative for CD133, CD146, and Tie2 (20). Most studies suggest that these short-term cultures of MNCs fail to differentiate themselves into functional endothelial cells, but predominantly promote vessel formation by activating resident endothelial cells through paracrine mechanisms (21–23).

Late EPCs are often called out growth EPCs and have a more mature phenotype. These cells lack hematopoietic and myeloid markers and are usually derived from long-term cultures of at least 2–4 weeks *in vitro* (24, 25). They are now commonly named as endothelial colony-forming cells. They generate mature endothelial progeny *in vitro* and it has also been observed that they physically contribute to formation of new capillaries (26, 27).

The exact definition and characterization of EPCs is still an on-going and unresolved issue. EPC populations represent a heterogeneous mix of progenitors, in terms of lineage, proliferative potential, and mechanism of action. Experimental models suggest that there is no evidence for the superiority of one cell type over another, and different signaling pathways co-work in regulating EPC commitment (28). Furthermore, Yoon et al. showed that *in vitro*, the angiogenic capability of the two cell types (early and late EPCs) was augmented by mutual interaction through cytokines and metalloproteinases. In addition, the injection of a mixture of the two cells resulted in a superior neovascularization than that obtained by using any one of the single-cell-types (29).

The characteristics of the *in vivo* environment may influence the fate and function of EPCs. In normal homeostatic conditions, there is a low number of circulating EPC populations in the PB. EPCs reside within a stem cell niche in the bone marrow (BM) and complex mechanisms regulate EPC trafficking from the BM to the bloodstream (30). In reality, mechanisms that trigger the regeneration process of EPCs are not well understood and are still under investigation. Tissue ischemia is believed to be the most powerful physiological stimulus for mobilizing EPCs from the BM to the site of new vessel growth (31, 32). EPC recruitment requires a coordinated sequence of multi-step adhesive and signaling events, including chemoattraction, adhesion, and migration. Once at the site of tissue repair, EPCs contribute to new vessel formation in different ways: direct incorporation into the neovessel wall, differentiation into mature endothelial cells, and production of paracrine signals including growth factors, such as VEGF, stromal derived factor, monocyte chemotactic protein 1, and platelet-derived growth factor. Altogether, these pro-angiogenic molecules might further activate resident endothelial cells toward proliferation and vascular repair (28, 33).

EPCs IN SSc

In spite of the on-going controversy over EPC identity, the clinical significance and therapeutic potential of EPCs in vascular regenerative applications has been extensively studied in the last few decades. The existence of postnatal vasculogenesis, mediated by a population of endothelial progenitors identified in both the BM and PB, represents an important tool to better understand the biological contribution of EPCs in different vascular diseases, including SSc.

Several studies have demonstrated that the number and function of EPCs (both in circulation and BM) may be impaired in some disorders characterized by prolonged chronic endothelial damage, such as chronic coronary artery disease, hypertension, congestive heart disease, and diabetes mellitus (34, 35). It has, therefore, been postulated that impaired vascular repair may contribute to the pathogenesis of these chronic disorders and that a small number of EPCs may also be a risk factor for atherosclerotic plaque instability (36). By contrast, an increased number of EPCs is often observed in patients experiencing a myocardial infarction (37) or acute vascular trauma (38). Several studies have suggested that EPCs have a role in the pathogenesis of different autoimmune diseases (34, 39). On average in rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), studies have found decreased peripheral counts of EPCs with altered function (39). The interpretation of these observations is quite difficult at the moment because of the presence of an intrinsic increased cardiovascular risk related to these inflammatory disorders that alone might be responsible for EPC involvement.

Several studies have shown that EPCs can be detected in the PB of patients with SSc and are impaired in their function (40–44). However, the numbers and functions of EPCs in SSc is still a matter of debate, since conflicting reports have been released. Whereas some studies reported a significant depletion in the count of $CD34^+/CD133^+/VEGFR2^+$ cells in SSc patients using flow-cytometry (40, 45–47), other studies found increased circulating EPC counts, mainly in the early and active stages of the disease (41–43). By contrast, lower EPC counts were associated with long disease duration, higher Medsger's severity scores, and ischemic digital ulcers (41–44). These different findings may be ascribed to difficulties in correctly assessing patients with different scleroderma subtypes, disease duration, and level of disease activity.

The complexity of this scenario is further increased by the identification of a subpopulation of multipotent circulating monocytes able to differentiate along the endothelial lineage and promote neovascularization *in vitro* and *in vivo* (48). Really, the current consensus is that these monocytic cells, termed monocytic pro-angiogenic hematopoietic cells (PHCs) and characterized by the positive expression of CD14, CD45, CD34 and type I collagen, do not give rise to endothelial cells *in vivo*, but can support vascular repair through their rapid recruitment to the site of endothelial injury, local secretion of pro-angiogenic factors, and differentiation into mural cells (49). Increased circulating PHCs have been found in patients with SSc (50, 51) and some correlation with the fibrotic clinical features of the disease was observed (50).

Recent studies showed that a specific T cell population, called angiogenic T cells (Tang) may contribute to the formation of new vessels enhancing endothelial cell proliferation and function. Hur et al. demonstrated that these T cell subtype, characterized by the surface phenotype $CD3^+CD31^+CXCR4^+$, constituted the center of EPC colonies during cultures of human PB mononuclear cells. These Tang were required for colony formation and differentiation of early EPCs, actively participating in postnatal vasculogenesis and vascular repair (52). Clinical studies showed an inverse relationship of Tang with cardiovascular risk factors, even in autoimmune diseases, such as RA, SLE, and ANCA-associated vasculitis (53–55). In SSc, Tang cell counts have been reported to be selectively increased in the PB of SSc patients with severe vascular complications like digital ulcers and there was an inverse correlation between circulating Tang and EPCs (defined as $CD34^+CD133^+VEGFR2^+$). Furthermore,

the presence of circulating Tang cells positively correlated with the levels of pro-angiogenic factors, namely VEGF and MMP-9 (56). Such Tang cell expansion has, therefore, been suggested as a possible ineffective attempt to compensate the decrease in EPC number (56).

Indeed, a growing body of evidence suggests that there is a large heterogeneity of EPC populations, which include both hematopoietic and non-hematopoietic lineages. Thus, different subtypes of circulating progenitor cells with vasculogenic and/or angiogenic potentialities have been identified in patients with SSc (see **Table 1**). However, the methods of characterization and quantification of EPCs are not standardized, and the protocols used to count EPCs vary in the different studies (57). Methodological issues are further increased by the difficulties in reliably identifying EPCs. In view of this complexity, the European League against Rheumatism Scleroderma Trials and Research (EUSTAR) recommendations appear to have a limited application (58).

Additional *in vitro* studies showed that, despite having a normal phenotype, EPCs isolated from SSc patients had an impaired ability to differentiate into mature endothelial cells (40). These functional changes have been further confirmed by the examination of BM aspirated from SSc patients that showed low numbers of EPCs characterized by impaired capacity for endothelial differentiation (59). Interestingly, only the BM EPCs from patients with early disease led to some degree of endothelial differentiation, thus suggesting a probable progressive exhaustion of the pool of the BM resident EPCs during the disease progression. The increased levels of VEGF observed in SSc sera (40, 59–62) and the high expression of VEGF receptor on the surface of BM EPCs generated the intriguing hypothesis of a powerful angiogenic "push" without an appropriate vessel formation in SSc patients. Really, no study has shown a direct evidence of correlation between VEGF levels and circulating EPCs in SSc patients. In addition, recent studies have shown that the "VEGF scenario" is rather more complex in SSc and cannot be simply explained by a general insufficient angiogenic/vasculogenic response to important promoters of new vessel formation. Indeed, the overexpression of the anti-angiogenic splice isoform $VEGF_{165}b$ in the plasma, microvascular endothelial cells dermis, and in the platelets of SSc, adds a new play actor in the set of the mechanisms of scleroderma dysfunctional angiogenesis (63–65).

However, the hypothesis of a direct link between EPCs and pro-angiogenic soluble factors has been further sustained by recent data showing a positive correlation between EPC mobilization and circulating levels of Fractalkine in a context of endothelial activation in SSc patients (66). Fractalkine, the only member of the CX3C chemokine family, has recently been described as an angiogenic chemokine. Previous studies have shown that fractalkine induced endothelial cell migration and proliferation, EPC migration and tube-like structure formation *in vitro*, and stimulated new blood vessel formation *in vivo* even in the context of ischemic diseases (67, 68).

An alternative to the hypothesis that prolonged and continuous endothelial cell recruitment may exhaust the BM reservoir of resident EPCs is that disease-related toxic mechanisms can negatively influence the half-life and mobilization of BM EPCs. Since EPCs share the phenotypic and functional properties of mature endothelial cells (15), it is possible that the same immuno-mediated mechanisms capable of inducing peripheral endothelial injury in SSc (i.e., apoptotic phenomena and/or anti-endothelial activity) might also be found in the BM environment. This hypothesis has been confirmed by the detection of significant titers of anti-endothelial cell antibodies (AECA) in the SSc BM plasma (69). Furthermore, their presence correlated

TABLE 1 | Circulating EPCs detected in systemic sclerosis (SSc).

Surface markers detected by FACS analysis	Alterations in EPC number	Association with SSc clinical features	Reference
$CD34^+/CD133^+/VEGFR2^+$	Decreased	Active digital ulcers and pitting scars	Kuwana et al. (40)
$CD34^+/CD133^+$	Increased in the early phase, decreased in the late phase	None	Del Papa et al. (41)
$CD34^+/CD133^+/VEGFR2^+$	Increased in the early phase, decreased in the late phase	None	Del Papa et al. (59)
$CD34^+/CD133^+$	Increased	European disease activity score	Allanore et al. (42)
$CD34^+/CD133^+$; $CD34^+/CD133^+/VEGFR2^+$ $CD34^+/VEGFR2^+$	Decreased	None	Zhu et al. (45)
$CD34^+/VEGFR2^+$ $CD133^+/VEGFR2^+$	Increased in the early phase, decreased in the late phase	Severity of peripheral vascular manifestations in the early, severe organ involvement in the late	Nevskaya et al. (44)
$CD34^+/VEGFR2^+$ $CD133^+/VEGFR2^+$	Decreased	None	Mok et al. (46)
$Lin^-/7AAD/CD34^+/CD133^+/VEGFR2^+$	Increased	Inverse correlation with Medsger's severity score and Digital Ulcers	Avouac et al. (43)
$CD34^+/CD133^+/VEGFR2^+$ $CD34^+/VEGFR2^+$ $CD133^+/VEGFR2^+$	Decreased	None	Andrigueti et al. (47)
$CD133^+/VEGFR2^+$	Decreased	ND	Patschan et al. (88)

with that of activated and apoptotic progenitors (59, 69). These findings were further confirmed by an *in vitro* assay in which the apoptosis of normal progenitors was induced by the addition of AECA$^+$ purified IgG (69). The role of apoptosis in SSc endothelial impairment, even at progenitor level, has been further elucidated by Zhu et al. (45). They reported an increase rate of apoptosis in EPCs isolated from PB of patients with SSc. Depletion of IgG fraction from SSc sera completely abolished the apoptotic effects, suggesting that EPCs may be destroyed upon their mobilization from the BM by serological factors, may be auto-antibodies, present in the SSc sera. As expected, SSc sera induced apoptosis even in human mature microvascular endothelial cells, although these cells were less susceptible than EPCs to the toxic factors present in the SSc sera (45). Similar to what happens in other cell types, the Akt-FOXO3a–Bim axis is the key pathway implicated in apoptotic process in SSc EPCs (45). In summary, these findings support the hypothesis that AECA may play a pathogenetic role by affecting the BM EPC machinery that should repair the peripheral vascular lesions.

Recently, Shirai et al. proposed Pentraxin 3 (PTX3) might represent another intriguing candidate as a contrasting mediator of EPC-mediated vasculogenesis in SSc (70). The expression of PTX3 is induced by inflammatory cytokines in response to inflammatory stimuli in several mesenchymal and epithelial cell types, particularly endothelial cells and mononuclear phagocytes. Additional properties of PTX3 other than those related to innate immunity and inflammation have been described, such as extracellular matrix deposition (ECM), tissue remodeling, and angiogenesis. PTX3 is involved in a variety of molecular mechanisms leading to vascular damage, and its elevated plasma levels were associated with endothelial dysfunction in different human diseases (71). Elevated plasma level of PTX3 have been found in patients with SSc and correlated with vascular manifestations such as digital ulcers and pulmonary hypertension (PH) (70, 72). Interestingly, EPC counts negatively correlated with circulating PTX3 levels (69). These observations suggest a potential role of PTX3 in regulating vascular homeostasis in SSc. In an experimental model, the exposure to a high concentration of PTX3 inhibited EPC differentiation. Based on these data, PTX3 seems to be an additional contributor to worsening the outcome of vascular EPC-mediated repair in SSc (70).

To further investigate these functional changes in SSc EPCs, the gene expression profiles were investigated by Avouac et al. (73). In this study, a different gene expression profile was observed in EPCs from SSc patients compared to control subjects. Interestingly, many of these genic alterations were associated with a proadhesive, proinflammatory, and activated phenotype. Furthermore, experimental hypoxia conditions modulated the gene expression profile of late-growth EPC-derived endothelial cells (73) showing a further upregulation of genes involved in inflammatory and immune response and a downregulation of HOXA9, a factor necessary for endothelial tube formation during angiogenesis (74) and a key regulator of adult progenitor cell commitment to the endothelial lineage (75).

Further studies on late-outgrowth EPC-derived endothelial cells from SSc patients have revealed that when compared with patient mature dermal microvascular endothelial cells, these EPC-derived cells may already present or not alterations in the expression of key regulators of vascular integrity and angiogenesis, such as epidermal growth factor-like domain 7 (EGFL7) (76),the key VEGF co-receptor neuropilin-1 (NRP-1), and Fli1 transcription factor (77). EGFL7 is an important pro-angiogenic molecule, almost exclusively expressed by and active on endothelial cells and their progenitors. EGFL7 expression is highest when the endothelium is in an active, proliferating state, and is a key actor in controlling vascular patterning and integrity (78). EGFL7 expression in progenitor and mature endothelial cells is deeply downregulated in SSc patients, suggesting its role in the mechanisms of defective vascular repair machinery characteristic of SSc (76). On the contrary, it has been demonstrated that SSc dermal microvascular endothelial cells exhibit impaired expression of NRP-1 due to Fli1 deficiency, while SSc late-outgrowth EPC-derived endothelial cells have a genuine phenotype characterized by normal expression levels of either Fli1 or NRP-1 (77). In another study, gene expression profiling of EPC-derived endothelial cells identified matrix metalloproteinase 10 (MMP10) as a novel candidate gene in SSc-associated PH (79). MMP10 seems to have a predominant role in pathologic conditions related to tissue repair and inflammation (80) and more recently its role in neovascularization has also been proposed (81, 82). Circulating serum proMMP10 concentrations were markedly increased in patients with SSc-associated PH compared to SSc patients without PH and healthy controls. Microarray experiments showed that the MMP10 gene was the top upregulated gene in EPC-derived ECs from patients with SSc-associated PH (79).

Another hypothesis has been made on the mechanisms underlying SSc pathogenesis. Once recruited at sites of vascular damage and after exposition to TGFβ, EPCs might transdifferentiate into myofibroblasts, which are the effector cells ultimately responsible for the severe fibrotic process in SSc. Thus, instead of promoting vasculogenic and angiogenic processes, EPCs and endothelial cells might undergo a phenotypic modification, called EndoMT (83, 84). In this context, recent studies showed the presence of cells in intermediate stages of EndoMT in vessels of lung and dermal tissues of patients with SSc (85–87). Furthermore, the treatment of human normal dermal microvascular endothelial cells with TGFβ and SSc sera induced a myofibroblast morphology and the expression of markers of myofroblasts (a-SMA and type I collagen) with downregulation of endothelial markers (CD31, VE-cadherin) (87). More recently, the occurrence of such a phenotypical change from endothelial cells to myofibroblasts has been further demonstrated in the early circulating EPCs from SSc patients (88).

EPC-BASED THERAPIES FOR SSc

In view of the fact that EPCs are defective in several chronic diseases, including SSc, different therapeutic strategies could be postulated to stimulate the production of EPCs or directly use these cells for vascular repair in these conditions. From a theoretical point of view, stem/progenitor therapies might be superior to pharmacological therapy not only because of their direct vasculogenic properties but also paracrine action related

to the secretion of multiple growth factors known to be effective in promoting angiogenic processes (89).

Pharmacological Approaches to Improve Endothelial Repair Mechanisms

Several pharmacological agents have been shown to impact on the number and function of EPCs in animal models and small clinical studies. Here, we focus on recent data concerning the effects of pharmacological agents in clinical use for the treatment of SSc vasculopathy.

3-Hydroxy-3 methylglutaryl coenzime A reductase inhibitors, or statins, have been developed as lipid lowering drugs, but besides this well-known effect, statins are capable of having anti-inflammatory and immunoregulatory effects (90). In particular, statin therapy improves endothelial function by decreasing platelet aggregation and increasing endothelial-derived nitric oxide production (91). Moreover, statins induce mobilization of EPCs from BM (92, 93), increase their functional activity and, probably, their homing to sites of vascular injury (94). The beneficial effect of statins in the treatment of different vascular diseases led several investigators to propose them as a potential treatment for SSc vasculopathy (95), although only a few studies have evaluated the clinical effect of statins in SSc patients. Two studies measured the impact of statin treatment on EPCs and mature endothelial cells (96, 97). They were both open-label studies in which all the participants received statins, and the EPC counts were made before therapy, upon completion of the trial, and throughout. Kuwana et al. treated 13 SSc patients with atorvastatin 10 mg/day for 12 weeks. The authors observed a significant improvement in peripheral vascular manifestations during the treatment period. Atorvastatin treatment resulted in a 1.7- to 8.0-fold increase in the EPC number from baseline, and the number returned to baseline after treatment with atorvastatin was stopped. Circulating levels of the angiogenic factors VEGF and basic fibroblast growth factor, which are believed to be upregulated in SSc to compensate for the inability EPCs to respond adequately to angiogenic stimuli, were significantly reduced during the atorvastatin treatment. In addition, the circulating levels of soluble vascular cell adhesion molecule-1 and E-selectin, which reflect the status of endothelial activation and injury, decreased (96).

Del Papa et al. compared the effects of simvastatin on EPC mobilization in a hypercholesterolemic group and in 20 normocholesterolemic patients suffering from the limited form of SSc. The therapy significantly increased the number of circulating EPCs in the hypercholesterolemic group, but failed to improve the EPC levels in the SSc patients, mainly in those with long-standing disease. In addition, baseline levels of mature circulating endothelial cells were significantly higher in SSc patients compared with controls and, at the end of the treatment, they were significantly decreased. Regarding other markers of endothelial activation, they found the levels of endothelial activation-related markers decreased in a statistically significant manner in the treated patients (97). The different results observed in the two studies could be partially ascribed to the different statins used and to a different selection of patients enrolled (96, 97). Anyway, the fact that EPC mobilization from BM is always reduced, in comparison with non-scleroderma controls, confirms the hypothesis that impaired vascular repair mediated by EPCs may have a role in the progression of the scleroderma vasculopathy. Different mechanisms may be postulated to explain the failure of EPC recruitment in the BM of SSc patients treated with statins. First, as observed in patients with cardiovascular risk factors, the prolonged request from the peripheral damaged vessels may induce the exhaustion of the BM reservoir of EPCs (36). Second, as other organ and tissues involved in SSc, the involvement of the BM microvascular set could interfere with the processes driving the mobilization of EPCs, including those mediated by statins.

Erythropoietin (EPO) was originally described as a hematopoietic cytokine, regulating proliferation and differentiation of erythroid precursor cells. However, several recent studies have suggested that EPO exerts other important anti-apoptotic and anti-inflammatory effects, beyond the regulation of hematopoiesis. EPO induces mobilization of EPCs from the BM as shown in animal models and humans. Heeschen et al. demonstrated that EPO treatment improved neovascularization in a murine hindlimb ischemia model, and this effect was associated with an increase in the number and proliferation of EPCs (98). These results have been confirmed in humans by the demonstration of a correlation between serum concentration of EPO and number and function of both BM-derived and circulating EPCs in patients with coronary artery disease (99). With regard to SSc vasculopathy, Ferri et al. reported an SSc patient with non-healing cutaneous ulcers successfully treated with recombinant human erythropoietin (rHuEPO). Before rHuEPO treatment, the BM sample from this patient contained reduced numbers of EPCs, which were functionally impaired. After a 6-month rHuEPO cycle, a marked increase in endothelial progenitor markers was seen, along with a significant reduction in their apoptotic rates (100).

Furuya et al. assessed EPC counts in a small group of patients with SSc and alveolitis. They showed that low-dose i.v. cyclophosphamide (CYC) plus corticosteroid, but not corticosteroid alone, increased the EPC levels. Moreover CYC-induced EPC recruitment was less efficient in SSc patients in comparison with those with other connective tissue disease, thus confirming the well-known impaired differentiation potential of EPC in scleroderma (101).

Other EPC mobilizing cytokines are under investigation in cardiovascular diseases (i.e., G-CSF, VEGF) (94). However, their use at present is hampered by the fact that the intrinsic mechanisms, whereby they alter number and function of EPCs, should be determined in more detail.

Transplantation of EPCs

Direct injection of EPCs into circulation or into the injury site (namely ischemic site) is a therapeutic option that has been shown effective and safe in animal models. However, although promising, little is known about the real benefit of EPC transplantation in several clinical trials. The heterogeneity of the definition and characterization of EPCs results in the use of different

cell populations for vascular repair. In this context, interpretation of results from human studies cannot be definitive. In addition, most trials are pilot studies, not controlled and involving small numbers of patients. Furthermore, the rate of homing, incorporation, survival, and long-term follow-up observations are still lacking, with no results regarding the potential cancer risk and/or immunoreactivity. Finally, the consistent evidence of the impairment and reduction of EPCs in diabetes, atherosclerosis, and autoimmune diseases, represents a theoretical obstacle against cell-based therapies with autologous cells. This feature is further evident with regard to SSc, as suggested either by the *in vitro* evidences (40, 58) and the experimental demonstration of an impaired *in vivo* neovascularization capacity of EPCs from SSc patients in SCID mice (102). On the other hand, the improvement of EPC isolation and amplification techniques or the use of heterologous cord blood-derived cells might represent a valid future direction of these strategies.

With regard to SSc, similarly to other vascular diseases, the use of autologous EPCs has been largely suggested as a therapeutic option for ulcer healing and other vascular complications related to scleroderma vasculopathy. Local injections of isolated $CD34^+$ cells obtained from PB after mobilization by G-CSF or isolated from BM, probably including EPCs, have been shown to be effective in inducing a rapid and evident beneficial effect on vascular symptoms and ulcer healing (103).

Adipose tissue has been also proposed as a cell source for therapeutic angiogenesis in ischemic diseases. Adipose tissue is mainly composed of two types of cells: mature adipocyte and their precursors, the so-called stromal vascular fraction (SVF) which contains multipotent mesenchymal stem cells, EPCs, pericytes, and macrophages. The ability of SVF to promote angiogenesis and neovascularization has major implications for diseases characterized by poor vascularization, ischemia, and necrosis. Its application has resulted in neovascular formation when applied to acute myocardial infarction, cosmetic procedures, burn wounds, diabetic foot ulcers, and ischemic muscle (104). Prompted by these results, the simple autologous fat grafting (AFG) and other evolved procedures have been used for the treatment of SSc cutaneous complications, such as perioral changes and hand involvement, including skin fibrosis and ulcers (105–107). The present data do not allow us to attribute the reported clinical benefit common to all the studies to a specific subpopulation of cells or a specific mechanism. However, these studies clearly provide evidence that AFG was able to induce neoangiogenesis in the lip and fingers after treatment as suggested by the significant improvement induced by lipofilling in microvascular patterns at labial and digital capillaroscopy (105, 106). Moreover, the AFG in the perioral area induced a neoproliferation of dermal capillaries and reduced the fibrotic changes with partial restoration of the dermal structure, suggesting that the local tissue improvement observed after AFG occurs *via* a pro-angiogenic process (105). To date, therapeutic mechanisms responsible for angiogenic properties of adipose tissue implantation have not been fully understood. It is likely that both the heterogeneous cellular mixture and growth factors may account for the robust angiogenic and vasculogenic potential confirmed in experimental and human studies (104).

CONCLUSION

Despite the presence of several stimuli that induce the formation of new vessels such as tissue hypoxia and increased levels of VEGF, appropriate vessel repair does not occur in SSc patients. In SSc, EPCs, usually involved in the mechanisms of vascular repair, have been deeply investigated, with consistent findings showing a significant dysfunction and/or altered cell counts in both PB and BM environment.

In this scenario, a possible therapeutic strategy could be the use of drugs able to induce mobilization, homing, and proliferation of EPCs or alternatively the local or systemic use of purified EPCs or their precursors to treat microvascular damage. Pharmacological interventions with statins or growth factors have shown partially positive results. Few but encouraging studies with different tissue sources of progenitors (including EPCs, mesenchymal stem cells, pericytes) have been carried out in a limited number of patients and so their results are far from conclusive (107–110). Adipose tissue is now under investigation as an alternative source of pro-angiogenic stem cells and pilot studies have demonstrated that these cells may be useful in improving scleroderma-related fibrotic and vascular complications when grafted locally.

AUTHOR CONTRIBUTIONS

NNDP designed the review organization and wrote it, based on her expertise in the field. FP helped in revising the literature on the subject and writing the manuscript.

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Prostaglandin E2 Stimulates the Expansion of Regulatory Hematopoietic Stem and Progenitor Cells in Type 1 Diabetes

*Moufida Ben Nasr[1,2†], Francesca D'Addio[2†], Amir Mohammad Malvandi[2], Silvia Faravelli[2], Eduardo Castillo-Leon[1], Vera Usuelli[1,2], Francesca Rocchio[2], Teresa Letizia[2], Abdel Basset El Essawy[3], Emma Assi[2], Chiara Mameli[4,5,6], Elisa Giani[4,5,6], Maddalena Macedoni[7], Anna Maestroni[2], Alice Dassano[2], Cristian Loretelli[2], Moira Paroni[8], Giuseppe Cannalire[9], Giacomo Biasucci[9], Marco Sala[10], Alessandra Biffi[11,12], Gian Vincenzo Zuccotti[2,4,5,6] and Paolo Fiorina[1,2,13]**

[1] Nephrology Division, Boston Children's Hospital, Harvard Medical School, Boston, MA, United States, [2] International Center for T1D, Pediatric Clinical Research Center Fondazione Romeo ed Enrica Invernizzi, Department of Biomedical and Clinical Science L. Sacco, University of Milan, Milan, Italy, [3] Medicine, Al-Azhar University, Cairo, Egypt, [4] Department of Pediatrics, Buzzi Children Hospital, Milan, Italy, [5] Pediatric Clinical Research Center Fondazione Romeo ed Enrica Invernizzi, Department of Biomedical and Clinical Science L. Sacco, University of Milan, Milan, Italy, [6] Department of Pediatrics, Children's Hospital Buzzi, Milan, Italy, [7] Department of Pediatrics, Diabetes Service Studies, University of Milan, Ospedale dei Bambini Vittore Buzzi, Milan, Italy, [8] Department of Bioscience, University of Milan, Milan, Italy, [9] Department of Pediatrics and Neonatology, Ospedale Guglielmo da Saliceto, Piacenza, Italy, [10] Department of Pediatrics, Tradate Hospital, Tradate, Italy, [11] Gene Therapy Program, Dana-Farber/Boston Children's Cancer and Blood Disorders Center, Boston, MA, United States, [12] Harvard Medical School, Boston, MA, United States, [13] Division of Endocrinology, ASST Sacco Fatebenefratelli-Sacco, Milan, Italy

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Kelen Cristina Ribeiro Malmegrim, Universidade de São Paulo, Brazil

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Adriane Feijo Evangelista, Barretos Cancer Hospital, Brazil
Daniela Carlos, Universidade de São Paulo, Brazil

***Correspondence:**
Paolo Fiorina
paolo.fiorina@childrens.harvard.edu

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

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Hematopoietic stem and progenitor cells (HSPCs) are multipotent stem cells that have been harnessed as a curative therapy for patients with hematological malignancies. Notably, the discovery that HSPCs are endowed with immunoregulatory properties suggests that HSPC-based therapeutic approaches may be used to treat autoimmune diseases. Indeed, infusion with HSPCs has shown promising results in the treatment of type 1 diabetes (T1D) and remains the only "experimental therapy" that has achieved a satisfactory rate of remission (nearly 60%) in T1D. Patients with newly diagnosed T1D have been successfully reverted to normoglycemia by administration of autologous HSPCs in association with a non-myeloablative immunosuppressive regimen. However, this approach is hampered by a high incidence of adverse effects linked to immunosuppression. Herein, we report that while the use of autologous HSPCs is capable of improving C-peptide production in patients with T1D, *ex vivo* modulation of HSPCs with prostaglandins (PGs) increases their immunoregulatory properties by upregulating expression of the immune checkpoint-signaling molecule PD-L1. Surprisingly, CXCR4 was upregulated as well, which could enhance HSPC trafficking toward the inflamed pancreatic zone. When tested in murine and human *in vitro* autoimmune assays, PG-modulated HSPCs were shown to abrogate the autoreactive T cell response. The use of PG-modulated HSPCs may thus provide an attractive and novel treatment of autoimmune diabetes.

Keywords: hematopoietic stem and progenitor cells, prostaglandins, autoimmune diseases, PD-L1, CXCR4

INTRODUCTION

Encouraging results of previous pilot trials suggest that autologous hematopoietic stem and progenitor cell transplantation (AHSCT) may be a relevant alternative therapeutic option to immunosuppressive drugs in the treatment of several refractory autoimmune disorders (1, 2). Over 3,000 transplants using AHSCT have been performed worldwide with a very high safety profile (2, 3). We recently demonstrated that AHSCT could induce long-term, drug free, and symptoms-free remission in patients newly diagnosed with type 1 diabetes (T1D). Insulin independence was achieved in nearly 60% of treated subjects at 6 months, with 40% showing sustained insulin-free remission over 4 years following the procedure (4). The aim behind the use of AHSCT is to suppress autoreactive immune cells, while allowing for *de novo* generation of a naïve immune compartment tolerant to pancreatic β cells antigens (5), thus preventing T cell infiltration into targeted organs (6). AHSCT trials showed that in treated patients, an overall resetting of the immune system toward a "regulatory"-like T cell landscape was evident, with an increase in $CD4^+Foxp3^+$ Tregs (7). Unfortunately, the use of immunosuppression during AHSCT limits the potential use of this therapy in T1D to experimental conditions, due to patients' potential exposure to adverse effects. Interestingly, the immunoregulatory properties of hematopoietic stem and progenitor cells (HSPCs) seem to be linked to their expression of the immune checkpoint-signaling molecule PD-L1 (or CD274) (8, 9). They further express CXCR4, which allows HSPCs to traffic to inflamed area/sites of injuries (10). Unlike mesenchymal or embryonic stem cells, which are associated with the potential development of tumorogenesis and formation of ectopic tissue (5, 11–13), HSPCs have been safely used for years (14–16). Several studies suggested that prostaglandin E2 (PGE2) might have anti-inflammatory effects through inhibition of several pro-inflammatory cytokines (17). Other investigators have demonstrated that the endogenous anti-inflammatory role of PGE2 is mainly mediated through it receptor EP4, thereby inhibiting macrophage derived pro-inflammatory chemokines production during atherogenesis (18, 19). While others have mainly studied in depth the mechanism by which PGE2 can control inflammation and demonstrated that PGE2 plays its regulatory role by limiting T cell activation thereby impairing T cell arrest and inhibiting T cells interactions with dendritic cells (DCs) (20). Previous reports have introduced and identified prostaglandins (PGs) as potentials HSPCs enhancing candidates capable of inducing/improving their long-term maintenance and engraftment faculties (21). We hypothesize that enhancing the immunoregulatory properties of HSPCs using pharmacological modulation with small molecules may create a novel powerful immunoregulatory tool for the treatment of T1D.

MATERIALS AND METHODS

Human Studies

Study Population Included in the AHSCT Clinical Trial

Two cohorts consisting of 36 T1D patients were enrolled in the AHSCT program and were also enrolled in three independent clinical trials as previously described (6). Autoantibodies were analyzed on serum by RIA (for insulin autoantibodies) and ELISA (for insulinoma-2-associated autoantibodies, glutamic acid decarboxylase autoantibodies, and Znt8) according to the standard of care clinical procedure. The study was performed in accordance with Institutional Review Board committee approval of each participant Institution, informed consent was provided by all individuals. All baseline demographic and clinical characteristics of the study population are reported in **Table 1**.

Study Population Included in the PG-Library Screening

Blood samples were obtained from long lasting T1D patients ($n = 24$) and healthy controls (CTRL) ($n = 5$) in accordance with Institutional Review Board committee approval of San Raffaele Hospital and of Boston children's Hospital (BCH 3851); informed consent was provided by all individuals included in the present study. Baseline characteristics of the study population are summarized in **Table 2**. Peripheral blood mononuclear cells (PBMCs) isolated from 20 ml blood samples using Lymphoprep (Stem Cell Technologies, Cambridge, MA, USA) were frozen in freezing

TABLE 1 | Baseline demographic and clinical characteristics of patients with T1D treated with autologous non-myeloablative hematopoietic stem cell transplantation in two AHCST cohorts.

Patient characteristics	
Number of patients included	$n = 36$
Age (years ± SEM)	22.4 ± 0.9
Gender (M/F)	27/9
BMI (kg/m^2 ± SEM)	20.7 ± 0.5
HbA1c (mmol/mol ± SEM)	86.6 ± 6.4
C-peptide (ng/ml ± SEM)	0.73 ± 0.06
Autoantibodies	(% of patients)
GAD	86
Other (IAA, IA-2A, ICA)	17
DKA or DK history	(% of patients)
No DKA/DK	67
DKA	28
DK	5

T1D, type 1 diabetes; AHSCT, autologous hematopoietic stem and progenitor cell transplantation; BMI, body mass index; GAD, glutamic acid decarboxylase autoantibodies; ICA, islet cell cytoplasmic autoantibodies; IA-2A, insulinoma-2-associated autoantibodies; IAA, insulin autoantibodies; DKA, diabetic ketoacidosis; DK, diabetic ketosis.

TABLE 2 | Clinical characteristics of patients with T1D and of healthy controls included in the PGs library screening.

Patient characteristics	
Number of patients included	$n = 24$
Age (years ± SEM)	58.2 ± 11.6
Gender (M/F)	16/8
BMI (kg/m^2 ± SEM)	20.7 ± 0.5
EIR (UI)	18.3 ± 5.4
Concomitant treatment	Levothyroxine ($n = 8$), statin ($n = 5$)
Healthy control characteristics	
Number of individuals included	$n = 5$
Age (years ± SEM)	40.8 ± 6.4
Gender (M/F)	2/3

T1D, type 1 diabetes; BMI, body mass index; EIR, exogenous insulin requirement units; PGs, prostaglandins.

medium (RPMI 1640 20% FBS and 8% DMSO) and stored at −80°C. After thawing, PBMCs were recovered in culturing medium consisting of RPMI 1640 (Life Technologies, Carlsbad, CA, USA) supplemented with 10% FBS, 2 mM L-glutamine (Life Technologies), 100 U/ml penicillin (Life Technologies), for 48 h, and $CD34^+$ cells were then isolated using a CD34 Positive Isolation Kit (Miltenyi Biotec, San Diego, CA, USA) according to the manufacturer's instructions.

Pharmacological Modulation of Human $CD34^+$ Cells

1×10^5 of isolated human $CD34^+$ HSPCs (purity 99%) were cultured in 200 µl of StemSpan SFEM II media (SEMCELL Technologies Inc., Cambridge, MA, USA), and each compound in the Prostaglandin Screening Library II (Cayman Chemicals, Ann Arbor, MI, USA), the detailed composition of the library is shown in Table S1 in Supplementary Material, was added individually at day 0 and at day 1 at a concentration of 10 µM as previously reported by our group and others (9, 21). In another assay, isolated $CD34^+$ cells from freshly isolated human PBMCs or from cryopreserved PBMCs, and processed as described earlier, were cultured in the presence of a cocktail of cytokines containing: 10 µg/ml heparin (SEMCELL Technologies Inc., Cambridge, MA, USA), 10 ng/ml human stem cell factor (SCF) (Miltenyi Biotec, San Diego, CA, USA), 20 ng/ml human thrombopoietin (TPO) (Miltenyi Biotec, San Diego, CA, USA), 10 ng/ml human FGF-1 (Miltenyi Biotec, San Diego, CA, USA), 100 ng/ml insulin-like growth factor-binding protein 2 (IGFBP2) (R&D Systems, Inc., Minneapolis, MN, USA), and 500 ng/ml angiopoietin-like 3 (R&D Systems, Inc., Minneapolis, MN, USA). PGE2 (PromoKine, PromoCell Gmbh, Germany) was added by pulsing the culture at 0, 24, 72 h and 6 days with 2 µl of diluted PGE2 (10 µM). Cells were cultured for 7 days at 37°C in 5% CO_2, and $CD34^+$ cells were then subjected to FACS analysis and were run on a FACSCelesta™ (Becton Dickinson, Franklin Lakes, NJ, USA). Data were analyzed using FlowJo software version 8.7.3 (Treestar, Ashland, OR, USA). The different cytokines used here and their related concentration as well as the choice of the incubation timing was used as previously reported in the literature (22).

Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

RNA was extracted from $CD34^+$ cells using Direct-zol™ RNA Kits (Zymo, Irvine, CA, USA) and Trizol Reagent (Invitrogen Carlsbad, CA, USA), RNA quality was assessed by Multiskan™ GO Microplate spectrophotometer and the ratios of absorbance at 260 and 280 nm were assessed for all the samples. Only samples with RNA ratios within 1.9 were included in the present study. cDNA synthesis was made from purified total RNA by reverse transcription using High capacity cDNA Reverse Transcription RETROscript® Kit (Thermo Fisher Scientific, Waltham, MA, USA) followed by a pre-amplification using Taqman PreAmp Kit (Applied Biosystems) according to the manufacturer's instructions. qRT-PCR analysis was performed using TaqMan assays (Life Technologies, Grand Island, NY, USA) containing PCR primers and TaqMan probes according to the manufacturer's instructions. Normalized expression values were determined using the ΔCt method. qRT-PCR data were normalized for the expression of GAPDH. qRT-PCR reactions were performed in triplicate in a 96-well format using an Applied Biosystems 7900HT fast real-time PCR instrument. Relative expression was calculated using the comparative threshold cycle method as previously described (23, 24). For two-groupcomparisons, a Student's *t* test was employed. Reported below are the main characteristics of the primers used:

Gene symbol	Assay ID	Refseq accession #	Band size (bp)	Reference position
CD274 (PD-L1)	Hs01125299_m1	NM_ 001267706.1	89	441
CD184 (CXCR4)	Hs00237052_m1	NM_ 001008540.1	153	973
IDO1	Hs00984148_m1	NM_ 0022164.5	66	651
GAPDH	Hs99999905_m1	NM_001289746.1	122	229

Human ELISPOT Assay

An ELISPOT assay was used to measure the number of IFN-γ-producing cells according to the manufacturer's protocol (BD Biosciences, San Jose, CA, USA) as previously shown by our group (2). 1×10^6 PBMCs isolated from T1D patients were cultured for 2 days in the presence of IA-2 peptide (Thermo Fisher Scientific Gmbh, Germany) (100 µg/ml) in RPMI medium supplemented with 10% FBS. At 24 h after stimulation, 500 µl of medium was added to the culture. Cells were collected at day 2 and added to plates coated with anti-IFN-γ antibody (eBioscience, Thermo Fisher Scientific, Waltham, MA, USA) with or without PGE2-modulated $CD34^+$ cells at ratios of 1:2 or 1:10 or 1:32 in RPMI medium supplemented with 10% FBS. Spots were counted using an A.EL.VIS Elispot Reader (A.EL.VIS GmbH, Hannover, Germany) or on an Immunospot Reader (C.T.L. Cellular Technology Ltd., Cleveland, OH, USA).

Immunofluorescence and Confocal Microscopy

Regulatory $CD34^+$ (PGE2-modulated) cells and unmodulated $CD34^+$ cells isolated from peripheral blood of healthy controls were fixed in 4% PFA for 1 h at 4 C, washed three times for 20 min in PBS, and cells were counterstained with blue fluorescent DAPI (1:10,000, BioLegend, San Diego, CA, USA) and anti-human PD-L1 (BD Biosciences). Cells were photographed under a 63× objective. Images were captured on a Leica SP5X system with an upright DM6000 microscope and A1R confocal microscope (Nikon Instruments, Melville, NY, USA). Histology was evaluated by at least two expert pathologist (9).

Migration Assay

Transwell migration assays were performed on PGE2-modulated HSPCs compared to unmodulated HSPCs in the presence of 0–50 ng/ml SDF-1 (R&D Systems, Minneapolis, MN, USA). In brief, cells were suspended in 0.5% BSA Phenol Red-Free RPMI and plated in the upper chambers of an HTS-Transwell-96-well permeable support plate (Corning, Acton, MA, USA) and incubated at 37°C in 5% CO_2 for 2 h. After 2 h incubation, migrated cells were counted using BD TruCount (BD Biosciences) by flow cytometry.

Murine Studies

Mice

Female NOD/ShiLtJ (NOD) or non-obese diabetic mice (NOD) which is the commonly used model for autoimmune T1D studies, NOD.FVB-Tg (CAG-luc,-GFP)L2G85Chco/FathJ (Luciferase NOD) mice which exhibit a widespread expression of the two cell tracers eGFP and firefly luciferase directed by the CAG promoter allowing thus an easily tracking of the cells and NOD. CgTg (TcraBDC2.5,TcrbBDC2)1Doi/DoiJ (BDC2.5 NOD) mice which has the particularity to carry a rearranged TCR a and eight genes from a diabetogenic T cell clone, BDC2.5 and is commonly used *in vitro* autoimmune assays; were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All mice were housed under specific pathogen-free conditions at an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility at BCH. Institutional guidelines and protocols were approved and adhered to the Institutional Animal Care and Use Committee.

Murine Regulatory KL Cell Modulation

Murine bone marrow KL (Lineage$^-$c-Kit$^+$) cells were isolated using magnetic beads and MACS® separation columns (Miltenyi Biotec, San Diego, CA, USA) and ~2 × 10^5 cells were plated in a U-bottomed 96-well plate with 200 µl of stem cell medium, Stemspan-SFEMII (STEMCELL Technologies, Cambridge, MA, USA) and PGE2 (PromoKine, PromoCell Gmbh, Germany) was added at day 0 and day 1, at a concentration of 10 µM.

Flow Cytometric Analysis and Intracellular Cytokine Staining

Flow cytometry was performed to analyze surface expression markers of PGE2-modulated HSPCs and dmPGE2 (16, 16-dimethyl PGE2)-modulated HSPCs. Anti-mouse PD-L1, PD-L2, PD-1, CD40, CD80, CD86, CD4, CD8, Ly-6G (Gr-1), B220, CD3, CXCR4, CCR2, CCR4, CCR5, CCR6, CCR7, CCR8, CXCR3, IL-4, IL-10, and IFN-γ were purchased from BD Biosciences, eBioscience (San Diego, CA, USA) and BioLegend. The following antibodies corresponded to isotype controls for the murine antibodies above: PE mouse IgG1, κ isotype ctrl, Armenian hamster IgG; APC mouse IgG2b, κ isotype ctrl, Armenian hamster IgG. Cells were subjected to FACS analysis and were run on a FACSCalibur™ (Becton Dickinson). Data were analyzed using FlowJo software version 8.7.3 (Treestar).

Intracellular Staining for Flow Cytometry

Naïve CD4$^+$CD25$^-$ T cells (5 × 10^5) were isolated from BDC2.5 TCR tg mice with a negative selection strategy using a CD4$^+$ CD25$^+$ Regulatory T cell isolation kit (Miltenyi Biotec) and were stimulated with BDC2.5 peptides and CD11c$^+$ DCs (2.5 × 10^5) previously isolated using CD11c$^+$ mAb-coated microbeads. DCs were added at a 1:2 ratio to T cells and were cocultured with PGE2-modulated KL cells (PGE2-KL) at ratios of 1:1, 5:1, and 10:1 (ratio of T cells to PGE2-KL) or alone (controls) or with untransduced KL cells for 24 h in RPMI 10% FBS in a humidified incubator at 37°C, 5% CO_2. After incubation, cells were collected, washed, and plated in RPMI 10% FBS, then stimulated with 50 ng/ml PMA (Sigma Aldrich, St. Louis, MO, USA), 750 ng/ml ionomycin (Sigma Aldrich) and the protein transport inhibitor BD GolgiStop (6 µl per 6 ml of RPMI as recommended by the manufacturer, BD Biosciences) for 5 h in a humidified incubator at 37°C, 5% CO_2. After incubation, cells were collected, washed, stained for surface marker CD4 APC, followed by washing and permeabilization using the BD Cytofix/Cytoperm Kit (BD Biosciences) and staining with anti-mouse IFN-γ (eBioscience). Finally, CD4$^+$ IFN-γ$^+$ cells were assessed by flow cytometric analysis.

Pancreas Digestion and Preparation for Flow Cytometry

Pancreata were collected in ice-cold IMDM medium, cut into small pieces, and digested with Collagenase D for 1 h at 37°C, with DNase I added after 30 min. Digested pancreata were passed through a 70-µm cell strainer to obtain single cell suspensions and then analyzed by flow cytometry. For tracking GFP$^+$ cells, biotinylated anti-GFP (BD Biosciences) was used at 20 µg/ml followed by staining with APC-conjugated streptavidin (BD Biosciences).

Statistical Analysis

Statistical analysis was performed using an unpaired Student's *t* test. A two-sided value of $P \leq 0.05$ was considered statistically significant. All graphs were generated using GraphPad Prism software version 5.0b (GraphPad Software, Inc., La Jolla, CA, USA). All statistical tests were performed at the 5% significance level.

RESULTS

AHSCT Improves β Cell Function in Treated T1D Patients

Two cohorts consisting of 36 T1D patients were enrolled in the AHSCT program and were also enrolled in three independent clinical trials as previously described (6). All baseline demographic and clinical characteristics of the study population are reported in **Table 1**. The patient group was predominantly male (27 males and 9 females) with a mean age of 22.4 years and a short history of disease duration (within 6 weeks of diagnosis), confirmed by the presence of autoantibodies to islet peptides [glutamic acid decarboxylase antibodies (anti-GAD) were detected in 86% of patients, while other autoantibodies were detected in 17% of patients]. Most of the patients studied (67%) had no previous history of diabetic ketoacidosis/ketosis. The mean body mass index of patients at diagnosis was 20.7 ± 0.5 (kg/m^2 ± SEM), and their mean glycated hemoglobin of (HbA1c) was 86.6 ± 6.4 (mmol/mol ± SEM). All patients underwent a stem cell mobilization protocol as previously described (6) with cyclophosphamide (2 g/m^2) and granulocyte colony-stimulating factor (5–10 µg/kg) daily, beginning the day after cyclophosphamide administration (6). A mean dose of 5.8 ± 0.8 × 10^6/kg cryopreserved CD34$^+$ cells was administered as a single infusion at day 0 (6). All patients showed improvement in β cell function, as revealed by an increase in C-peptide levels over time, which reached a persistent and

stable median value >2.5 ng/ml at 12 months of follow-up and lasted until 24 months after treatment (**Figure 1A**). Interestingly, compared to pre-AHSCT treatment levels, 2 h postprandial peak-stimulated C-peptide levels increased significantly at 6 months post-AHSCT treatment and reached ~2.7 ng/ml at 24 months of follow-up (**Figure 1A**). Furthermore, a significant positive correlation, albeit weak, was found between the number of $CD34^+$ cells infused and C-peptide levels at 6 months after treatment (**Figure 1B**). Statistical analysis revealed a significant negative correlation between the number of $CD34^+$ cells injected during AHSCT and exogenous insulin requirement units evaluated at 8 months (**Figure 1C**) and 12 months (**Figure 1D**) of follow-up. T1D patients treated with autologous hematopoietic stem cell transplantation showed an overall improvement in glycometabolic control and maintenance of β cell function.

PG Library Screening

Prostaglandin E2 has been described as a small molecule known to enhance the homing and engraftment of HSPCs. We therefore sought to screen all known types of PGs using the Prostaglandin Screening Library II, which contains 64 small molecules. We first screened each small molecule contained in the library for its capacity to upregulate PD-L1 in human $CD34^+$ cells isolated from T1D patients (**Figures 2A,B**). $CD34^+$ cells isolated from T1D patients were cultured in StemSpan SFEMII and pulsed with each PG small molecule contained in the aforementioned library at a concentration of 10 μM at 24 and 48 h. Using FACS analysis, we calculated the mean fluorescence intensity of PD-L1 expression following treatment with each small molecule used to modulate $CD34^+$ cells as compared to untreated/unmodulated $CD34^+$ cells (**Figure 2B**). We generated a heat map depicting the degree to which each PG small molecule affected PD-L1 expression on $CD34^+$ cells (**Figure 2B**), thus allowing us to evaluate PG candidates. Based on its ability to modulate PD-L1 expression, we selected PGE2 as a candidate for further study; in addition, PGE2 has been described in the literature and has been tested in clinical trials as a potential therapy for enhancing HSPC engraftment following cord blood transplantation (21). Isolated $CD34^+$ cells from healthy control patients (CTRL) and from T1D patients were cultured in StemSpan SFEMII and pulsed with 10 μM of PGE2 at 24 and 48 h. We first tested the effect of pharmacological modulation with PGE2 by FACS analysis, and our data revealed a slight increase in PD-L1 expression, although not significant, in cultured $CD34^+$ cells from T1D and healthy

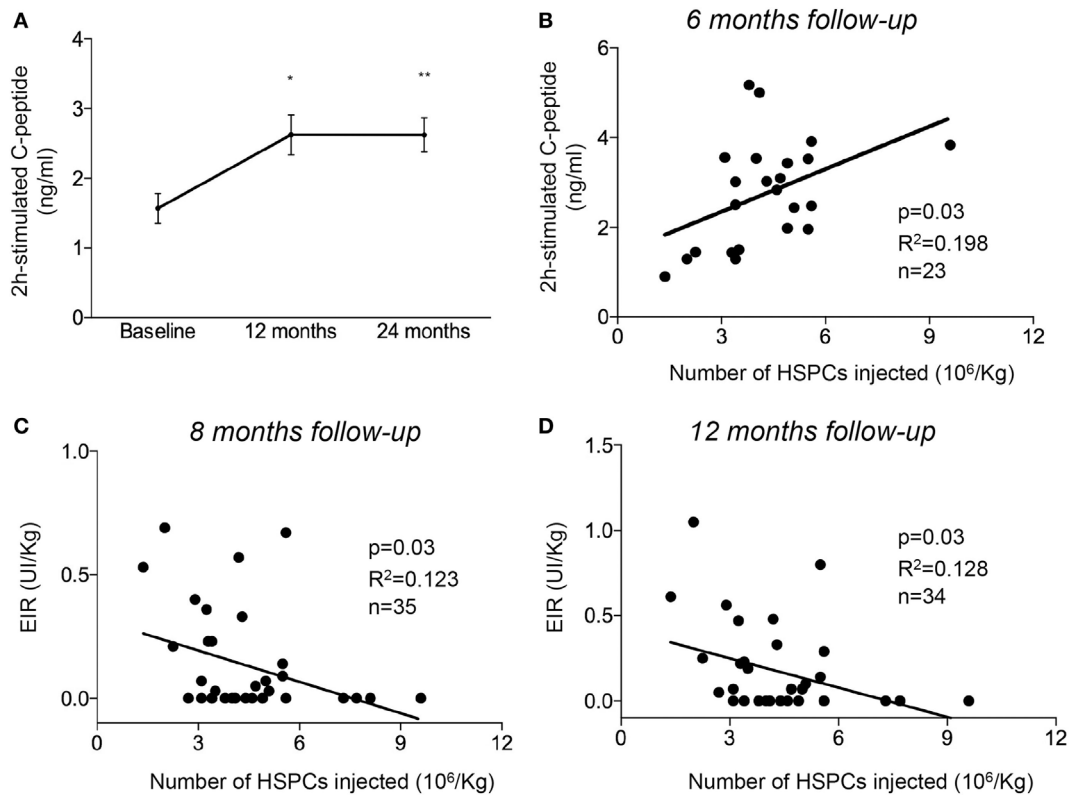

FIGURE 1 | Non-myeloablative AHSCT preserved pancreatic β cell function in patients newly diagnosed with T1D. **(A)** Line graph showing 2 h peak-stimulated C-peptide levels after a mixed meal tolerance test following AHSCT, at 12 and 24 months of follow-up. **(B)** Correlation between the number of HSPCs injected during AHSCT and 2 h peak-stimulated C-peptide levels evaluated at 6 months of follow-up. **(C,D)** Correlation between the number of hematopoietic stem cells (HSPCs) injected during AHSCT and EIR evaluated at 8 months **(C)** and 12 months **(D)** of follow-up. All data are expressed as mean ± SEM. All parameters examined were statistically significantly different when comparing baseline values vs. those at 6, 8, and 12 months. $^*P < 0.05$, $^{**}P < 0.001$. Abbreviations: AHSCT, autologous hematopoietic stem and progenitor cell transplantation; UI, units of insulin; EIR, exogenous insulin requirement units; HSPCs, hematopoietic stem and progenitor cells; T1D, type 1 diabetes.

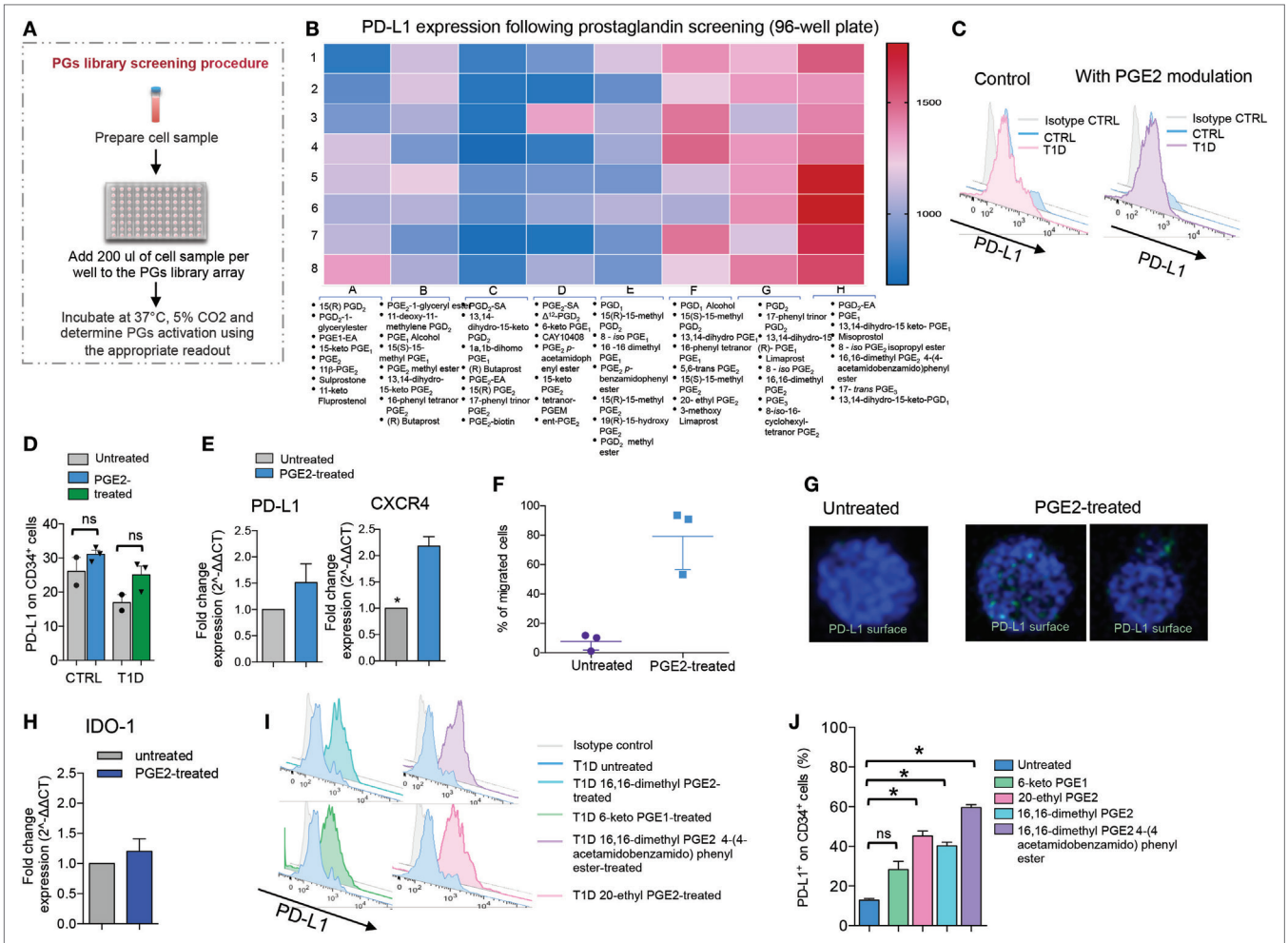

FIGURE 2 | Prostaglandins (PGs) enhance PD-L1 expression on human CD34⁺ cells. **(A,B)** Results of screening of a PG small molecule library tested for the ability to upregulate PD-L1 (MFI) on CD34⁺ cells obtained from T1D patients. The schematic of the experimental design is shown in **(A)**. The 3-color coding shown in **(B)** represents lowest PD-L1 MFI values (blue), median PD-L1 MFI values (pink), and highest PD-L1 MFI values (red). **(C,D)** Representative flow cytometric analysis and quantitative bar graph of PD-L1 expression on CD34⁺ cells from T1D patients pre- and post-pharmacologic modulation with PGE2 as compared to CD34⁺ cells obtained from healthy controls. **(E)** PD-L1 and CXCR4 expression (mRNA) fold change was quantified for CD34⁺ cells pre- and post-modulation with PGE2. **(F)** Migration assay using CD34⁺ cells pre- and post-modulation with PGE2. **(G)** Confocal imaging of CD34⁺ cells pre- and post-modulation with PGE2, showing DAPI (in blue) and PD-L1 (in green) staining. 63× magnification. Scale bar, 50 μm. **(H)** IDO-1 expression (mRNA) fold change was quantified for CD34⁺ cells pre- and post-modulation with PGE2. **(I,J)** Representative flow cytometric analysis **(I)** and quantitative bar graph **(J)** of PD-L1 expression on CD34⁺ cells from T1D patients pre- and post-pharmacologic modulation with four small molecule PGE2 agonists. All data are expressed as mean ± SEM. *$P < 0.05$, **$P < 0.001$. Abbreviations: MFI, mean fluorescence intensity; T1D, type 1 diabetes; PGE2, prostaglandin E2.

control patients, with the latter showing a higher percentage of PD-L1 expression as compared to cells from T1D patients (**Figures 2C,D**). This pattern was further confirmed by confocal imaging, in which PD-L1 surface expression was upregulated in PGE2-treated CD34⁺ cells as compared to untreated (**Figure 2G**). Similar results were obtained by RT-PCR, which demonstrated a slight increase (although not significant) in PD-L1 expression in CD34⁺ cells upon modulation with PGE2 (**Figure 2E**). Notably, RT-PCR showed a twofold increase of CXCR4 gene expression, a protein required for the homing of HSPCs, in PGE2-treated CD34⁺ cells as compared to untreated (**Figure 2E**). We therefore sought to perform a migration assay in order to assess the homing properties of PGE2-treated CD34⁺ cells (**Figure 2F**). Our data confirmed a substantial increase in the homing potential of PGE2-treated CD34⁺ cells (**Figure 2F**). The expression of another relevant immunoregulatory protein, IDO-1, remained unchanged post-pharmacologic modulation with PGE2 (**Figure 2H**). We next selected the four PG small molecules [16,16-dimethyl PGE2, 16,16-dimethyl PGE2 4-(4-acetamidobenzamido) phenyl ester, 6-keto PGE1 and 20-ethyl PGE2] that showed the strongest capacity to upregulate PD-L1, based on the results obtained from library screening (**Figures 2I,J**). By a PGE2-analog and two competitive inhibitors of 15-hydroxy PG dehydrogenase, which possess a prolonged half-life *in vivo* [16,16-dimethyl PGE2, 16,16-dimethyl PGE2 4-(4-acetamidobenzamido) phenyl ester and 20-ethyl PGE2]. FACS analysis of PD-L1 protein expression following pharmacologic modulation with these four PGs (**Figures 2I,J**) demonstrated robust upregulation of PD-L1

expression. These results were further confirmed by RT-PCR, which showed a marked upregulation of PD-L1 mRNA following pharmacological modulation with 16,16-dimethyl PGE2, 16,16-dimethyl PGE2 4-(4-acetamidobenzamido) phenyl ester and 20-ethyl PGE2 (data not shown).

PGE2 Highly Augments PD-L1 Expression in Human HSPCs When Supplemented With Hematopoietic Cytokines

In order to improve the strategy used for HSPC expansion and to enhance the function of PGE2-modulated HSPC, we added hematopoietic cytokines (SCF, TPO, FGF-1, IGFBP-2, and Angptl-3 proteins) known as a potent cocktail for HSPC maintenance, into our established culture conditions (22). Isolated CD34$^+$ cells (HSPCs) obtained from T1D patients and from healthy controls were cultured using StemSpan SFEMII supplemented with the aforementioned human stem cell growth factors (STFIA medium) and pulsed with PGE2 (10 μM) at 24, 96 h and at 7 days at 37°C 5% CO_2. PD-L1$^+$ HSPCs were then quantified by FACS analysis at different time points post-culture. After 7 days, a ~5-fold increase in the percentage of PD-L1$^+$CD34$^+$ cells was evident in human HSPCs obtained from T1D, with a similar albeit much less pronounced increase in the percentage of PD-L1$^+$CD34$^+$ cells obtained from healthy control patients (~2-fold increase) (**Figures 3A–E**). We next determined whether freezing/cryopreservation has any effect on PD-L1 expression, by comparing freshly isolated HSPCs with frozen

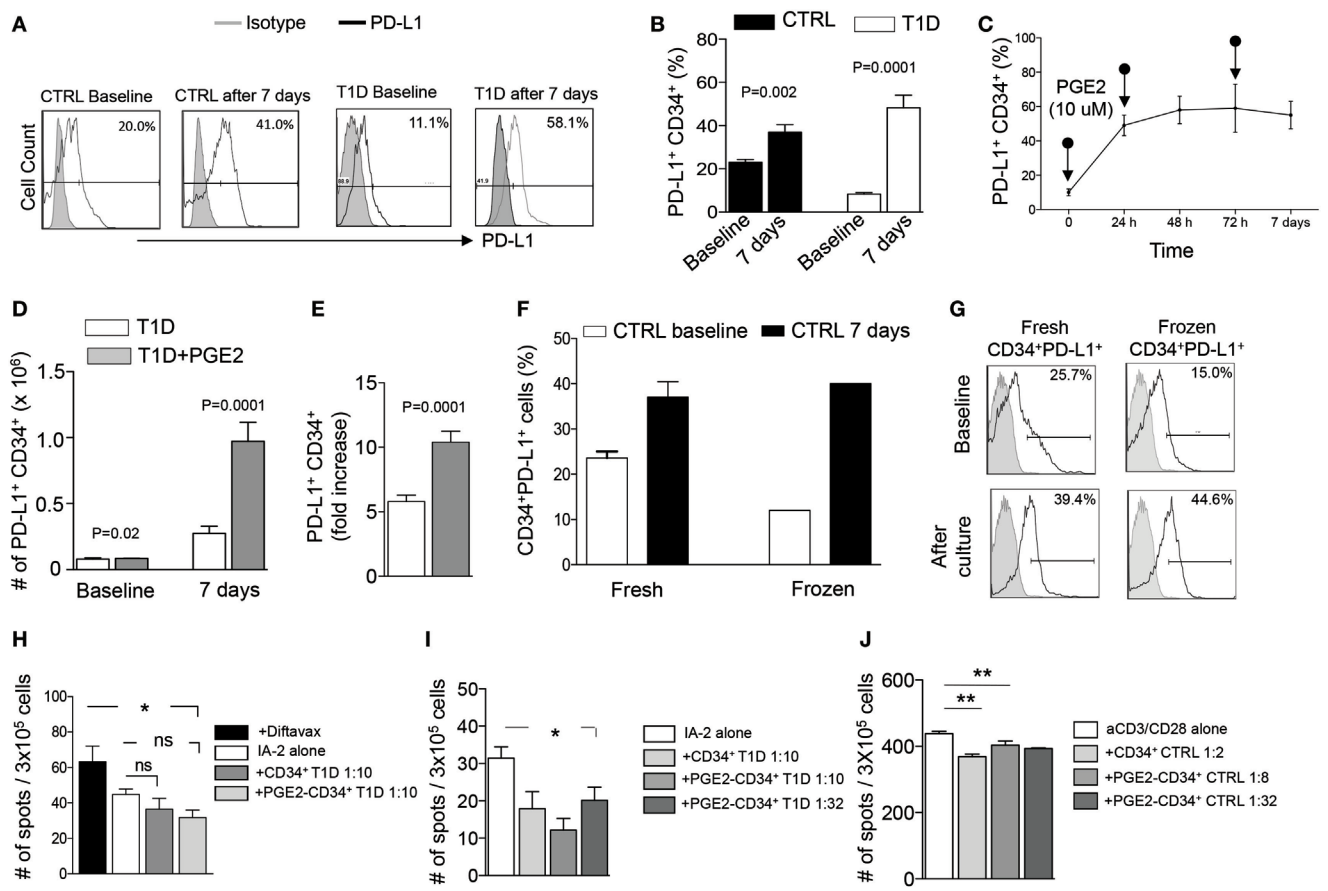

FIGURE 3 | Effects of human PGE2-modulated and cytokine-treated CD34$^+$ cells. **(A,B)** Sustained and robust upregulation of PD-L1 upon culture for 7 days with PGE2 and a cocktail of cytokines (heparin, human SCF, human TPO, human FGF-1, IGFBP2, and Angptl3) on CD34$^+$ cells obtained from T1D patients as compared to those from healthy controls. **(C)** Effect of PGE2 pulsing on CD34$^+$ cells cultured for 0, 24, 72 h and 6 days on PD-L1 expression on CD34$^+$ cells. **(D,E)** Bar graphs showing an increase in the number of PD-L1$^+$ CD34$^+$ cells and fold increase in cell number after 7 days of culture with PGE2 supplemented with cytokines. **(F,G)** Upregulation of PD-L1 expression following culture with PGE2 (shown as percentage) on CD34$^+$ cells was not altered by the freeze/thawing process. **(H)** PGE2-modulated CD34$^+$ cells abrogate the IFN-γ autoimmune response to insulin-associated 2 (I-A2) autoantigen *in vitro*, as measured *via* the quantification of IFN-γ-producing cells in an Elispot assay; Diftavax refers to a vaccine including immunization against tetanus toxoid, difteria, and hemophilus. **(I)** PGE2-modulated CD34$^+$ cells and PGE2-modulated CD34$^+$ cells cultured for 7 days in STFIA media abrogate the IFN-γ autoimmune response toward insulin-associated 2 (I-A2) autoantigen *in vitro*, as measured *via* the quantification of IFN-γ-producing cells in an Elispot assay, even when added at low dose. **(J)** PGE2-modulated CD34$^+$ cells abrogate the anti-CD3/CD28-stimulated PBMC response *in vitro* as measured *via* the quantification of IFN-γ-producing cells in an Elispot assay. Data are expressed as mean ± SEM. Data are representative of at least two independent experiments. *$P < 0.05$; **$P < 0.01$. Abbreviations: SCF, stem cell factor; TPO, thrombopoietin; hFGF-1, human fibroblast growth factor 1; IGFBP2, insulin-like growth factor-binding protein 2; Angptl3, angiopoietin-like 3; PBMCs, peripheral blood mononuclear cells; PGE2, prostaglandin E2; T1D, type 1 diabetes.

HSPCs, after 7 days of culture using STFIA media pulsed with PGE2 (**Figures 3F,G**). We observed sustained and conserved PD-L1 expression pre- and post-cryopreservation, suggesting that storage of HSPCs has no detrimental impact on their *ex vivo* expansion.

PGE2-Modulated Human HSPCs Abrogate the Autoimmune Response *Ex Vivo*

To study the *ex vivo* immunoregulatory effects of PGE2 modulation as well as whether cytokine treatment enhances these effects, we performed an autoimmune assay using unmodulated CD34$^+$ cells, PGE2-modulated CD34$^+$ cells, or PGE2-modulated HSPCs cultured for 7 days in STFIA medium. CD34-depleted PBMCs were cocultured with control CD34$^+$ cells (unmodulated), PGE2-modulated CD34$^+$ cells, or STFIA medium-cultured PGE2-modulated human CD34$^+$ cells in the presence of insulin-associated autoantigen-2 (I-A2) peptide at different cell ratios (1:2, 1:8, and 1:32 CD34$^+$ cells to PBMCs), and the number of IFN-γ-producing cells was quantified using an ELISPOT assay (**Figures 3H,I**). Interestingly, addition of PGE2-modulated human CD34$^+$ cells resulted in a significant decrease in the number of IFN-γ-producing cells (**Figure 3H**), suggesting that PGE2-modulated CD34$^+$ cells are endowed with immunoregulatory activity *ex vivo*. Addition of PGE2-modulated

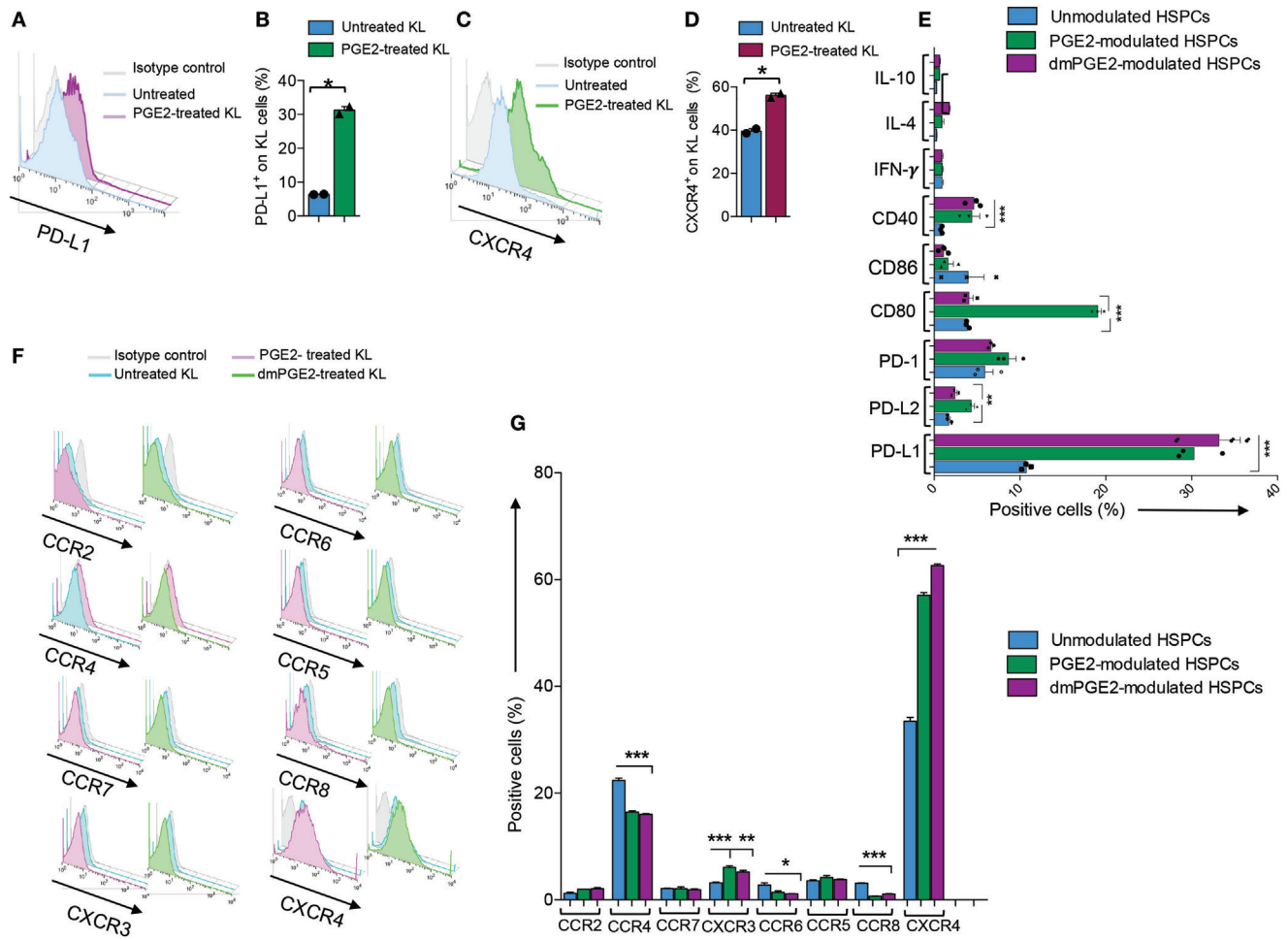

FIGURE 4 | Profile of murine KL cells. **(A,B)** Representative flow cytometric analysis and quantitative bar graph of PD-L1 expression on Lineage$^-$c-kit$^+$ (KL) cells from NOD mice pre- and post-pharmacologic modulation with PGE2. **(C,D)** Representative flow cytometric analysis and quantitative bar graph of CXCR4 expression on KL cells from NOD mice pre- and post-pharmacologic modulation with PGE2. **(E)** Quantitative bar graph of flow cytometric expression of positive and negative costimulatory molecules (CD40, CD80, CD86, PD-L1, PD-L2, and PD-1) and of select pro-inflammatory and anti-inflammatory cytokines (IFN-γ, IL-10, and IL-4) in PGE2-modulated KL cells (PGE2-KL) from NOD mice as compared to those modulated with a selected PGE2 clinical grade agonist (dmPGE2) and compared to unmodulated KL cells isolated from the bone marrow of normoglycemic NOD mice. **(F)** Flow cytometric expression of selected chemokine receptors (CXCR4, CCR2, CCR4, CCR5, CCR6, CCR7, CCR8, and CXCR3) in PGE2-KL from NOD mice as compared to those modulated with a selected PGE2 clinical grade agonist (dmPGE2) and to unmodulated KL cells isolated from the bone marrow of normoglycemic NOD mice. **(G)** Quantitative bar graph of flow cytometric expression of chemokines receptors (CCR2, CCR4, CCR5, CCR6, CCR7, CCR8, CXCR3, and CXCR4) in PGE2-KL from NOD mice as compared to those modulated with a selected PGE2 clinical grade agonist (dmPGE2) and compared to unmodulated KL cells isolated from the bone marrow of normoglycemic NOD mice. Data are expressed as mean ± SEM. Data are representative of at least two independent experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Abbreviations: KL, Lineage$^-$c-kit$^+$ cells; PGE2, prostaglandin E2.

$CD34^+$ cells cultured for 7 days in STFIA medium showed a further abrogation of the autoimmune response, and this effect was observed even when cells were added at a very low ratio (1:32) to PBMCs (**Figure 3I**). The effects of PGE2-modulated $CD34^+$ cells cultured for 7 days in STFIA media were further confirmed in a nonautoimmune-specific anti-CD3/anti-CD28 assay (**Figure 3J**).

Murine PGE2-Modulated HSPCs Abrogate the Autoimmune Response *In Vitro*

We next explored the feasibility of pharmacological modulation of PD-L1 with PGE2 in murine HSPCs. FACS analysis showed an upregulation of PD-L1 post-PGE2 modulation in KL ($Lineage^-c\text{-}Kit^+$) cells isolated from bone marrow of NOD mice (**Figures 4A,B**). Furthermore, we assessed PD-1 on T cells from NOD mice as compared to those from C57BL/6 and our data showed a significant defect in PD-1 expression on CD4 T cells isolated from NOD mice (**Figure 5**). Interestingly, another protein, CXCR4, primary involved in the homing of HSPCs, was markedly upregulated (**Figures 4C,D**). We then analyzed the expression of costimulatory molecules, as well as pro-inflammatory and anti-inflammatory cytokines by flow cytometry, which demonstrated upregulation of PD-L2, PD-1, CD80, and CD40 in PGE2-KL as compared to unmodulated KL cells. The expression of these molecules in KL cells modulated with dmPGE2 (known as 16, 16-dimethyl PGE2, a molecule which exerts a prolonged effect *in vivo* as compared to PGE2) was similarly upregulated (**Figure 4E**). Moreover, we explored the chemokine receptor profile of PGE2-KL as compared to dmPGE2-modulated KL cells and unmodulated KL cells in order to assess which chemokines are potentially involved in the homing of PGE2-modulated and dmPGE2-modulated KL cells. Consistent with the results in **Figure 4C**, CXCR4 was the most expressed chemokine in both groups of KL cells treated with PGE2 and dmPGE2 (**Figures 4F,G**). We next explored the immunoregulatory properties of PGE2-KL in an autoimmune setting *in vitro*. PGE2-KL generated from normoglycemic NOD mice were cocultured at ratios of 1:1, 1:5, and 1:10 (KL cells to T cells) with $CD11c^+$ DCs and BDC2.5 transgenic $CD4^+CD25^-$ T cells in the presence of BDC2.5 peptide. Quantification by flow cytometry revealed a pronounced and significant decrease in the percentage of IFN-γ^+CD4^+ T cells when PGE2-KL were added to the assay as compared to when unmodulated KL cells were used (**Figures 6A,B**). Indeed, PGE2-KL exerted a robust immunoregulatory effect even if added at low ratios (1:5 PGE2-treated KL cell to T cells). A less pronounced effect was observed when unmodulated KL were added to the assays. Interestingly, the percentage of activated $CD4^+CD25^+$ T cells declined upon coculture with KL or PGE2-KL (**Figure 6C**). PGE2-modulated HSPCs are thus endowed with immunoregulatory properties and are capable of abrogating the autoimmune response *in vitro*.

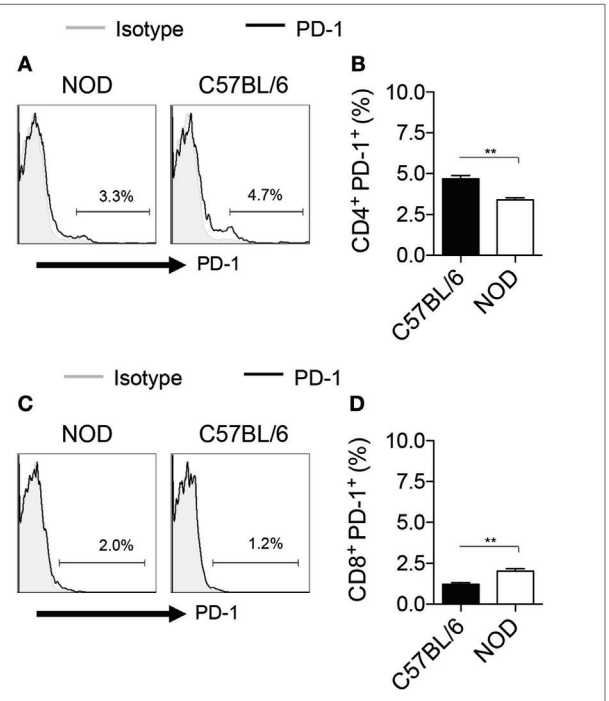

FIGURE 5 | PD-1 expression on murine T cells. **(A,B)** Representative flow cytometric analysis **(A)** and quantitative bar graph **(B)** for PD-1^+CD4^+ T cells isolated from splenocytes of NOD mice as compared those from C57BL/6 mice. **(C,D)** Representative flow cytometric analysis **(C)** and quantitative bar graph **(D)** for PD-1^+CD8^+ T cells isolated from splenocytes of NOD mice as compared those from C57BL/6 mice.

Adoptively Transferred Murine PGE2-Modulated HSPCs Traffic to Inflamed Areas

To examine the trafficking properties of GFP^+ PD-L1-expressing KL cells in an *in vivo* inflammatory setting, we performed a set of tracking experiments in NOD mice. Following infusion of GFP^+ KL cells extracted from the bone marrow of Luciferase NOD-GFP mice and treated with PGE2 as previously described, the pancreas and pancreatic draining lymph nodes (PLN) of NOD mice were harvested at 24 h. GFP^+ cells were quantified in the aforementioned tissues by flow cytometry and were detectable in the PLN (**Figure 6D**) and in the pancreata of NOD mice (**Figure 6E**). The phenotype of the GFP^+ cells after adoptive transfer and after homing to the pancreata of NOD mice showed Gr-1 expression, indicative of myeloid lineage, while very few cells were $CD3^+$ (**Figure 6E**). These GFP^+ PD-L1-expressing cells might probably interact with autoreactive CD4 and CD8 T cells.

DISCUSSION

The prospect of successful cell therapy has recently gained greater footing in the medical landscape in the past 2 years with the arrival of many cell-based products. Recently, many AHSCT-related clinical trials have demonstrated a beneficial effect in the treatment of several autoimmune diseases, and AHSCT is now considered one of the few therapies capable of reversing T1D in humans (6, 14, 25–27). In our study, we observed preservation of

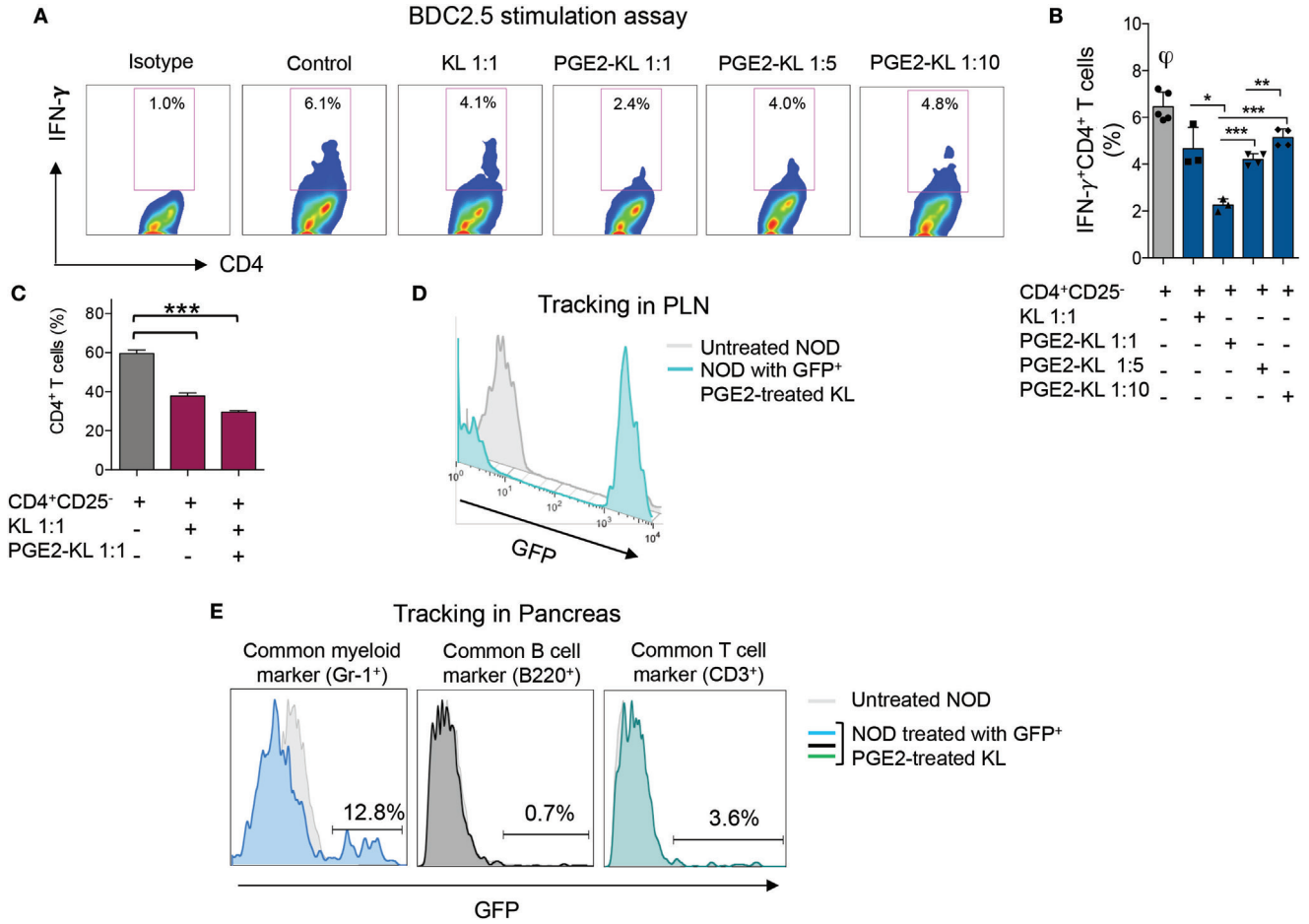

FIGURE 6 | Effects of murine KL cells. **(A,B)** Representative flow cytometric analysis **(A)** and quantitative bar graph **(B)** for IFN-γ$^+$CD4$^+$ T cells isolated from NOD-BDC2.5 TCR Tg mice and stimulated with BDC2.5 peptide in the presence of DCs (Control) or upon coculture with unmodulated KL or with PGE2-KL (at different ratios). **(C)** Quantitative bar graph of the percentage of CD4$^+$ T cells upon coculture with KL or PGE2-KL. **(D)** Representative flow cytometric analysis of GFP$^+$PGE2-KL in the PLN of treated NOD mice following 24 h of treatment with GFP$^+$PGE2-KL, demonstrating that they traffic to the PLN following adoptive transfer into NOD mice. **(E)** Representative flow cytometric analysis of GFP$^+$PGE2-KL in the pancreas of NOD mice 24 h post-infusion with GFP$^+$PGE2-KL, demonstrating the surface phenotype of GFP$^+$ cells. Abbreviations: KL, Lineage$^-$c-kit$^+$ cells; PGE2-KL, PGE2-modulated KL cells; HSPCs, hematopoietic stem and progenitor cells; GFP, green fluorescent protein; PGE2, prostaglandin E2; DCs, dendritic cells.

β cell function following AHSCT, as most patients included in our study population exhibited a sustained and adequate postprandial C-peptide response. The majority of these patients achieved and maintained peak-stimulated C-peptide levels higher than 0.6 ng/ml for at least 2 years of follow-up. Sustained C-peptide secretion is known to be associated with reduced prevalence (~30%) of hypoglycemic events and with a slower progression of diabetes complications, as reported by the DCCT Trial (28). Several patients also experienced reversal of the disease or a decrease in the exogenous insulin daily requirement. Although these are very encouraging results, many investigators have reported various complications and adverse effects associated with AHSCT in T1D patients, primarily related to the effects of immunosuppression (6). Some patients experience only temporary remission, and thus achieving prolonged remission of the disease remains the foremost goal for future clinical trials. Recently, much progress has been made with regard to the identification of small molecules and growth factors capable of both enhancing HSPC proliferation (15, 16) and further expanding the immunomodulatory subsets of HSPCs, in order to capitalize on their immunosuppressive properties. Interestingly, a screening study performed in zebrafish embryos showed that PGE2 enhances HSPC expansion and facilitates HSPC engraftment after bone marrow transplantation (21). Investigating and determining the effects of *ex vivo* modulation of HSPCs with PGE2 in an autoimmune setting may provide insight with regard to how to robustly enhance their immunoregulatory properties. Our screening results performed on ~64 known PGs allowed us to select four PGs, which are analogs to PGE2 and which we show induce relatively high upregulation of PD-L1 expression on human CD34$^+$ cells. We therefore sought to test the ability of PGE2-modulated HSPCs to affect the autoimmune response *in vitro*. Compared to unmodulated HSPCs, HSPCs overexpressing PD-L1 successfully abrogated the human autoimmune response *in vitro*. Next, we

sought to explore whether refining the *ex vivo* culture approach by including a cocktail of potent cytokines important for HSPC maintenance and extending the length of culture to 7 days could enhance the effects observed. Importantly, this approach remarkably enhanced the immunoregulatory properties of HSPCs and induced more pronounced PD-L1 expression. This expression appeared to be stable, unaffected by the freeze/thaw process, and resulted in a potent abrogation of the autoimmune response by modulated HSPCs, even when added at a very low ratio to T cells. Paralleling the human data, these preclinical murine studies also confirmed that PGE2-modulated HSPCs similarly exhibited immunoregulatory effects, as they markedly abrogated CD4-restricted autoimmune responses *in vitro*. *In vivo* tracking studies suggested that PGE2-modulated HSPCs home to the inflamed pancreas and PLN of NOD mice, most likely due to their substantial expression of CXCR4 (9). Based on the data herein, *ex vivo* expansion strategies with PGE2 combined with hematopoietic cytokines could generate a novel immunoregulatory HSPC-based approach potentially useful in the treatment of autoimmune T1D, without the detrimental effect of immunosuppressive agent toxicity, which is observed with standard immunotherapy. The recent discovery that a pre-established suicide genetic system may control survival and prevent toxicity of HSPCs undergoing *ex vivo* expansion will implement their use in clinical settings, allowing for easier manipulation of HSPCs and for a cell therapy-based approach in immune-mediated disorders (29).

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Institutional Review Board committee approval of San Raffaele Hospital, Milan-Italy (P2X7-T1D/01 and PD-L1-T1D/01) and BCH (3851).

AUTHOR CONTRIBUTIONS

MN designed and performed experiments, analyzed data, and wrote the paper; FD, AMM, SF, EC-L, FR, TL, AE, EA, AM, EG, AD, VU, CL, CM, EG, and MM performed experiments; GC, GB, MS, and GZ coordinated research; PF designed the study, wrote and edited the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01387/full#supplementary-material.

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Autologous Hematopoietic Stem Cell Transplantation for Autoimmune Diseases: From Mechanistic Insights to Biomarkers

Kelen Cristina Ribeiro Malmegrim[1,2]*, *João Rodrigues Lima-Júnior*[2,3], *Lucas Coelho Marlière Arruda*[4,5], *Júlia Teixeira Cottas de Azevedo*[4,6], *Gislane Lelis Vilela de Oliveira*[7] and *Maria Carolina Oliveira*[2,4]

[1] Department of Clinical Analysis, Toxicology and Food Science, School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Ribeirão Preto, Brazil, [2] Center for Cell-based Therapy, Regional Hemotherapy Center of Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil, [3] Biosciences Applied to Pharmacy Program, School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Ribeirão Preto, Brazil, [4] Division of Rheumatology, Allergy, Immunology and Immunotherapy, Department of Internal Medicine, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil, [5] Department of Clinical Science, Intervention and Technology, Karolinska Institutet, Stockholm, Sweden, [6] Basic and Applied Immunology Program, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil, [7] São Paulo State University (UNESP), Institute of Biosciences, Humanities and Exact Sciences (IBILCE), São Jose do Rio Preto, São Paulo, Brazil

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Anne Mary Dickinson, Newcastle University, United Kingdom

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Adriane Feijo Evangelista, Barretos Cancer Hospital, Brazil
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Femke Van Wijk, University Medical Center Utrecht, Netherlands

***Correspondence:**
Kelen Cristina Ribeiro Malmegrim
kelenfarias@fcfrp.usp.br

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Phase I/II clinical trials of autologous hematopoietic stem cell transplantation (AHSCT) have led to increased safety and efficacy of this therapy for severe and refractory autoimmune diseases (AD). Recent phase III randomized studies have demonstrated that AHSCT induces long-term disease remission in most patients without any further immunosuppression, with superior efficacy when compared to conventional treatments. Immune monitoring studies have revealed the regeneration of a self-tolerant T and B cell repertoire, enhancement of immune regulatory mechanisms, and changes toward an anti-inflammatory milieu in patients that are responsive to AHSCT. However, some patients reactivate the disease after transplantation due to reasons not yet completely understood. This scenario emphasizes that additional specific immunological interventions are still required to improve or sustain therapeutic efficacy of AHSCT in patients with AD. Here, we critically review the current knowledge about the operating immune mechanisms or established mechanistic biomarkers of AHSCT for AD. In addition, we suggest recommendations for future immune monitoring studies and biobanking to allow discovery and development of biomarkers. In our view, AHSCT for AD has entered a new era and researchers of this field should work to identify robust predictive, prognostic, treatment-response biomarkers and to establish new guidelines for immune monitoring studies and combined therapeutic interventions to further improve the AHSCT protocols and their therapeutic efficacy.

Keywords: hematopoietic stem cell transplantation, immune reconstitution, autoimmune diseases, biomarkers, immune tolerance, immunoregulation

INTRODUCTION

More than 20 years ago, autologous hematopoietic stem cell transplantation (AHSCT) was proposed as an alternative and innovative treatment for severe and refractory autoimmune diseases (AD) (1, 2). This therapeutic approach has been successfully used to treat several AD and over the years phase I/II clinical studies have led to increased safety and efficacy of the procedure (1, 2). More recently, phase III randomized trials have proven greater therapeutic efficacy of AHSCT than conventional therapies for some AD, such as multiple and systemic sclerosis. In addition, these trials have demonstrated that AHSCT can induce long-term disease remission without further use of immunosuppression (3–11).

Despite the overall positive outcomes of the procedure, subgroups of patients fail to remain in remission after AHSCT and reactivate the autoimmune disease (1, 2). The factors associated with disease reactivation, however, remain to be investigated. They may range from patient-specific (e.g., disease physiopathology, age, co-morbidities) to more general factors (e.g., infections, resurgence of autoreactive T, and B cells) (1, 12).

In the last decade, immune monitoring analyses have shown that AHSCT is able to regenerate a new auto-tolerant immune T and B cell repertoire, increase immune regulatory mechanisms, and induce changes to an anti-inflammatory milieu in patients with different AD (13–44). Most of the established immune mechanisms of AHSCT are common to AD in general, while others may be disease-specific (1, 2, 12). Few studies have analyzed the mechanistic results by comparing data of autoimmune patients with different outcomes after AHSCT (17, 18, 26, 31). **Table 1** lists all 39 mechanistic studies of AHSCT for AD already published since 2004 and the most common immune mechanisms reported.

In this context, understanding of the immune mechanisms that lead to different clinical outcomes is essential to refine AHSCT protocols or to propose adjuvant/combined immunotherapy. **Figure 1** indicates future directions to establish biomarkers of AHSCT for AD, including sample biobanking of multicenter randomized clinical studies and development of adjuvant/combined immunotherapy for patients that did not respond to AHSCT as single therapy.

In this perspective, we discuss the current knowledge about the immune mechanisms involved in AHSCT for AD, suggest recommendations for further immune monitoring studies and propose future directions in the field.

IMMUNE MECHANISMS AFTER AHSCT FOR AD: CURRENT STATUS

The rationale of AHSCT is the eradication of the autoreactive immunological memory and regeneration of the immune system. Ablation of the immune system, including depletion of autoreactive memory T and B cells, is followed by the reestablishment of immune tolerance (51). However, exactly how AHSCT corrects a deregulated immune system is not yet completely understood (1, 2).

Since 2004, and over the past 14 years, several immune mechanisms after AHSCT for AD have been described (**Table 1**). Many groups have investigated how the immunological renewal after AHSCT for ADs may reset a deregulated immune system into a self-tolerant status, inducing long-term remission (1, 2). Patients have been prospectively evaluated in studies that elucidate some immune mechanisms (**Table 1**). Currently, we may consider that these results, already reproduced on different patient cohorts, diseases, conditioning regimens, and laboratories around the world, are quite robust and consistent. Nevertheless, we still need to extend and worldwide standardize the immune monitoring evaluations in AD patients to enable the discovery of biomarkers, which will ultimately help to improve AHSCT protocols and their therapeutic efficacy (**Table 2**).

Common Immune Mechanisms After AHSCT For AD

Reactivation of Thymus Function and Renewal of the TCR Repertoire

Thymus function translates into production of naive T cell populations and their exportation to the periphery (52). Generation of a diverse T cell repertoire throughout life is essential for immunity against pathogens. During development, T cells undergo central tolerance mechanisms, where positive selection ensures that T cells recognize self-MHC molecules and negative selection eliminates most T cells specific to autoantigens (52–54).

Thymic production of early naive T cells, named recent-thymic emigrants (RTE), can be determined through analysis of signal-joint T cell receptor excision circles (sjTRECs) (51). TRECs are DNA byproducts of T cell receptor (TCR) gene rearrangements that take place during T cell development in the thymus, and are reliable markers of RTE production and thymic function (55).

In the context of AHSCT, thymic rebound, which is defined by volumetric enlargement and functional reactivation of the thymus following lymphoid ablation, has been reported (14, 32, 55). Lymphoid and myeloid cells may be partially or completely depleted, depending on the intensity of the AHSCT conditioning regimen. Shortly after AHSCT, immune reconstitution predominantly relies on peripheral expansion of T and B cells that survived the highly immunosuppressive regimen or that were re-infused with the stem cell graft (15, 56).

Muraro et al. (14) were the first to demonstrate the so-called "immune resetting" mechanism in multiple sclerosis (MS) patients treated with AHSCT. Increased TREC levels indicated that peripheral T cells underwent thymic maturation after transplantation (14, 51). In addition, deep sequencing analysis demonstrated extensive replacement of a pre-existing TCR repertoire with new T cell clones that have emerged after transplantation. In fact, greater TCR repertoire diversity was found in patients with complete clinical response, indicating an interesting clinical correlation (18). In the following years, other researchers have also demonstrated, in different AD, that T cells could regenerate in adults with inactive thymuses (13–15, 18, 27, 31, 34, 35, 40, 41) and that the TCR repertoire after

TABLE 1 | Described immune mechanisms of AHSCT in patients with autoimmune diseases.

Disease	References	Immune mechanism								
		Renewal of the TCR Repertoire	Reactivation of thymic function	Modulation of gene expression	Regulatory T expansion	Regulatory B expansion	Homeostatic proliferation/ $CD8^{+}CD28^{-}CD57^{-}$ expansion	Increased PD-1 expression on T and B cells	Changes in cytokine patterns	Decreased autoreactivity early post-AHSCT
Multiple Sclerosis	Sun et al. (13)	■	■						■	■
	Muraro et al. (14)	■	■				■			
	Dubinsky et al. (25)	■								
	Darlington et al. (36)		■		■					
	Abrahamsson et al. (39)				■		■			■
	Muraro et al. (40)	■								
	de Paula Sousa et al. (41)			■						
	Arruda et al. (42)			■	■		■	■		
	Arruda et al. (45)				■		■	■		
	Oliveira et al. (44)			■						
	Karnell et al. (15)				■					
	Cull et al. (46)								■	
	Darlington et al. (36)			■					■	
Type 1 Diabetes	Li et al. (16)								■	
	Ye et al. (38)			■						
	de Oliveira et al. (17)			■					■	
	Zhang et el. (47)			■						
	Malmegrim et al. (18)	■	■		■		■	■		■
Systemic Sclerosis	Farge et al. (19)	■	■							
	Bohgaki et al. (20)		■						■	
	Tsukamoto (21)								■	
	Baraut et al. (22)				■					
	Farge et al. (7)	■								
	Arruda et al. (48)	■	■		■	■				
	Arruda et al. (23)						■	■	■	
Systemic Lupus Erythematosus	Alexander et al. (24)				■					
	Zhang et al. (26)				■		■	■		
	Wada et al. (27)									
	Alexander et al. (28)	■	■							
Crohn's Disease	Burt et al. (29)	■								■
	Clerici et al. (30)				■				■	
	Le Bourhis et al. (49)	■								
Juvenile Idiopathic Arthritis	de Kleer et al. (31)				■				■	
	Brinkman et al. (32)*									
	Wu et al. (33)	■								
	Delemarre et al. (34)	■			■					
Juvenile Dermatomyositis	Szodoray et al. (35)				■					
	Enders et al. (37)				■				■	
Other Rheumatic Diseases	Szodoray et al. (35)				■					
	Váróczy et al. (50)*									

*These studies included only peripheral blood immunophenotyping analyses.

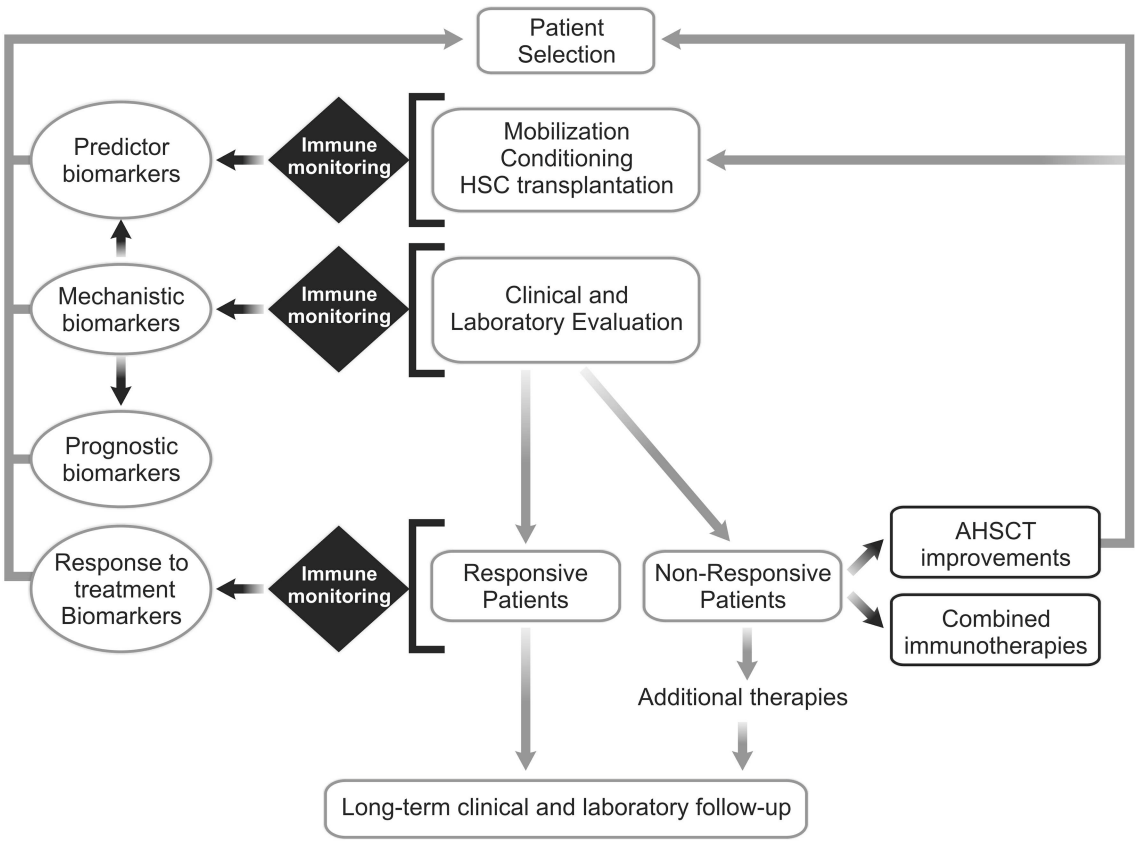

FIGURE 1 | Future directions for establishment of biomarkers of autologous hematopoietic stem cell transplantation for autoimmune diseases. Patients with autoimmune disease (AD) must be are selected to undergo autologous hematopoietic stem cell transplantation (AHSCT). Based on the clinical, laboratory and immune monitoring results, patients will be classified in "responsive" or "non-responsive" to AHSCT. Well-performed immune monitoring evaluations are essential to settle mechanistic biomarkers and reveal new prognostic, predictor and/or response to treatment biomarkers. Further, additional therapeutic interventions can be proposed to treat patients who do not respond sufficiently to transplantation as a single treatment or reactivate the disease after some period of remission. New approaches for improving AHSCT efficacy in AD patients and/or combined immunotherapies are warranted.

AHSCT was indeed renewed (13, 14, 16, 18, 26–28, 31, 34) (**Table 1**).

In summary, thymus reactivation and renewal of the TCR repertoire following AHSCT are the most significant mechanisms of action of this therapy so far described. Therefore, we should strongly recommend routine analyses of TREC levels in patients with AD undergoing AHSCT (**Table 2**). In addition, whenever possible, TCR repertoire evaluation by next generation sequencing should also be performed (**Table 2**).

Modulation of Gene Expression

Transcriptional analyses are currently used to describe disease signatures, evaluate response to treatments and to define patient subgroups, among other applications. However, to date few studies have evaluated modulation of gene expression of immune cells in the context of AHSCT for AD. Our group has evaluated the gene and microRNA expression profiles of reconstituted immune cells after AHSCT in MS patients. de Paula Sousa et al. (41) analyzed the global gene expression profiling of peripheral $CD4^+$ or $CD8^+$ T cells from MS patients at pre-transplantation and periodically after AHSCT. Hierarchical gene clustering analysis revealed that at 2 years after AHSCT, $CD8^+$ T cells from MS patients were more similar to samples from healthy controls (19).

Other studies demonstrated normalization of deregulated gene expression following AHSCT for multiple sclerosis (20, 22, 25). Arruda et al. (23) demonstrated post-transplant normalization of the expression of mir-16, mir-155 and mir-142-3P, which have immmunoregulatory functions and are abnormally expressed in MS patients (20). As expected, expressions of their putative target genes, FOXP3, FOXO1, PDCD1, and IRF2BP2, were increased at 2 years post-transplantation (20) (**Table 1**).

In transplanted type 1 diabetes patients, Ye et al. (38) observed increased IL10, TGFβ, and FOXP3 mRNA expression, despite no significant regulatory T cell expansion (44).

In conclusion, few studies have so far explored transcriptomic analyses to understand the immune mechanisms of AHSCT for AD. One important challenge is to perform global transcriptome or single gene expression analysis for specific genes in purified T and B cell subsets from patients that undergo AHSCT, before and after disease reactivation. Lack of proper study design for

TABLE 2 | Requirements for immune monitoring analyses in patients with AD undergoing AHSCT.

BIOLOGICAL SAMPLE	TIME-POINTS OF ANALYSES	RECOMMENDED ANALYSES	METHODS
Minimum requirements for immune monitoring, biobanking and biomarker identification			
Serum/Plasma	At baseline (before mobilization) and at 1, 3, 6, 9, 12, 18, 24, 30, 36 months after AHSCT and annually thereafter Serum and plasma samples storage at−80°C	Total immunoglobulin levels (IgG, IgA, IgM)	ELISA
		Soluble biomarkers (TNF-α, IFN-γ, IL-2, IL-4, IL-6, IL-8, IL-17, IL-18, IL-10, TGF-β)	ELISA, multiplex
Total peripheral blood or PBMCs	At baseline (before mobilization) and at 1, 3, 6, 9, 12, 18, 24, 30, 36 months after AHSCT and annually thereafter PBMC samples cryopreservation and storage at N_2 liquid for future functional assays	Blood cell counts (essential to calculate absolute numbers of immune cell subsets)	Hematology Analyzer
		Immunophenotyping of T, B, NK cell subsets (see **Table 3**) on fresh blood samples	Flow Cytometry, CyTOF (mass cytometry)
DNA (from PBMC)	At baseline (before mobilization) and at 3, 6, 9, 12, 24, 30, 36 months after AHSCT and annually thereafter DNA samples storage at−20°C	TREC and KREC levels	Multiplex real-time PCR
RNA (from PBMC)	At baseline (before mobilization) and at 6, 12, 18, 24 months after AHSCT and annually thereafter cDNA samples storage at −20°C	–	–
Additional recommendations for immune monitoring and biomarker discovery			
GrafT cells	At graft collection	Immunophenotyping of T, B, NK cell subsets (see **Table 3**) on fresh samples	Flow Cytometry, CyTOF (mass cytometry)
RNA (from PBMC)	At baseline (before mobilization) and at 6, 12, 18, 24 months after AHSCT and annually thereafter	B cell receptor (BCR) and/or T cell receptor (TCR) repertoire	NGS
		Gene expression, MicroRNA expression	Microarrays, PCR arrays, Real-time PCR
PBMCs or sorted cell subset	At baseline and at 1, 3, 6, 9, 12, 18, 24 months after AHSCT and annually thereafter Protein, DNA and/or RNA extraction	Proteomics Genomics (genome-wide association studies of polymorphisms) and epigenomics (epigenetic modifications) Transcriptomics (transcriptional signatures of tissues, cell population or single-cell)	Mass spectrometry, protein or peptide microarrays, aptamers High-Throughput DNA sequencing RNA sequencing, Microarrays
Disease-specific recommendations for immune monitoring and biomarker discovery			
Serum/plasma	At baseline (before mobilization) and at 1, 3, 6, 9, 12, 18, 24, 30, 36 months after AHSCT and annually thereafter	Specific autoantibody titers	ELISA
		Complement component levels	ELISA
		Specific disease surrogate soluble biomarkers	ELISA, multiplex
		Proteomics of extracellular microvesicles	Mass spectrometry
Total peripheral Blood (in EDTA) or PBMCs	At baseline (before mobilization) and at 1, 3, 6, 9, 12, 18, 24, 30, 36 months after AHSCT and annually thereafter PBMC samples cryopreservation at N_2 liquid for future functional assays	Immunophenotyping of specific cell subsets (such as innate lymphoid cells; gut-homing T cells; skin-homing T cells; specific cell subset already demonstrated as surrogate/mechanistic biomarkers) Expression of PD-1, Lag-3, Tim-3, and TIGIT (co-inhibitory receptors with specialized functions in immune regulation) on T cells	Flow Cytometry, CyTOF (mass cytometry)
		Autoantigen-specific T cells (autoreactive cells)	Tetramer staining by flow cytometry
PBMCs or sorted cell subset	At baseline (before mobilization) and at 1, 3, 6, 9, 12, 18, 24 months after AHSCT and annually thereafter Protein, DNA and/or RNA extraction	Proteomics Genomics (genome-wide association studies of polymorphisms) and epigenomics (epigenetic modifications) Transcriptomics (transcriptional signatures of tissues, cell population or single-cell)	Mass spectrometry, protein or peptide microarrays, aptamers High-throughput DNA sequencing RNA sequencing, Microarrays
RNA from PBMC	At baseline (before mobilization) and at 6, 12, 18, 24 months after AHSCT and annually thereafter	MicroRNA expression	PCR arrays, Real-time PCR

(Continued)

TABLE 2 | Continued

BIOLOGICAL SAMPLE	TIME-POINTS OF ANALYSES	RECOMMENDED ANALYSES	METHODS
Tissue biopsies (e.g., gut, skin)	At baseline (before mobilization) and at 6, 12, 18, 24 months after AHSCT and annually thereafter Protein and RNA extraction	Protein expression Gene expression	Immunofluorescence, Immunohistochemistry PCR arrays, Real-time PCR
Other biological fluid (e.g., cerebrospinal fluid)	At baseline (before mobilization) and at 6, 12, 18, 24 months after AHSCT and annually thereafter	Oligoclonal bands	Isoelectric focusing, followed by immunoblotting
		Immunophenotyping of specific cell subsets	Flow Cytometry, CyTOF (mass cytometry)

PBMC, Peripheral Blood Mononuclear Cells; NGS, Next Generation Sequencing; TREC, T-cell receptor excision circles; KREC, κ-deleting recombination excision circles.

appropriate cell separation/sorting and RNA isolation and/or technology cost are the most probable reasons for researchers not having so far explored this approach. In our opinion, this is a powerful method that should be explored in future studies. Transcriptional signatures of diseases for diagnosis, mechanisms and response to treatment are currently needed. Besides transcriptomics, other advanced new tools and technologies may be also useful to identify therapeutic biomarkers, such as proteomics, cytomics, lipidomics, and metabolomics (57–61).

Changes in Cytokine Patterns

Many mechanistic studies have demonstrated that AHSCT decreases the inflammatory status of patients with different AD (13, 24, 28, 37, 38, 43, 51–53) (**Table 1**). Sun et al. (13) reported the first study that deeply evaluated the immune reconstitution in MS patients treated with AHSCT (13). Serum levels of IL-12, IFN-γ, TNF-α, IL-10, and IL-4 were modulated after transplantation, however they decreased to baseline levels at 12 months (13). The authors also reported significant transient increase of IFN-γ, TNF-α, and IL-10 serum levels between 3 and 6 months after AHSCT, compared to the pre-transplant period. On the other hand, IL-12 serum levels consistently decreased in all patients following AHSCT (13).

Bohgaki et al. (20) evaluated IFN-γ and IL-4 production in T cells of SSc patients by intracellular staining. At inclusion, the cytokine production profile between transplanted SSc patients and healthy individuals was not significantly different (28). Frequencies of $CD3^+CD8^-$ and $CD3^+CD8^+$ cytokine-producing T cells were not different between poor or good response groups. Notably, IFN-γ producing $CD8^+$ T cells increased after transplantation in both groups (28).

Tsukamoto et al. (21) showed that while AHSCT was effective in controlling disease activity of systemic sclerosis (SSc) patients, Th1/Th2 ratio was significantly increased for at least 3 years after transplantation. The authors showed that the IFN-γ^+/$CD4^+$ T cells to IL-4^+/$CD4^+$ T cell ratio increased early after transplantation, reaching a plateau 6 months after AHSCT (29).

In addition, Crohn's disease patients who fully responded to treatment with AHSCT had higher numbers of regulatory T cells (Treg) and lower IFN- γ and IL-12 serum levels than non-responders (37). In juvenile arthritis patients, de Kleer et al. (31) demonstrated increased expression of mRNA IL-10 and decreased expression of mRNA IFN-γ in hsp60-specific T cells after transplantation (38). Therefore, authors suggest that AHSCT promotes a shift in autoreactive cells from pro-inflammatory to a more tolerant phenotype (38).

Type 1 diabetes (T1D) patients showed decreased levels of serum autoantibodies and of pro-inflammatory cytokines IL-1, IL-17, and TNF-α after AHSCT (24). Enders et al. (37) reported that patients with active juvenile dermatomyositis had elevated levels of three pro-inflammatory biomarkers (CXCL10, TNFR2, and Galectin-9) that highly correlated with disease activity. Notably, levels of these biomarkers decreased in two patients after transplantation (43).

In summary, the above-described reports showed interesting modulation of cytokine/chemokine/other soluble markers (13, 28, 29, 38, 43, 46, 50) after AHSCT. However, these post-transplantation changes only make sense if related to disease pathogenesis and/or activity. In our opinion, serum evaluation of the main classical cytokines (TNF-α, IFN-γ, IL-2, IL-4, IL-6, IL-8, IL-17, IL-18, IL-10, TGF-β) is interesting, but not essential for most AD post-AHSCT follow-up (**Table 2**). In organ-specific AD, serum concentration of most general cytokines is low and heterogeneous. However, in systemic AD serum levels of important soluble factors (such as cytokines chemokines, growth factors, and surrogate disease-specific markers) may be evaluated at pre- and post-transplantation periods to search for predictive biomarkers and/or treatment-response biomarkers of AHSCT for systemic AD (**Table 2**).

Regulatory T Cell Expansion

Autoreactive T cells that escape negative selection in the thymus are found in the peripheral blood of healthy individuals (53). However, peripheral tolerance mechanisms control autoimmunity and prevent development of autoimmune disease (62, 63). T cell-intrinsic (anergy, clonal deletion, or immunological ignorance) and extrinsic mechanisms (mediated by suppressor/regulatory cells) are essential to peripheral tolerance. The $CD4^+CD25^+Foxp3^+$ Treg, the main natural regulatory T cell subset, are very important for extrinsic control of peripheral tolerance, once they modulate autoreactive T and B cell responses.

Almeida et al. (64) demonstrated that in lymphopenic environments $CD8^+$ T-cell subsets impact on each other during expansion (64). $CD4^+CD25^+$ Treg suppress and control repopulation of $CD8^+$ T cells, leading to balanced repopulation of central-memory and effector-memory T cell subsets (64). Therefore, $CD4^+CD25^+$ Treg may have a major role in

regulating the expansion of $CD8^+$ T cell subsets during repopulation of the lymphopenic environment after AHSCT in AD patients (55, 64).

In the context of AHSCT, de Kleer et al. (31) were first to demonstrate that AHSCT for AD induces immunologic self-tolerance by restoring the $CD4^+CD25^{hi}$ immune regulatory network (**Table 1**). AHSCT was able to normalize the frequency of $CD4^+CD25^{hi}$ Treg in patients with idiopathic juvenile arthritis (IJA). Recovery of normal Treg levels after transplantation was due to both, homeostatic expansion during the lymphopenic phase of immune reconstitution and thymic generation of naive Treg $CD4^+CD25^{hi}$ expressing FOXP3 mRNA (38). Since then, many groups have shown increased Treg numbers as an important and common immune mechanism of AHSCT for AD (16, 20, 23, 26, 31–33, 36–38, 41, 63) (**Table 1**). Delemarre et al. (34) demonstrated that remission of AD after transplantation involves renewal of the Treg TCR repertoire during thymic reactivation (41).

During immune reconstitution, the lack of $Foxp3^+$ Treg results in development of "gaps" in the TCR repertoire and inappropriate responses to foreign antigens. Conversely, the presence of $Foxp3^+$ Treg optimizes TCR repertoire diversity and foreign antigen responsiveness (64, 65). These studies provide an example of Treg activity that actually enhances immunity, which is very important in transplanted AD patients, contrasting with their generally accepted immunosuppressive function (64, 65).

The peripheral expansion of $CD4^+CD25^{hi}Foxp3^+$ Treg and other regulatory populations during the lymphopenic phase post-transplantation is a very intriguing mechanism of AHSCT for AD. During this early phase of immune reconstitution, the total number of $CD4^+$ T cells is very low. Therefore, expanded $CD4^+CD25^{hi}Foxp3^+$ Treg cells correspond to the majority of $CD4^+$ T cells in this phase. The expansion of cells with regulatory phenotype and function may allow the achievement of a "fine immune balance" in the patient until new naive T cells are produced by the reactivated thymus. Therefore, all future immune monitoring studies should assess the absolute number of phenotypically well-characterized Treg subsets and their immunosuppressive potential in well-designed assays (**Tables 2**, **3**).

Regulatory B Cell Expansion

Regulatory B cells (Breg) are immunosuppressive cells that support immunological tolerance (70, 71). They modulate the immune responses mainly via IL-10 secretion (70, 71). Abnormalities in the Breg number or function have been described in many immune-mediated disorders. Therefore, Breg are considered essential to maintain immune homeostasis (70, 71). $CD19^+CD24^{hi}CD38^{hi}$ B-cell populations are well-characterized Breg in humans, playing an important role in the control of autoreactivity (72).

Recently, our group (31) showed that Breg frequencies transiently increased after AHSCT, tending to remain higher than pre-transplant values for at least 2 years (**Table 1**). We believe that Breg may be involved in the reestablishment of auto-tolerance after AHSCT, as suggested by persistently increased Breg/memory B cell ratio, as well as higher IL-10 production in SSc patients after transplantation (31). Notably, we found that Breg expansion occurs early post-transplantation [(31) and unpublished observation of ongoing studies]. Therefore, peripheral blood samples should be collected at 1, 3, and 6 months after transplantation (**Tables 2**, **3**). In our opinion, whenever possible, besides the classical $CD19^+CD24^{hi}CD38^{hi}$ Breg, other regulatory B cell subsets (71) should be evaluated. In addition, functional assays to evaluate the immunosuppressive capacity of reconstituted Breg populations are also desirable (72).

Homeostatic Proliferation and $CD8^+CD28^-CD57^-$ Expansion

After transplant-induced immunological depletion, lymphocytes repopulate the immune space both through enhanced T cell neogenesis from the thymus and proliferation of residual non-depleted peripheral lymphocytes, in a biological process named homeostatic proliferation. At this condition, $CD8^+$ T cells proliferate more than the $CD4^+$ T cell population (73).

Abrahamsson et al. (39) showed that $CD8^+CD28^-CD57^+$ T cell subsets were persistently increased in MS patients after AHSCT and were able to suppress $CD4^+$ T cell proliferation with variable potency (17). $CD8^+CD28^-CD57^+$ T cells have regulatory properties and their numbers are usually found decreased in AD (74) (**Table 1**). In this context, we suggest that $CD8^+CD28^-CD57^+$ T regulatory T cells expand preferentially following peripheral immune homeostasis disruption, such as in the early post-AHSCT period. In addition, many other mechanistic studies have also demonstrated that this regulatory/suppressor T cell subset expands after transplantation, indicating its important role in AHSCT for AD (**Table 1**).

We have also recently found in SSc patients that homeostatic proliferation after AHSCT results in transient telomere attrition and increased numbers of senescent and exhausted $CD8^+CD28^-CD57^+$ T cells. In addition, high expression of programmed cell death protein 1 (PD-1) in peripheral T cells is associated with better clinical outcomes after AHSCT (48).

In our opinion, this T cell population plays and important role in controlling autoimmunity early post-AHSCT. Future immune monitoring studies should better characterize their phenotype, their immunosuppressive capacity and possibly other mechanisms of action in AD patients undergoing AHSCT (**Tables 2**, **3**). Our studies have suggested that these $CD8^+CD28^-CD57^+$ T cells express PD-1 (73), and perhaps other immunoregulatory molecules, as supported by recent literature from other researcher groups (75).

Increased PD-1 Expression on T and B Cells

During T cell activation, the PD1 (or CD279) molecule is expressed on the cell surface and may engage its ligand, the programmed cell death ligand 1 (PDL1; also known as CD274) and/or PDL2 (also known as CD273). Upon binding, positive signals are generated through TCR and CD28 (76). These co-inhibitory receptors act as immune checkpoints for effector T cells, which regulate the adaptive immune responses. In fact, mice deficient in PD-1 molecules are susceptible to development of autoimmunity and AD, as reviewed by Sharpe and Pauken (77). Moreover, PD-1 polymorphisms detected in

TABLE 3 | Immunophenotyping of peripheral blood cell subsets in patients with AD undergoing AHSCT.

CELL SUBSET	PHENOTYPE
Minimum panel for immune reconstitution analyses	
Total CD3, CD4, or CD8 T cells	$CD3^+CD4^+CD8^-$; $CD3^+CD4^-CD8^+$
Recent thymic emigrants	$CD3^+CD4^+CD31^+CD45RA^+CD45RO^-$
Naive T cells[a,b]	$CD3^+CD4^+(CD8^+)CD45RA^+CD45RO^-CCR7^+CD62L^+$
Central memory T cells[a,b]	$CD3^+CD4^+(CD8^+)CD45RA^-CD45RO^+CCR7^+CD62L^+$
Effector memory T cells[a,b]	$CD3^+CD4^+(CD8^+)CD45RA^-CD45RO^+CCR7^-CD62L^-$
Effector T cells[a,b]	$CD3^+CD4^+(CD8^+)CD45RA^+CD45RO^-CCR7^-CD62L^-$
Exausted T cells[c]	$CD3^+CD4^+CD8^-PD\text{-}1^+$; $CD3^+CD4^-CD8^+PD\text{-}1^+$
Naive B cells	$CD19^+CD27^-IgD^+$
Switched memory B cells	$CD19^+CD27^+IgD^-$
Non-Switched memory B cells	$CD19^+CD27^+IgD^+$
Plasma cells	$CD19^+CD27^{high}IgD^-CD38^{high}$
Regulatory B cells (Breg)	$CD19^+CD24^{high}CD38^{high}$ or $CD19^+CD24^{high}CD38^{high}IgM^{high}IgM^{high}CD5^+CD10^+CD20^+CD27^-CD1d^{high}$
Regulatory T cells (nTreg)	$CD4^+CD25^{high}CD127^-Foxp3^+$
Suppressor T cells (CD8)	$CD8^+CD28^-CD57^+PD1^+$
NK cells (cytotoxic)	$CD3^-$ $CD56^{dim}CD16^+$
NK cells (cytotoxic)	$CD3^-$ $CD56^{bright}CD16^{+/-}$
iNKT cells	$CD19^-CD3^+V\alpha24^-J\alpha18$ TCR^+
Stem cell-like memory T cells (TSCM)	$CD3^-CD4^+(CD8^+)CD45RO^-CD45RA^+CCR7^+CD27^+CD28^+CD95^+CD122^+$
Other cell subsets for immune reconstitution analyses	
Follicular T helper (Tfh)	$CD3^+CD4^+CXCR5^+PD\text{-}1^+Bcl\text{-}6^+FoxP3^-$ or $CD3^+CD4^+CXCR5^+PD\text{-}1^+ICOS^+$
Th1 cells	$CD3^+CD4^+CXCR3^+CCR6^-Tbet^+$ or $CD3^+CD4^+CXCR5^-CXCR3^+$
Th2 cells	$CD3^+CD4^+CCR4^+CCR6^-GATA3^+$ or $CD3^+CD4^+CXCR5^-CCR4^+CCR6^-$
Th17 cells	$CD3^+CD4^+CCR6^+CCR4^+ROR\gamma t^+$ or $CD3^+CD4^+CXCR5^-CCR4^+CCR6^+CCR10^-$
Th22 cells	$CD3^+CD4^+CCR10^+CCR4^+AHR^+$ or $CD3^+CD4^+CXCR5^-CCR4^+CCR6^+CCR10^+$
Monocytes (classic)	$CD14^+CD16^-$
Monocytes (inflammatory)	$CD14^+CD16^+CD64^{high}$ $CD32^{low}$
Monocytes (patrolling)	$CD14^{low}CD16^+$
Myeloid-derived suppressor cells (MDSCs) granulocytic[d]	$Lin\text{-}CD14^-HLADR^-CD33^+CD11b^+$
Myeloid-derived suppressor cells (MDSCs) monocytic[d]	$Lin\text{-}CD14^+HLADR^-CD11b^+$
Plasmocytoid dendritic cells[d]	$Lin^-CD14^-CD123^+CD11c^-$
Conventional dendritic cells[d]	$Lin^-CD14^-CD123^-CD11c^+$
Upon *in vitro* t or b cell activation	
Signaling Pathways (Erk, p38MAPK)[e]	$CD3^+CD4^+CD8^-$ERK; $CD3^+CD4^+CD8^-$p38MAPK
Cytokine-producing $CD4^+$ ou $CD8^+$ T cells	$CD3^+CD4^+(CD8^+)$ $IL2^{-/+}IL4^{-/+}IL17^{-/+}TNF\alpha^{-/+}IL10^{-/+}IFN\gamma^{-/+}$
IL-10-producing Breg cells	$CD19^+CD24^{high}CD38^{high}IL10^+$
IL-10-producing Treg cells	$CD4^+CD25^{high}CD127^-Foxp3^+IL10^+$

[a] *$CD4^+(CD8^+) = CD4^+$ or $CD8^+$ T cells.*
[b] *CD45RO and CD62L are not essential to the panel.*
[c] *Other exhaustion markers (such as TIM-3, LAG-3) or activation markers (CD69, HLA-DR) can be used.*
[d] *Lineage (Lin) cocktail: CD3/CD19/CD20/CD56. Immunophenotyping panels were based on previous reports (12, 66–69).*

human autoimmune disorders support a role for the PD-1 pathway in self-tolerance mechanisms (77). Of note, during viral infections, the PD-1 molecule is upregulated on the surface of T cells during acute homeostatic proliferation (78, 79).

Thangavelu et al. (80) showed that recent thymic emigrants deficient in PD-1, which were generated after transfer of PD-1 deficient hematopoietic stem cells into lymphopenic adult Rag-deficient mice, induced a systemic inflammatory disease (80). Therefore, under lymphopenic conditions, PD-1 signaling is essential for systemic self-tolerance. In addition, Ellestad et al. (81) demonstrated that the most important role of PD-1 pathway is not to promote Treg expansion, but to control T cell activation and proliferation in response to self-antigens (81). Therefore, in lymphopenic environments, the PD-1 pathway is essential to regulate T cell activation and proliferation and consequently to avoid development of autoimmunity and AD.

In this context, Arruda et al. (43) showed that MS patients treated with AHSCT had expansion of $PD\text{-}1^+CD19^+$ B-cells and

PD-1$^+$CD8$^+$ T-cells early post-transplantation. Therefore, the PD-1 inhibitory pathway is considered an immune regulatory mechanism by which AHSCT restores auto-tolerance in patients with MS (45) and other AD (**Table 1**).

Our group has recently shown increased expression of PD-1 in T cells from SSc patients who responded to AHSCT. As a general mechanism to keep potentially autoreactive CD8$^+$ T-cell clones under control after AHSCT, PD-1 expression may be a reliable immune marker of clinical response in SSc and MS patients after AHSCT (48) (**Table 1**). Based on these recent data, we recommend the evaluation of expression of PD1 on T and B cell subsets in future immune monitoring studies (**Tables 2**, **3**). In addition, we suggest to evaluate the expression of Tim-3 (T cell immunoglobulin-3), Lag-3 (Lymphocyte activation gene-3 or CD223) and TIGIT (T cell immunoglobulin and ITIM domain), other co-inhibitory receptors with specialized functions in immune regulation, on T cells (82).

Decreased Autoreactivity Early Post-AHSCT

Elimination of autoreactive effector memory T cells in patients with AD is supposed to ameliorate the autoimmune aggression and diminish disease activity. Sun and colleagues showed reduced T cell responses to myelin basic protein in the reconstituted immune system of MS patients after AHSCT, when compared to pre-transplant levels (13) (**Table 1**).

Recently, we showed that pre-transplant frequencies of autoreactive CD8$^+$ T cells in T1D patients predicted the duration of insulin independency after AHSCT (26). Additionally, T1D patients that remained insulin free for longer periods also had persistently lower frequencies of autoreactive CD8$^+$ T cells compared with patients who resumed insulin after transplantation (26) (**Table 1**).

In our opinion, monitoring of antigen-specific autoreactivity should be performed routinely in immune monitoring studies, as reported previously (13, 17, 26). We acknowledge the enormous difficulties to quantify peripheral autoreactive CD4$^+$ or CD8$^+$ T cells based on the variety of autoantigens/peptides and class-I or class-II HLA molecules. However, collaborations should be settled with autoimmunity expert groups for monitoring autoreactive T cells on frozen PBMC patient samples or researchers could use commercially available HLA-tetramers.

We believe it would be very important to also investigate if late reactivation of the AD after AHSCT coincides with the increase of new autoreactive naive T cells produced by the reactivated thymus or/and with a decrease in immune regulatory networks (Treg and Breg cell numbers and/or function), triggered by environmental factors, leading to loss of the once achieved "fine immune balance."

Current data indicate that more intense immunoablation is associated with more favorable clinical outcomes after AHSCT (26, 31, 32, 35, 45) due to greater depletion of autoreactive T and B cells. However, complete eradication of the existing autoreactive immunologic memory may not be possible, even under high-intensity myeloablative conditioning regimens, since memory T and B cells reside in the bone marrow, are present in other body tissues and may survive immunoablative regimens (51, 83).

Currently, it is not known if immunosuppressive agents can infiltrate the tissues beyond the bone marrow. In experimental models, it has been shown that T cell depletion by ATG is more efficient in the blood compared with peripheral lymphoid organs (84). Park and Kupper (83) have demonstrated that tissue resident memory T cells are not totally depleted in non-barrier tissues after high dose immunosuppressive therapy (83). Curiously, in patients with refractory Crohn's disease, it was recently shown that the TCR repertoire diversifies after AHSCT in their intestinal tissue. The authors showed significant resetting of the TCR repertoire, since only 20% of TCR sequences were detected pre-transplantation and also 6 and 12 months post-AHSCT periods (49), prior to the production of any new naive T cells.

DISEASE-SPECIFIC IMMUNE MECHANISMS AFTER AHSCT FOR AD

So far, few disease-specific immune mechanisms of AHSCT have been reported. These mechanisms include quantification of disease-specific autoreactive T cells and their characterization (cytokine-profile, mRNA expression) (13, 16, 38); identification/quantification/functional characterization of different regulatory T cell subsets (33); and quantification of cytokine and other soluble markers related to disease pathogenesis (16, 17).

Darlington et al. (36) demonstrated that myelin-specific T cells reconstitute after AHSCT in all transplanted MS patients. These autoreactive T cells may expand from residual cells or may be newly generated by the thymus. Re-emerged myelin-specific T cells had the same Th1 and Th2 baseline profile, but Th17 cells decreased after transplantation, as demonstrated by lower RORγ expression and IL-17A serum concentration. In addition, levels of IL-1β and IL-6 were also decreased in MS patients after AHSCT (16). In MS patients, Muraro et al. (14) showed increased expression of Fas on CD4$^+$ and CD8$^+$ T cells after AHSCT (14). Moreover, the same group has shown that the pro-inflammatory CD8$^+$CD161^{high} mucosal-associated invariant T cell (MAIT) subset was depleted from the peripheral blood of MS patients after AHSCT (17) (**Table 1**).

Most reported studies have evaluated classical regulatory T cell subsets (**Table 1**) in the context of AHSCT for AD, but Zhang et al. (26) identified peripheral CD8$^+$ T cells from SLE patients with sustained high FoxP3 expression and increased expression of CTLA-4, PD-1, PD-L1, latency-associated peptide (LAP), and CD103, when compared with pre-transplant CD8$^+$ T cells. The CD8$^+$ Treg subset reconstituted post-transplantation presented increased suppressive activity, both autoantigen-specific and non-specific, which was predominantly TGF-β dependent and contact-independent. Therefore, the generation of a new LAP^{high}CD103^{high}CD8$^+$ Treg subset was reported, improving the immune regulatory deficiency and correlating with clinical remission (33) (**Table 1**).

In summary, we cannot conclude that the immune mechanisms cited above are disease-specific because they have not been yet evaluated in all AD subtypes currently treated

with AHSCT. For future immune monitoring studies, we suggest the evaluation of validated disease-specific biomarkers (closely related to disease pathogenesis) in patients with AD undergoing AHSCT therapy (85–93).

ESTABLISHING BIOMARKERS OF AHSCT FOR AUTOIMMUNE DISEASES

Recent mechanistic studies of AHSCT for AD are more complete and comprehensive. Higher numbers of transplanted patients, longer follow-up, different methodologies, and modern experimental approaches have contributed to the understanding of the reconstituting immune system. This mechanistic knowledge can be now reverted to the clinic, improving transplantation protocols and clinical outcomes. Moreover, the identification of new biomarkers may further enhance transplantation efficacy.

Biomarkers are urgently needed to improve disease diagnosis, to monitor disease activity and therapeutic responses, and to allow validation of new therapies (94–96). A biomarker is defined as a biological substance that can be quantified and dynamically evaluated in the context of normal biological or pathogenic processes and their progression, or in the context of clinical responses to therapeutic interventions. Biomarkers must be reproducible, robust and validated (94–96).

Mechanistic biomarkers may inform and validate how a particular treatment works. Over the past years, many mechanistic biomarkers of AHSCT for AD have been described and validated, based on different clinical outcomes (response and lack of response to treatment). In this context, increased PD-1 expression and expansion of Treg and Breg cells associate with a favorable clinical response to AHSCT in SSc, MS, and T1D patients (**Table 1**, **Figure 1**).

In addition, predictive biomarkers are disease-associated and indicate whether there is a chance of disease activation. They can also measure, at time of enrollment, the expected patient responsiveness to a specific treatment. Karnell et al. (15) proposed the increased numbers of memory $CD4^+$ and $CD8^+$ T cells at pre-transplantation as predictive biomarkers for response in MS patients. In addition, cumulative frequency of autoreactive islet-specific $CD8^+$ T cells at pre-transplantation predicts the clinical outcome of AHSCT in T1D patients (26). After AHSCT, T1D patients with higher autoreactivity before transplantation reactivated disease earlier than patients with lower autoreactivity (26).

Biomarkers that predict the future outcome of a patient with a specific disease, and also the overall survival rates and clinical benefits from a therapeutic intervention, are called prognostic biomarkers. Several clinical studies demonstrated that remission of AD after AHSCT associates with increased TCR repertoire diversity due to thymic reactivation and to the decrease of effector and memory T cell numbers (14, 27, 32, 39, 40). In this context, thymic reactivation may be considered a prognostic biomarker, associated with positive clinical outcomes (**Table 1**, **Figure 1**).

Additional prognostic biomarkers have been defined for AHSCT in AD, such as increased Treg frequencies and reduced inflammatory cytokine levels (IFN-γ and IL-12) in responsive Crohn's disease patients (37), increased frequencies of T and B cells expressing PD-1 in responsive MS patients (62), reduced levels of inflammatory markers (TNFR2, CXCL10, and GAL-9) in responsive juvenile dermatomyositis patients, and increased Treg numbers in long-term responsive T1D patients (26). More recently, Arruda et al. (48) demonstrated that clinical improvement of SSc patients is related to increased numbers of newly generated Treg and Breg after AHSCT, as a result of coordinated thymic and bone marrow rebounds (31) (**Table 1**, **Figure 1**).

CONCLUSIONS AND FUTURE DIRECTIONS

Since the publication of Sun et al. (13), we have increased our knowledge on mechanistic biomarkers of AHSCT (**Table 1**). It is now clear that most immune mechanisms are common to several AD and depend more on the conditioning regimen and quality of immune reconstitution, rather than only on disease pathogenesis (**Table 1**). Every parameter that affects the quality of immune reconstitution (e.g., infections, patient age, conditioning regimen, previous treatments) may affect clinical outcomes after AHSCT. Most patients respond to AHSCT and achieve long-term disease remission, however a subset of patients reactivate the AD after transplant (1, 4, 7–10) (**Figure 1**). Therefore, additional immune interventions are urgently warranted to improve AHSCT protocols.

In our opinion, we have reached another level of investigation in the field of AHSCT for AD. Future perspectives are protocol improvements (e.g., modifications in the conditioning regimens) and combined therapies (e.g., infusion of *in vitro* expanded immune regulatory cells, immune modulatory drugs). These may vary according to AD pathogenesis (**Figure 1**).

Strategies can be directed to improve specific immune regulatory mechanisms of AHSCT. For example, to increase the number and/or function of regulatory $CD4^+$ or $CD8^+$ T cell subsets in patients who did not respond sufficiently to the AHSCT as a single treatment, we could propose combined therapies with similar immune mechanisms of action, such as administration of intravenous immunoglobulin (IVIG) (97), vitamin D (98) or low-dose rapamycin (99), infusion of autologous expanded Treg (100), or infusion of autologous or allogeneic mesenchymal stromal cells (101).

The ultimate importance of immune monitoring evaluations in patients undergoing AHSCT for AD is their impact in the clinical setting and consequent contributions to improve safety and efficacy of clinical protocols. Here, we provide three examples of how immune monitoring evaluations have already impacted the AHSCT scenario.

In 2004, Sun et al. (13) showed that myelin-specific T cells were not detected in MS patients at early periods post-AHSCT but that they reconstituted 1 year post-transplantation. Similarly, our group has shown that type 1 diabetes patients with higher frequencies of autoreactive islet-specific T cells before transplantation reactivate the disease earlier than patients with

lower frequencies of these cells (26). These data indicate that conditioning regimens might have different efficacy on different AD and/or patients depending on their immune status and/or level of autoreactivity before transplantation. Indeed, our study has shown that high dose immunosuppression was not strong enough to sufficiently deplete autoreactive islet-specific T cells (26). Based on these observations, our center has increased the immunoablative intensity of the conditioning regimen protocol from 200 mg/Kg of cyclophosphamide plus 4.5 mg/Kg of rabbit anti-thymocyte globulin (ATG), to 120 mg/Kg of cyclophosphamide plus 150 mg/Kg of fludarabine and 4.5 mg/Kg of ATG, to further increase T and B cell ablation.

In the second example, Dubinsky et al. (25) demonstrated that T-cell clones persisting in the peripheral blood after autologous hematopoietic SCT were undetectable in the $CD34^+$ selected graft (16). We can therefore assume that the residual autoreactive cells, responsible for disease reactivation in some patients, did not emerge from the $CD34^+$ autograft, but most probably survived the immunoablative regimen. In this context we recommend, for future mechanistic investigations, the evaluation of graft composition (all T and B naive and memory subsets, regulatory T and B cell subsets, autoreactive cells) after $CD34^+$ cell mobilization for each transplanted AD disease, as well as its correlation with clinical outcomes.

Third, some studies have evaluated how $CD34^+$ graft selection influences the immune reconstitution after AHSCT (1). Recently, Keever-Taylor et al. (102) showed that the manufacturing of autologous $CD34^+$ cells in the "High-Dose Immunosuppression and Autologous Transplantation for Multiple Sclerosis" (HALT MS) and "Scleroderma: Cyclophosphamide or Transplantation" (SCOT) protocols was comparable across all transplantation centers and allowed successful granulocyte and platelet recoveries. On the other hand, Oliveira et al. (44) have demonstrated that $CD34^+$ selection does not add benefit to the outcomes of transplanted SSc patients. These findings should be further confirmed by prospective randomized trials (22).

Immune monitoring and biobanking guidelines for AHSCT in patients with AD patients have been established by the European Blood and Marrow Transplantation (EBMT) group, in 2015 (12). They have provided very reliable recommendations for comparative research studies envisioning biomarker discovery (12). We believe EBMT guidelines for immune monitoring are still valid, especially for disease-specific evaluations. However, new data on the topic have been made available in the past 3 years, and we believe it is important to add them to this perspective, as additional/complementary recommendations (12) (**Figure 1**, **Tables 2**, **3**).

Here, we encourage standardization of immune monitoring studies throughout transplantation centers worldwide associated with data registries of clinical and laboratory results, aiming to empower statistical analyses. Nowadays, with modern biobanking infrastructure and internationalization trends it should be easier to manage multicenter immune monitoring studies in order to identify potential and consistent biomarkers.

Discovery and development of biomarkers are definitely an unmet need in the field of AHSCT for AD. Some biomarkers can serve as early surrogates of eventual clinical outcomes or guide therapeutic decisions by enabling identification of individuals likely to not respond to this therapy (50, 85–87, 103).

In recent years, both traditional and next-generation applications, including large-scale transcriptomic, epigenomic, genomic, lipidomic, cytomic, and proteomic technologies have yielded a huge amount of new candidate biomarkers that correlate with different clinical phenotypes of AD. Researchers should now focus on discovering and developing such biomarkers that would allow the improvement of clinical protocols and therapeutic efficacy of AHSCT for AD (57–61).

We encourage the scientific community to discuss future therapeutic directions and to establish updated guidelines of immune monitoring studies in order to expand the field. The following points should be addressed in the future, to better understand and subsequently improve AHSCT-AD outcomes:

- Establish a standardized immune monitoring platform for AHSCT-AD clinical trials: (a) to harmonize immune reconstitution results allowing centers to gather data/results and perform meta-analysis; (b) to invest in biomarker discovery by modern technologies and appropriate biobanking logistics (see recommendations on **Tables 2**, **3**, and **Figure 1**);
- Conduct additional multicenter clinical trials to harmonize clinical and also immune monitoring data, allowing significant, and conclusive results about AHSCT-AD efficacy and immune mechanisms;
- Establish standardized conditioning regimens for each AD, based on the recent experiences and clinical achievements from each group. Since conditioning regimens have great impact on immune reconstitution and potentially on clinical outcomes, there is an urgent need for standardization. Later, more personalized conditioning approaches may be allowed, based on the AD disease and patient immune status.
- Develop combined therapies to improve therapeutic efficacy of AHSCT in AD patients who do not respond sufficiently to transplantation as a single treatment (**Figure 1**).

ETHICS STATEMENT

This study has been approved by the Brazilian institutional review board, where the patients were enrolled, and complied with country-specific regulations. The study was conducted according to the Declaration of Helsinki and Good Practice Guidelines. All patients read and signed informed consents.

AUTHOR CONTRIBUTIONS

KM and MO wrote the manuscript. LA, JL-J, JdA, GdO performed literature review data analysis. All authors revised the manuscript and contributed to the final editing. All authors agreed with the content of the manuscript.

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