IMMUNOLOGICAL TOLERANCE IN TRANSPLANTATION: MORE THAN DELETION

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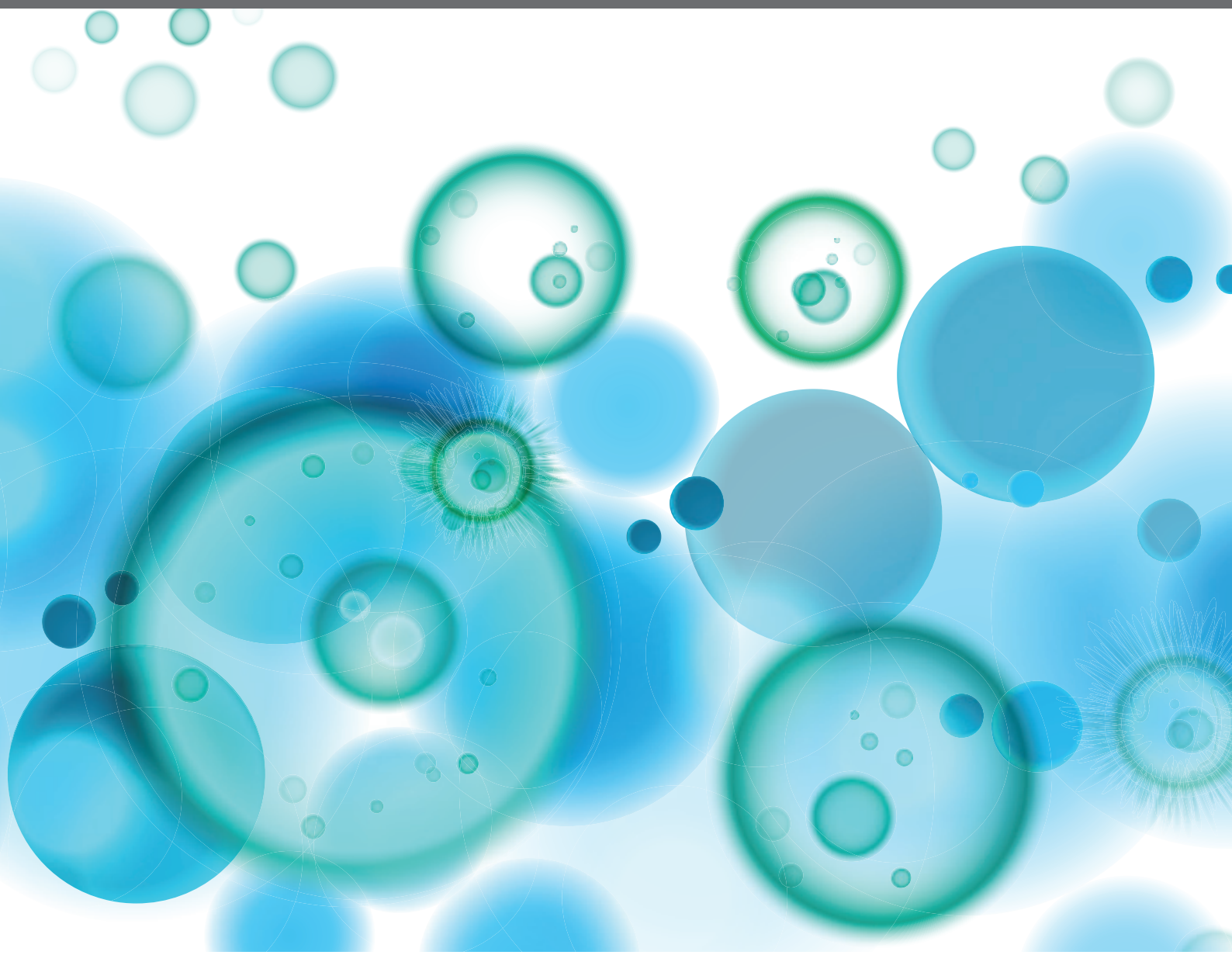

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IMMUNOLOGICAL TOLERANCE IN TRANSPLANTATION: MORE THAN DELETION

Topic Editors:
Nina Pilat, Medical University of Vienna, Austria
Edward Geissler, University of Regensburg, Germany
Thomas Wekerle, Medical University of Vienna, Austria

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EDITORIAL
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Editorial: Immunological Tolerance in Transplantation: More Than Deletion

Nina Pilat[1]*, *Thomas Wekerle*[1], *Edward K. Geissler*[2] and *Jonathan Sprent*[3,4]

[1] Division of Transplantation, Department of General Surgery, Medical University of Vienna, Vienna, Austria, [2] University Hospital Regensburg, Department of Surgery, University of Regensburg, Regensburg, Germany, [3] Immunology Division, Garvan Institute of Medical Research, Darlinghurst, NSW, Australia, [4] St Vincent's Clinical School, University of New South Wales, Sydney, NSW, Australia

Keywords: immunological tolerance, transplantation, mixed chimerism, Tregs, tolerance approaches, intragraft tolerance

Editorial on the Research Topic

Immunological Tolerance in Transplantation: More than Deletion

INTRODUCTION

In transplantation medicine, induction of donor-specific tolerance remains the "holy grail" to protect organs from rejection. Indeed, clinicians and immunologists still struggle to prevent rejection and improve long-term graft survival, with an acceptable safety profile. Transplant recipients still require life-long chronic immunosuppression, which results in drug-specific side effects and complications from nonspecific suppression of the immune response.

Tolerance is presumed to be an active equilibrium between allo/auto-reactive and regulatory immune mechanisms. Antigen-specific T regulatory cells (Tregs) as well as other regulatory cell subsets are critical for the maintenance of self-tolerance and have been recognized as promising and potent therapeutic tools in transplantation. However, although numerous tolerance approaches have been developed in preclinical animal studies (mostly rodents), translation into large animal models or clinical application has almost always failed the ultimate test.

This Research Topic brings together 9 articles that aim to tackle the most important questions about tolerance mechanisms and their translation into the clinic.

ARTICLE COLLECTION

The mixed chimerism approach, which involves co-transplantation of hematopoietic stem cells (HSCT) and a solid organ from the same donor, is the only tolerance approach which has been successfully translated into a clinical setting. However, widespread clinical application of this approach is still impeded by the often severe effects of cytotoxic recipient pretreatment and the risk of graft-versus-host disease (GVHD). To this point, Fehr et al recently reported on the first patients in Europe receiving combined kidney and HSCT for successful tolerance induction. Three patients receiving kidney and HSCT from an HLA-identical sibling donor were successfully weaned from immunosuppression without rejection or GVHD episodes. At the time of this writing, these three patients have been off immunosuppression for 4 years, 19 and 8 months. Importantly, all three

Edited and reviewed by:
Lucienne Chatenoud, Université Paris Descartes, France

***Correspondence:**
Nina Pilat
nina.pilat@meduniwien.ac.at

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patients showed excellent responses to vaccinations against SARS-CoV-2 in the ongoing pandemic with strong humoral and cellular specific immunity. The protocol used was previously developed by the Stanford group, who have decades of preclinical and clinical experience with the chimerism tolerance approach in HLA-identical siblings. Lowsky and Strober have contributed an excellent review on this topic, pointing out differences in the mechanisms of organ graft acceptance in mixed chimeras versus full chimeras.

Another excellent review about tolerance mechanisms induced by HSCT was contributed by Podesta and Sykes. Whereas tolerance is mainly maintained by central deletion of graft-reactive donor T cells in full chimeras, tolerance mechanisms in transient mixed chimeras rely largely on Treg-mediated suppression plus peripheral (intragraft) deletion of donor-reactive recipient T-cell clones. Although these mechanisms are not fully understood, a better understanding of intragraft tolerance mediated by regulatory mechanisms will be important for devising new approaches for preventing chronic rejection and allo-sensitization, and eventually achieving the ultimate goal of "one transplant for life". Currently, however, universally accepted and validated biomarkers for clearly defining tolerance in transplant patients have remained elusive. Here, the authors summarized the most important mechanistic studies on tolerance induction as well as cutting-edge methods to identify patients worth considering for safe immunosuppression withdrawal.

In another comprehensive review by Hall et al (Clonal deletion and T cell mechanisms that mediate transplant tolerance), the authors sum up historical aspects that have led to development of the clonal deletion theory before the discovery of T cells, and an improved understanding of the immune responses. Furthermore, models of operational tolerance, the concept of split tolerance, and the importance of regulatory mechanisms are summarized. Here, the authors discuss the role of clonal deletion in transplant tolerance in light of regulatory T cells and their ability to mediate antigen-specific tolerance.

With regard to inducing tolerance, extracorporal photopheresis (ECP) is an apheresis procedure involving the removal of peripherally circulating white blood cells, addition of a light sensitizer, exposure to UV light, and return of the cells to the patient. By mechanisms that are yet not fully understood, ECP can promote the transition from an inflammatory state to a pro-tolerance state in some settings. Originally approved for cutaneous T cell lymphoma, ECP is used for prevention/treatment of GVHD after HCST, but also to reduce rejection in solid organ transplantation. Dieterlen et al reported on a novel immune monitoring assay for ECP treatment after heart transplantation. Based on flow cytometric measurements of dendritic cell subsets and Tregs, the authors proposed classification criteria to identify patient-specific immunological improvement. This tool may allow optimization of ECP treatment duration in heart transplant patients; however, a multicenter study would be needed for validation of clinical use.

Solid organ and cellular grafts contain donor-derived bone marrow-derived hematopoietic cells. The role of these so-called "passenger leukocytes" in transplantation immunology is complex. Thus, although it has long been known that these cells can initiate graft rejection, in certain settings they can also contribute to graft acceptance. In this respect, Hitz et al aimed to characterize the expression of killer cell immunoglobulin-like receptors (KIR) on donor and recipient myeloid cells in recipient blood in double-lung transplant patients. For these patients, the authors report enhanced frequencies of donor NK and T cells expressing regulatory KIR; in addition, they find evidence suggestive of pre-activation of donor cells during the ischemic phase. In view of these findings, donor NK and T cells in lung transplant recipients may both play a role in regulating alloresponses and contributing to allograft tolerance.

In a murine model of Treg-mediated skin graft survival, Steiner et al investigated the mechanisms by which graft-resident leucocytes impact skin allograft survival. In this model, diminution of graft-resident leucocytes leads to a decreased infiltration of recipient T cells into the graft, thereby switching the intragraft cell composition to a more tolerogenic milieu. This study demonstrates the importance of donor-derived leucocytes in recipient sensitization and allograft rejection, and suggests that long-term graft survival could be improved by targeting the early stages of T cell allorecognition.

With regard to initial stem cell engraftment, Bhat et al aimed to evaluate immune reconstitution following haplo-HSCT for treatment of sickle cell disease. The authors performed longitudinal flow cytometric analysis of leucocyte subsets in PBMCs and cytokine analysis in serum of patients post non-myeloablative haplo-HSCT. Their data suggest that successful HSCT engraftment may depend upon an early increase in numbers of myeloid-derived suppressor cells. Notably, successful engraftment correlated with elevated levels of suppressive cytokines. Hence, the latter could serve as potential prognostic markers in predicting successful engraftment after HSCT.

Endoribonuclease Regnase-1 is expressed in T and B cells and is known to be an important feedback mechanism for negative regulation of immune responses. Here, Kong et al developed a transgenic mouse model expressing mutant Regnase-1 with improved *in vivo* stability. These transgenic mice are lymphopenic and show a marked decrease in numbers of mature T cells, leading to a failure to reject fully MHC-mismatched skin grafts. The authors demonstrated that the paucity of T cells reflected impaired T cell development in the thymus due to disrupted TCR signaling during positive selection. This study suggests Regnase-1 as a therapeutic target for the modulation of T cell function.

CONCLUSION

This Research Topic highlights recent preclinical and clinical findings on allotransplantation and provides new insights on the complex immunoregulatory mechanisms that induce and impede immunological tolerance towards allografts. Further work on this topic may lead eventually to the realization of the

ultimate goal of achieving permanent graft survival without the need for immunosuppression.

AUTHOR CONTRIBUTIONS

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

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ORIGINAL RESEARCH
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Transgenic Expression of a Mutant Ribonuclease Regnase-1 in T Cells Disturbs T Cell Development and Functions

Gangcheng Kong[1,2], *Yaling Dou*[1], *Xiang Xiao*[1], *Yixuan Wang*[1], *Yingzi Ming*[2] *and Xian C. Li*[1,3]*

[1] Immunobiology & Transplant Science Center, Houston Methodist Hospital, Texas Medical Center, Houston, TX, United States, [2] Transplantation Center of the 3rd Xiangya Hospital, Central South University, Changsha, China, [3] Department of Surgery, Weill Cornell College of Cornell University, New York, NY, United States

Regnase-1 is an RNA-binding protein with ribonuclease activities, and once induced it controls diverse immune responses by degrading mRNAs that encode inflammatory cytokines and costimulatory molecules, thus exerting potent anti-inflammatory functions. However, Regnase-1 is extremely sensitive to degradation by proteases and therefore short-lived. Here, we constructed a mutant Regnase-1 that is resistant to degradation and expressed this mutant *in vivo* as a transgene specifically in T cells. We found that the mutant Regnase-1 transgenic mice exhibited profound lymphopenia in the periphery despite grossly normal spleen and lymph nodes, and spontaneously accepted skin allografts without any treatment. Mechanistic studies showed that in the transgenic mice thymic T cell development was disrupted, such that most of the developing thymocytes were arrested at the double positive stage, with few mature $CD4^+$ and $CD8^+$ T cells in the thymus and periphery. Our findings suggest that interfering with the dynamic Regnase-1 expression in T cells disrupts T cell development and functions and further studies are warranted to uncover the mechanisms involved.

Keywords: Regnase-1, T cell development, positive selection, lymphopenia, skin transplantation, tolerance

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Nina Pilat,
Medical University of Vienna, Austria

Reviewed by:
Vigo Heissmeyer,
Helmholtz-Gemeinschaft Deutscher Forschungszentren (HZ), Germany
Jonathan Sprent,
Garvan Institute of Medical Research, Australia

***Correspondence:**
Xian C. Li
xcli@houstonmethodist.org

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INTRODUCTION

Regnase-1 (also known as Zc3h12a and MCPIP1) is an RNase that is widely expressed in various immune cells, including macrophages, B cells and T cells (1). In the immune system, most of the mRNAs encoding cytokines and costimulatory molecules are short lived, which constitutes an important negative regulatory mechanism of immune responses (2, 3). Regnase-1 degrades mRNAs by recognizing a specific stem loop structure at 3'UTR of mRNA in concert with the helicase UPF1 (4, 5). Thus, Regnase-1 serves as an anti-inflammatory role by breaking down mRNAs that encode potent inflammatory cytokines, and consequently inhibits immune activation. Some of the known targets of Regnase-1 include mRNAs for *Il2, Il6*, *Il12b*, *c-Rel* and *Ox40* (5). As the immune responses are often tightly controlled in order to prevent collateral damage due to sustained immune responses (6, 7), Regnase-1 acts as an important feedback regulatory mechanism to negatively control immune responses.

However, Regnase-1 is also short lived *in vivo* and often degraded rapidly after immune activation (2, 8). For example, T cell antigen receptor (TCR) stimulation leads to the induction CARMA1-Bcl10-Malt1 signaling complex (CBM complex) in T cells, which is critical in mediating T cell activation and effector differentiation, primarily by activating the NF-κB and MAPK pathways (9–11). Importantly, activation of the Malt1 complex also cleaves the Regnase-1 (8, 12), which allows the prolonged expression of survival and key signaling molecules in activated T cells. Specifically, Malt1, which has proteolytic activities, cleaves Regnase-1 at the Arginine 111 site, leading to inactivation of Regnase-1 (8). On the other hand, TCR signaling can also upregulate the expression of Regnase-1, which is important in limiting persistent T cell activation (8). Thus, Regnase-1 plays a dynamic role in fine-tuning the activation of T cells.

In addition to activating T cells, signals transduced by TCR are critical for T cell development in the thymus (13). In developing thymocytes, the interactions between TCR and peptide-MHC complex trigger dynamic changes of gene expression in thymocytes in supporting cell survival and further maturation (14, 15). In fact, only after productive rearrangement of *TCR-β* gene and signaling *via* the pre-TCR can thymocytes progress forward beyond the DN3 stage (16, 17). In fact, recognition of the peptide-MHC complex on thymic stromal cells by the αβ TCR on developing thymocytes is vital for T cell survival and differentiation from DP to mature SP stage (18). The affinity of the interaction of the TCR and peptide-MHC complex determines thymocytes fate decisions. Weak interactions protect thymocytes from apoptotic death and promote the positive selection (19). Only a small proportion of DP thymocytes with functional TCR and proper affinity for the MHC complex can survive from thymic selection, and the majority of thymocytes with high affinity for the MHC complex and therefore strong TCR signaling undergo apoptosis (20).

In our study, we constructed a mutant Regnase-1, in which the arginine 111 was replaced with alanine (i.e., R111A), and expressed this mutant in T cells as a transgene to study how Regnase-1 affects TCR signaling, T cell development and functions. We found that this mutant mouse had profound lymphopenia in the periphery due to a developmental defect in the thymus. In a skin transplant model, we observed long term skin allograft survival in the mutant mice without any treatment. Our results highlight the importance of dynamic regulation of Regnase-1 in T cell activities and further suggest that Regnase-1 may be targeted to modulate T cell functions.

MATERIALS AND METHODS

Mice

To create the R111A mutant transgenic mice, we inserted the CAG-LoxP-STOP-LoxP-Mcpip1(R111A)-P2A-EGFP cassette into the mouse *Rosa26* locus (21). *Cd4*-Cre, BALB/c mice were purchased from Jackson Laboratory (Bar Harbor, MA). *Mcpip1 (R111A)* knock-in mice were crossed to *Cd4*-Cre mice to conditionally overexpress Regnase-1 in $CD4^+$ T cells (Reg-1^{CD4KI}). *Mcpip1*^{KI}*Cd4*-WT mice were considered as WT littermate control. Mice at age of 6-8 weeks were used for all the experiments. All animals were maintained in specific pathogen free facility. All animal experiments in this study were approved by the Houston Methodist Animal Care Committee, in accordance with institutional animal care and use committee guidelines.

Cell Line

HEK293 cells were obtained from the ATCC and were maintained in DMEM supplemented with 10% FBS and 1% penicillin streptomycin.

Antibodies

Fluorescein conjugated antibody to mouse CD45 (Clone: 30-F11), CD3 (Clone: 17A2), CD4 (Clone: GK1.5), CD8 (Clone: 53-6.7), CD44 (Clone: IM7), CD25 (Clone: PC61), TCR-β (Clone: H57-597), CD69 (Clone: H1.2F3), CD62L (Clone: MEL-14), TNF-α (Clone: MP6-XT22), IFN-γ (Clone: XMG1.2), OX40 (Clone: OX-86), KLRG1 (Clone: 2F1/KLRG1) from BioLegend; CD5 (Clone: 53-7.3), Bcl-2 (Clone: 10C4), ICOS (Clone: 7E.17G9), PD-1 (Clone: J43), Foxp3 (Clone: FJK-16s) from Thermo Fisher were used in this study. Antibody for immunoblot in this study included the following: anti-Flag (F7425, Sigma), c-Myc antibody (9E10, Santa Cruz), anti-β-actin (C4, Santa Cruz), anti-Mcpip1 (604421, R&D), anti-Malt1 (cat #2494, Cell Signaling Technology).

Mouse Skin Transplantation and Histology Staining

BALB/c tail skin allografts were transplanted onto the fully MHC-mismatched WT B6 or Reg-1^{CD4KI} recipients as previously described (22). More than 80% necrosis of the donor skin tissue was considered as rejection. Hematoxylin & eosin (H&E) staining was performed on paraffin sections of thymus and skin grafts.

Flow Cytometry

The thymus and spleen were harvested to obtain single lymphocytes suspension. Red blood cells were lysed with ACK Lysing Buffer (Gibco). Live cell numbers were counted and the obtained samples (1×10^6 cells) were then stained with fluorescein conjugated antibody in 100 μl MACS buffer (Miltenyi Biotec) for 30 min at 4°C in the dark for surface staining. For intracellular staining, total thymocytes were fixed and permeabilized using the Foxp3/Transcription factor staining kit according to the procedure recommended by ThermoFisher. For cytokine staining, splenocytes were stimulated for 5 h with phorbol 12-myristate 13-acetate (50 ng/ml) and ionomycin (500 ng/ml; Sigma-Aldrich) in the presence of GolgiStop (BD PharMingen). After surface marker staining, splenocytes were fixed and permeabilized with Cytofix/Cytoperm solution (BD PharMingen), and then stained with fluorescein conjugated TNF-α and IFN-γ antibody following the manufacturers' instruction. All samples were acquired with LSRII (Beckton

Dickinson) and the data were analyzed with FlowJo v10 software (Tree Star).

Cell Sorting

Single thymocytes suspension was obtained as above. $CD4^+CD8^+$ DP thymocytes were sorted by an FACSAria cell sorter (BD Biosciences).

RNA Isolation, cDNA Synthesis and RT-qPCR

Total RNAs from sorted DP thymocytes were prepared using Trizol reagent (ThermoFisher). The supernatants containing total RNAs were further purified with Direct-zol RNA MicroPrep Kit (Zymo Research). 0.1-1 μg of total RNAs were then reverse transcribed with iScript Reverse Transcription Supermix (BIO-RAD) according to the manufacturers' instruction. The obtained cDNAs were tenfold-diluted and subjected into RT-qPCR experiments by using CFX96 Touch Real-Time PCR Detection System (BIO-RAD) and SsoAdvanced Universal SYBR Green Supermix (BIO-RAD). Primers for RT-qPCR were synthesized by Integrated DNA Technology (**Supplementary Table 1**). Expression was normalized to HPRT. The data were analyzed using the delta-Ct method.

Retrovirus-Mediated Gene Transfer

The cDNA fragments encoding mouse Regnase-1, Malt1, Bcl10 were amplified by PCR and further cloned into the pMYs-IRES-EGFP retroviral vector (Cell Biolabs). The Arginine at 111 of Regnase-1 was mutated to Alanine using Q5 Site-Directed Mutagenesis Kit (NEB) following the manufacturers' instruction. For transfection of HEK293 cells, Transporter 5 Transfection Reagent (Polysciences) was used according to the manufacturers' instruction.

Immunoblot Analysis

Protein extracts from HEK293 cells and thymocytes were prepared by washing the cells with cold PBS and then lysed in Pierce IP lysis buffer (87787, ThermoFisher Scientific) containing proteases and phosphatase inhibitor cocktail (78440, ThermoFisher) for 10 min. After 15,000g centrifugation for 10 min, the supernatants were transferred to new Eppendorf tubes. Whole cell lysates were subjected to immunoblot assay using standard procedures.

Luciferase Assay

HEK293 cells were transfected with luciferase reporter plasmid pmiGLO containing the 3'UTR of *Il6* or empty vector, together with expression plasmid for Regnase-1 or empty (mock) plasmid. After 24 hours cultivation, cells were lysed and relative luciferase activity in lysates was detected using Dual-Luciferase Reporter Assay system (Promega). The gene encoding Renilla luciferase on pmiGLO plasmid was used as an internal control.

Ex Vivo Thymocytes Cell Death Assay

1×10^5 total thymocytes were cultured with PMA (50 ng/ml) and ionomycin (500 ng/ml; Sigma-Aldrich) in 200 μl of RPMI1640 media (10% FBS, 1% penicillin streptomycin, 50 μM 2-mercaptoethanol) in a 96-well flat bottom plate for 4h. Viability was measured by Annexin V and 7-AAD staining using Annexin V FLUOS staining kit (Roche).

Ex Vivo TCR Stimulation Assay

96-well plates were coated with anti-CD3ε (5 μg/ml; Clone: 145-2C11, eBioscience) for 2h at 37 °C. After 1 time-wash with 100 μL PBS, 1×10^5 total thymocytes were cultured in 200 μl of RPMI1640 media (10% FBS, 1% penicillin streptomycin, 50 μM 2-mercaptoethanol) with anti-CD28 (1 μg/ml; Clone: 37.51, eBioscience) for 24h. Viability was measured by Annexin V and 7-AAD staining using Annexin V FLUOS staining kit (Roche). Total RNA from DP thymocytes was extracted after 3h TCR stimulation.

Statistical Analysis

The unpaired two-tailed Student's *t* test was used for comparison between two groups. One-way ANOVA was used to generate *P* value between multiple groups. The Log-rank test was used to determine the *P* value of skin-graft survival time. Data were represented as mean ± SD and analyzed with Prism version 8 (GraphPad Software). The $P < 0.05$ was considered statistically significant, as shown in the figure legends.

RESULTS

Enhanced Stability of the Regnase-1 Mutant Without Interference of Its Rnase Activity

To investigate the stability and function of Regnase-1, we constructed a wild type and a mutant Regnase-1 where the arginine 111 was replaced by alanine (i.e., R111A). As shown in Fig 1A, co-transfection of wild type Regnase-1 with Malt1 and Bcl10 into HEK293 cells led to the cleavage of Regnase-1, resulting in a ~20 kDa and a ~53 kDa fragment. In contrast, the R111A mutant, when expressed in HEK293 cells, showed resistant to Malt1 mediated degradation (**Figure 1A**). We also performed luciferase assay to test the Rnase activity of the mutant Regnase-1. We transfected HEK293 cells with pmiGLO empty vector and Regnase-1 expressing plasmid. In the absence of the 3'UTR, luciferase activity of HEK293 cells was not influenced by the expression of Regnase-1. We then generated a luciferase reporter construct with 3'UTR of *Il6* mRNA, which has been shown to be a target of Regnase-1. Addition of *Il6* 3'UTR resulted in reduction of the luciferase activity in response to wild type and R111A mutant Regnase-1. In contrast, the overexpression of Rnase-inactive form (D141N) Regnase-1 failed to inhibit luciferase expression (**Figure 1B**) (8). Our results indicate that Rnase activity of Regnase-1 was not impacted by R111A mutant.

To further investigate the function of Regnase-1 in T cells, we generated the mutant Regnase-1 conditional knock-in mice ($Reg\text{-}1^{CD4KI}$) by insertion of R111A mutant *Regnase-1* into *Rosa26* locus (**Figure 1C**) and crossed these mice with *Cd4*-cre

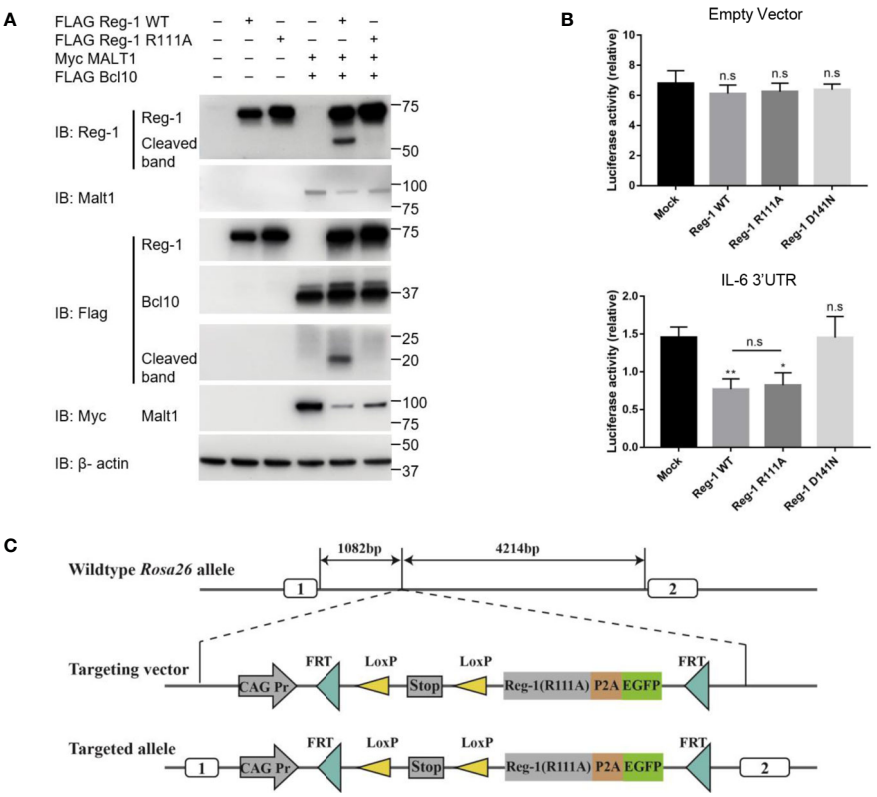

FIGURE 1 | The effects of R111A mutant on Regnase-1 and the strategy of generating Regnase-1(R111A) transgenic mice. **(A)** Immunoblot analysis of lysates from HEK293 cells transfected with Flag-Regnase-1 (WT or R111A) and/or Myc-Malt1 and Flag-Bcl10 expression plasmids. **(B)** Relative luciferase activity of HEK293 cells transfected with pmiGLO plasmids and Regnase-1 expression plasmids. **(C)** Strategy for insertion of the R111A mutant Regnase-1 into the mouse *Rosa26* allele. Data are mean ± SD (n=3) from one experiment, representative of three independent experiments. n.s not significant; *$P < 0.05$; **$P < 0.01$ (one-way ANOVA test).

mice (**Supplementary Figure 1A**). *EGFP* was the reporter gene to track cells expressing the mutant Regnase-1. The genotype of mouse was confirmed using genotyping (**Supplementary Figure 1B**). As compared to wild type B6 control mice, the Reg-1^{CD4KI} mice bred well, appeared normal, and born in accordance with Mendel's fashion. Of note, we did not observe any non-lymphocytic abnormalities in the Reg-1^{CD4KI} mice.

The Mutant Regnase-1 Transgenic Mice Exhibit Lymphopenia and Accept Skin Allografts

We examined the T cell compartment in peripheral tissues of Reg-1^{CD4KI} mice, total splenic cells, mature CD4$^+$ and CD8$^+$ T cells were both significantly decreased in spleen (**Figures 2A, B**), despite the normal size of the spleen (**Supplementary Figure 1C**). The number of Tregs in spleen was decreased, but the proportion of Tregs in splenic CD4$^+$ T cell was comparable (**Supplementary Figure 2A**). Furthermore, the expression level of TCR-β on CD4$^+$ or CD8$^+$ T cell in spleen was also significantly lower in Reg-1^{CD4KI} mice, as compared to in WT mice (**Figure 2C**). And there were few mature T cells expressing EGFP (**Figure 2D**). Thus, in naïve mice, overexpressing Regnase-1 appeared to inhibit the maturation of T cells. In addition, peripheral CD4$^+$ or CD8$^+$ T cells from Reg-1^{CD4KI} mice had increased frequency of memory (CD44^{hi}CD62L^{lo}) cells than those in WT mice (**Figures 2E, F**), which was consistent with the observation that mice with T cell depletion therapies had more T cells with a memory phenotype.

To investigate the T cell-mediated activities, we transplanted tail skins (0.8 cm × 0.8 cm) from BALB/c mice onto Reg-1^{CD4KI} and WT B6 recipients. The survival time of tail skin grafts on Reg-1^{CD4KI} recipients (mean survival time = 74.0 ± 12.9 days; n = 6) was significantly prolonged than that on WT recipients (mean survival time = 9.3 ± 0.3 days; n = 6) (**Figure 3A**). There were no signs of rejection of skin grafts on Reg-1^{CD4KI} recipients, but all skin grafts were rejected by WT recipients at day 10 (**Figure 3B**). In addition, the skin allografts from three Reg-1^{CD4KI} recipients survived more than 100 days (**Figures 3A, B**). H&E staining of skin grafts at day 10 showed that the skin grafts from WT recipients were destroyed and there were large number of lymphocytes infiltrating the graft tissue. In contrast, the skin grafts from Reg-1^{CD4KI} recipients at day 10 and day 100 were intact, with minimal infiltrating lymphocytes (**Figure 3C**). Taken together, overexpressing Regnase-1 in T cells led to the acceptance of allogeneic skin graft.

We analyzed the phenotype of splenocytes at 10 days post-grafting. The number of CD4$^+$ or CD8$^+$ T cells were significantly

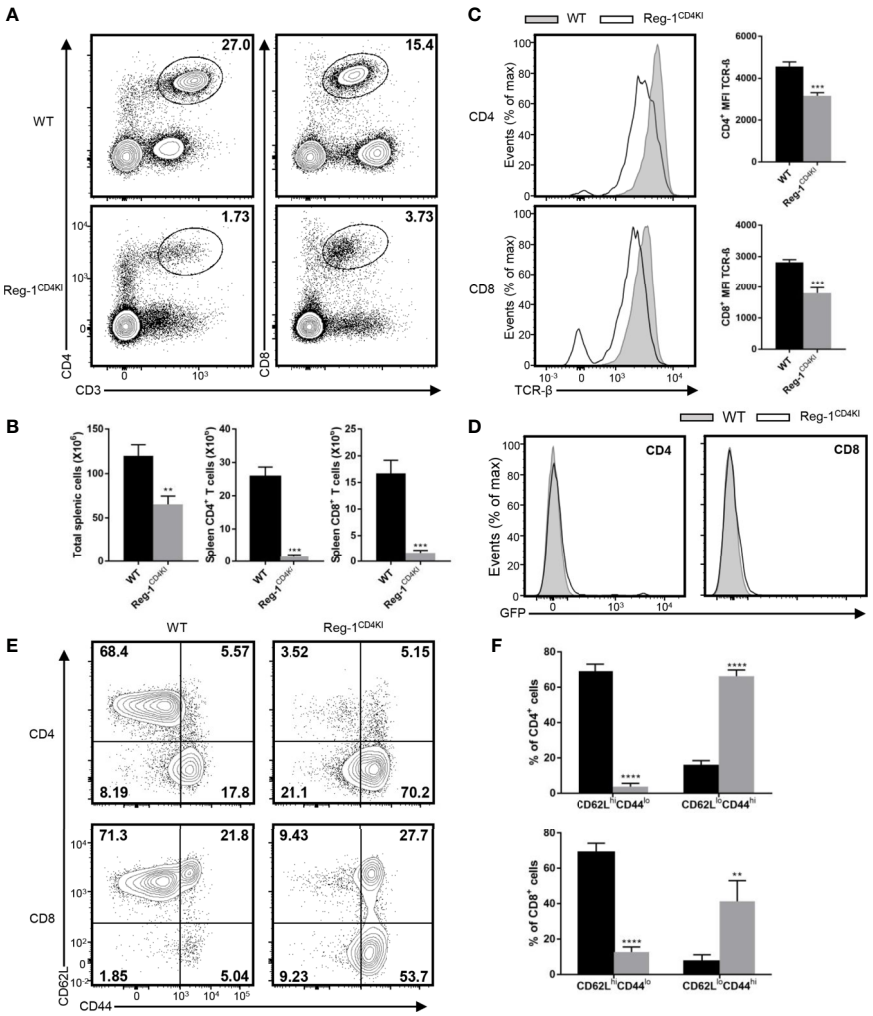

FIGURE 2 | Phenotype of peripheral T cells in Reg-1^{CD4KI} mice. **(A)** Surface staining of CD3, CD4 and CD8 on WT or Reg-1^{CD4KI} splenocytes. The percentage of CD4$^+$ and CD8$^+$ T cell in CD45$^+$ alive splenocytes. **(B)** The number of total splenocyte, CD4$^+$ and CD8$^+$ splenocytes from WT or Reg-1^{CD4KI} mice. **(C)** Flow cytometric analysis and mean fluorescence intensity of TCR-β on CD4$^+$ or CD8$^+$ splenocytes. **(D)** The analysis of EGFP expression in spleen cells from Reg-1^{CD4KI} mice or WT littermates at age of 6-8 weeks. **(E)** Expression of CD62L and CD44 on CD4$^+$ or CD8$^+$ splenocytes from WT or Reg-1^{CD4KI} mice. **(F)** The frequency of CD62L^{hi}CD44^{lo} and CD62L^{lo}CD44^{hi} subpopulations in the CD4$^+$ or CD8$^+$ cells. Data are mean ± SD (n=3) from one experiment, representative of two independent experiments. $^{**}P<0.01$; $^{***}P<0.001$; $^{****}P<0.0001$ (unpaired two-tailed Student's *t* test).

lower in the spleen of Reg-1^{CD4KI} recipients (**Figure 4A**) as compared to that in WT mice. The number of activated CD4$^+$ and CD8$^+$ T cells was also decreased in Reg-1^{CD4KI} recipients, but a higher proportion of CD4$^+$ T cells displayed an activated CD44^{hi}CD62L^{lo} phenotype was observed (**Figure 4B**). In addition, there were no significant differences between the expression level of activation markers on CD44^{hi} effect T cells, such as ICOS, OX40, PD-1 and KLRG1 (**Supplementary Figure 2B**). We also assessed the effector functions of T cells by measuring intracellular expression of TNF-α and IFN-γ. As shown in **Figure 4C**, CD4$^+$ and CD8$^+$ T cells from Reg-1^{CD4KI} recipients producing TNF-α and IFN-γ decreased significantly, as compared to those from WT mice. With the stimulation of alloantigen, the clone expansion of effector T cells was inhibited by mutant Regnase-1. Taken together, Reg-1^{CD4KI} mice failed to reject allografts most likely due to lymphopenia and impaired T effector functions.

Impaired T Cell Development in the Mutant Regnase-1 Transgenic Mice

Considering that the number of mature T cells reduced dramatically in Reg-1^{CD4KI} mice, we focused on the inhibitory effect of Regnase-1 on T cell development. In the thymus, very few CD4$^+$CD8^{int} thymocytes were observed in Reg-1^{CD4KI} (**Figure 5A**), and the number of total thymocytes was significantly lower in Reg-1^{CD4KI} mice (**Figure 5B**). In addition, the numbers of DP, CD4$^+$CD8^{int} and CD4^{int}CD8$^+$ thymocytes were also significantly decreased in Reg-1^{CD4KI}

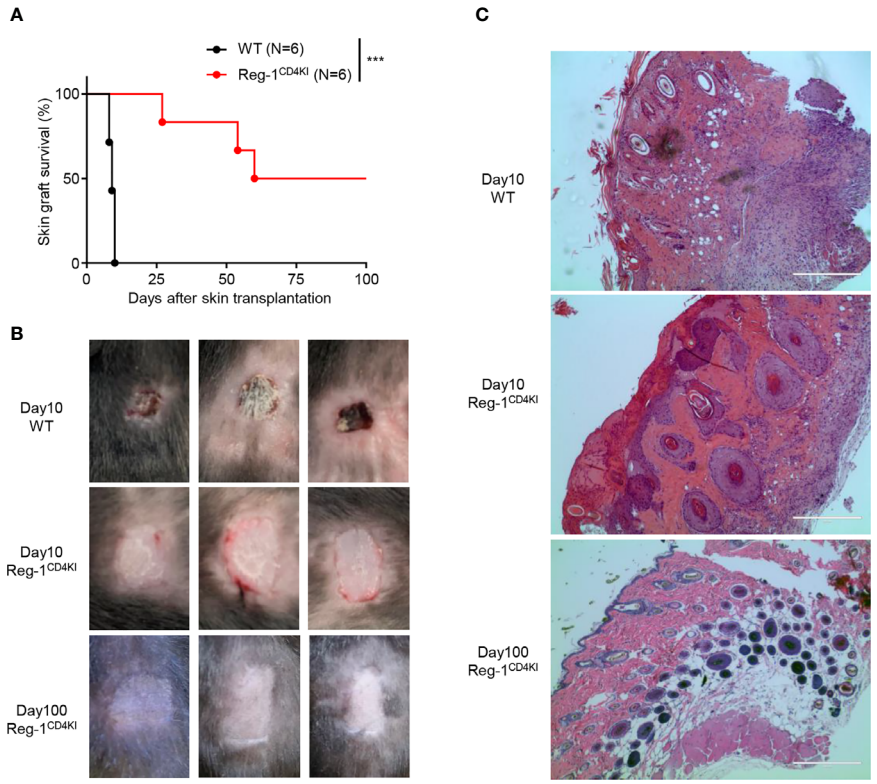

FIGURE 3 | Regnase-1 promotes graft survival in skin transplantation model. **(A)** The percentage of skin allograft survival on WT or Reg-1^{CD4KI} recipients. ***P < 0.001; Log-rank test. **(B)** Representative images of BALB/c skin allografts at day 10 on WT recipients, and day 10 and 100 on Reg-1^{CD4KI} recipients. **(C)** Representative H&E staining images (×100) of BALB/c skin grafts at day 10 from WT recipients, and day 10 and day 100 from Reg-1^{CD4KI} recipients.

thymus, but the number of DN thymocytes were comparable (**Figure 5C**). According to the expression of EGFP, R111A mutant Regnase-1 started to express at DP stage (**Figure 5D**). During thymocytes maturation, T cells migrate from thymic cortex to thymic medulla. The architecture of the thymus was also altered in Reg-1^{CD4KI} mice, as characterized by much smaller areas of the medulla (**Figure 5E**). These observations suggested that Regnase-1 likely disrupts maturation of T cells in the thymus and led to fewer mature T cells in peripheral tissues.

Impaired Positive Selection of Thymocytes in the Mutant Regnase-1 Transgenic Mice

Next, we investigated the specific developmental stage at which thymocytes were blocked in Reg-1^{CD4KI} mice. The mutant Regnase-1 was not expressed during DN stage, hence had little influence on DN thymocytes. The development of thymocytes from DN1 to DN4 was normal in Reg-1^{CD4KI} mice (**Supplementary Figure 3A**). Then, we quantified cells at five different stages based on the expression level of TCR-β and the activation marker CD69 (23). We found that the proportion and the number of thymocytes in population 1 (TCR^{lo}CD69^{lo}) or population 2 (TCR^{int}CD69^{lo}) were comparable between Reg-1^{CD4KI} mice and their WT littermates. But Reg-1^{CD4KI} mice had significantly fewer cells in population 3 (TCR^{int}CD69^{hi}), population 4 (TCR^{hi}CD69^{hi}), and population 5 (TCR^{hi}CD69^{lo}) (**Figures 6A, B**). We further investigated the expression of CD4 and CD8 gated on each population. Thymocytes in population 1 were mostly DN and DP cells; in population 2 were most preselection DP cells; in population 3 were cells undergoing selection; in population 4 were post-positive-selection thymocytes; in population 5 were SP cells ready for migrating to the periphery (**Supplementary Figure 3B**) (23).

We stained surface and intracellular TCR-β separately and observed that the levels of total TCR-β on DP thymocytes were comparable between Reg-1^{CD4KI} mice and their WT control, but the surface expression of TCR-β was impaired on CD4 single positive cells in Reg-1^{CD4KI} mice (**Supplementary Figure 3C**). Immature thymocytes upregulate CD69 and TCR-β during the process of positive selection (13). We detected the expression of TCR-β and CD69 in thymocytes at DP stage. We found that the population of post-selection DP T cells (TCR^{hi}CD69^{hi}) were extremely fewer in Reg-1^{CD4KI} mice (**Figures 6C, D**). According to prior studies, we employed a staging scheme to show the changes of several markers during the process of T cell development (**Figure 6E**) (14, 15). CD5 can be used as an indicator for TCR signal strength during thymic development (13), and the anti-apoptotic factor Bcl-2 can be induced by TCR signal to promote the survival of developing thymocytes (14, 23). We analyzed the expression levels of TCR-β, CD69, CD5 and Bcl-2 in CD4^{+}CD8^{int} thymocytes. Results showed that the

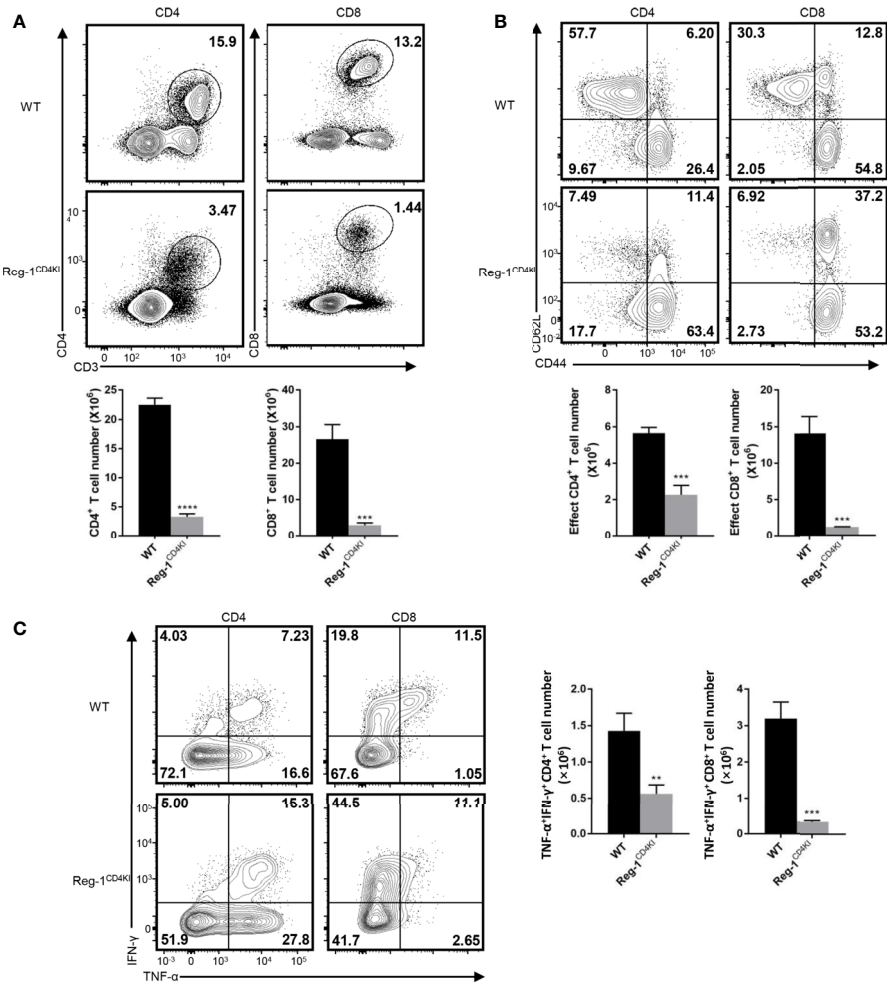

FIGURE 4 | Reduced T effector cells in Reg-1^{CD4KI} recipient after skin transplantation. **(A)** The percentage and number of CD4$^+$ or CD8$^+$ T cells in spleen based on the expression of CD3, CD4 and CD8. **(B)** The percentage and number of effect T cells in CD4$^+$ or CD8$^+$ splenocytes, based on the expression of CD44 and CD62L. **(C)** The percentage and number of IFN-γ$^+$TNF-α^{hi} T cells in CD4$^+$ or CD8$^+$ splenocytes. Data are mean ± SD (n=3) from one experiment, representative of three independent experiments. $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$ (unpaired two-tailed Student's *t* test).

expression of these markers were significantly lower in Reg-1^{CD4KI} mice (**Figure 6F**). These data indicated that TCR signaling during positive selection was disrupted by Regnase-1, which might contribute to the blockage of thymocytes during positive selection.

Thymocytes That Express the Mutant Regnase-1 Are More Susceptible to Apoptosis

We further investigated the influence of mutant Regnase-1 on thymocyte apoptosis. We sorted DP thymocytes and detected the mRNA and protein of Regnase-1. We confirmed that the mutant Regnase-1 was expressed in DP cells from Reg-1^{CD4KI} mice (**Figure 7A**). We treated thymocytes with anti-CD3 and anti-CD28 to stimulate TCR on thymocytes, and more DP thymocytes in Reg-1^{CD4KI} mice were undergoing apoptosis (**Figure 7B**). However, cell viability of DP thymocytes was not affected in the absence of TCR stimulation (**Supplementary Figure 4A**), indicating the susceptibility of DP T cells to apoptosis was due to the inappropriate TCR signaling. In addition, PMA and ionomycin, the nonspecific agonists to stimulate mature T cells, could lead to more apoptosis of CD4$^+$CD8^{int} thymocytes in Reg-1^{CD4KI} mice (**Figure 7C**). For DN thymocytes, cell viability was not influenced (**Supplementary Figures 4B, C**). Regnase-1 is known as an RNase that destabilizes a set of mRNAs (1, 24). We stimulated DP thymocytes with anti-CD3 and anti-CD28 for 3h and detected the expression of apoptosis-related genes. We found that the mRNA of anti-apoptotic genes *Bcl-2* and *Bcl2L1* were significantly lower in Regnase-1 knock-in DP thymocytes, but the level of pro-apoptotic gene *Bax* mRNA was comparable (**Figure 7D**). Taken together, these data suggested the mutant Regnase-1 could downregulate the expression of anti-apoptotic genes in thymocytes, which resulted in their apoptosis in thymus.

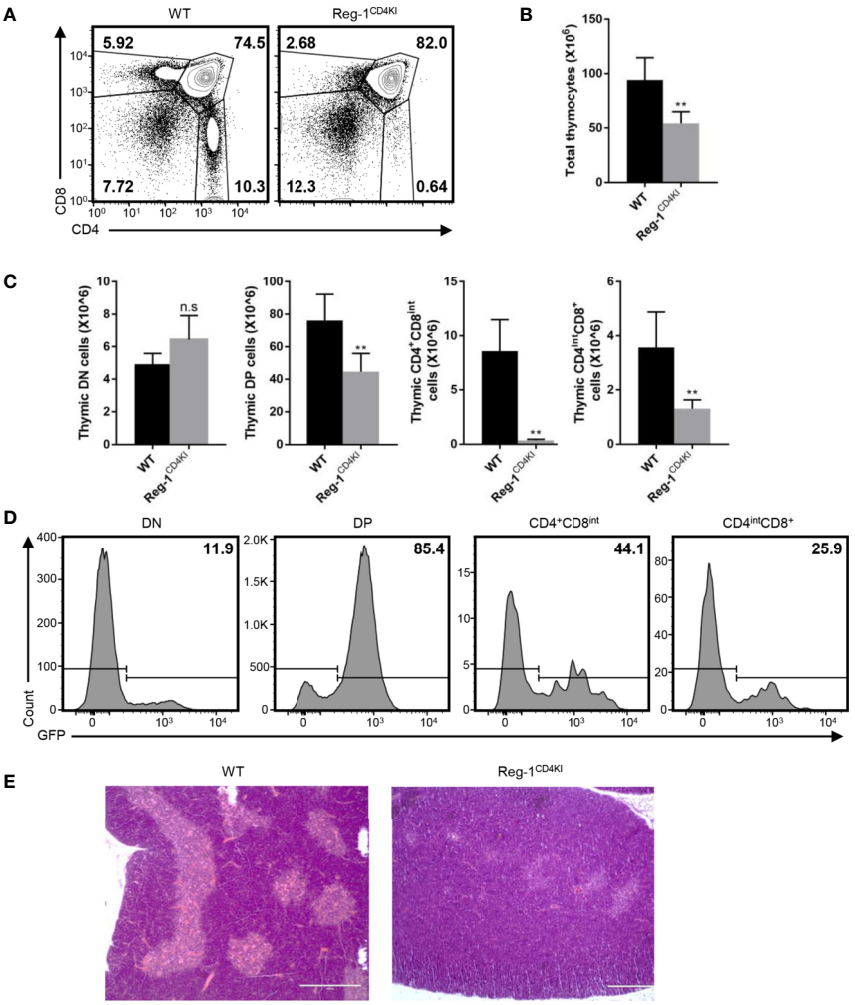

FIGURE 5 | Defective T cell development in Reg-1^{CD4KI} mice. **(A)** Surface staining of CD4 and CD8 on WT or Reg-1^{CD4KI} thymocytes, gated on CD45^{+} alive cells. **(B)** Bar graphs representing the number of total thymocyte in WT or Reg-1^{CD4KI} mice. **(C)** Bar graphs representing the number of DN, DP, CD4^{+}CD8^{int} or CD4^{int}CD8^{+} thymocyte subpopulations. **(D)** The percentage of GFP^{+} cells in DN, DP, CD4^{+}CD8^{int} or CD4^{int}CD8^{+} thymocytes subpopulations in Reg-1^{CD4KI} mice. **(E)** Representative H&E staining images (×100) of thymus from WT or Reg-1^{CD4KI} mice. The cortex (darker) and medulla (lighter) can be distinguished by the intensity of staining. Data are mean ± SD (n=5) from one experiment, representative of three independent experiments. n.s not significant; $^{**}P < 0.01$ (unpaired two-tailed Student's *t* test).

DISCUSSION

Regnase-1 is an RNA binding protein with potent endoribonuclease activities and traditionally thought to play a vital role in regulating mRNA stability (2, 25). In the present study, we created a Regnase-1 mutant and expressed this mutant *in vivo* as a transgene in an attempt to examine the impact of Regnase-1 on the T cell response. We found that the Regnase-1 mutant transgenic mice displayed profound lymphopenia, with few mature T cells in the periphery. Among the T cells in the periphery, however, most showed an effector memory phenotype, as they downregulated CD62L and expressed high CD44. Interestingly, those mutant mice failed to reject skin allografts, a response that depends on T cells, suggesting that such peripheral effector memory T cells are not sufficient to reject allografts. In fact, those peripheral T cells express much reduced levels of the effector cytokines TNF-α and IFN-γ. Mechanistically, the mutant Regnase-1 impaired T cell thymic positive selection, most likely *via* its inhibitory effect on TCR-β expression and TCR signaling. As a consequence, thymocytes that expressed mutant Regnase-1 were susceptible to apoptosis due to the lack of TCR-triggered survival signal.

In our transgenic mice, we replaced the arginine 111 of Regnase-1 with alanine to improve its stability *in vivo*. Our data suggested that R111A mutant Regnase-1 was resistant to Malt1 mediated cleavage without interfering with its endoribonuclease activities. Mino, T. et al. reported that Regnase-1 fails to degrade mRNAs in the absence of UPF1 (4). In our luciferase assay, the R111A mutant Regnase-1 had normal Rnase activity, indicating that the interaction

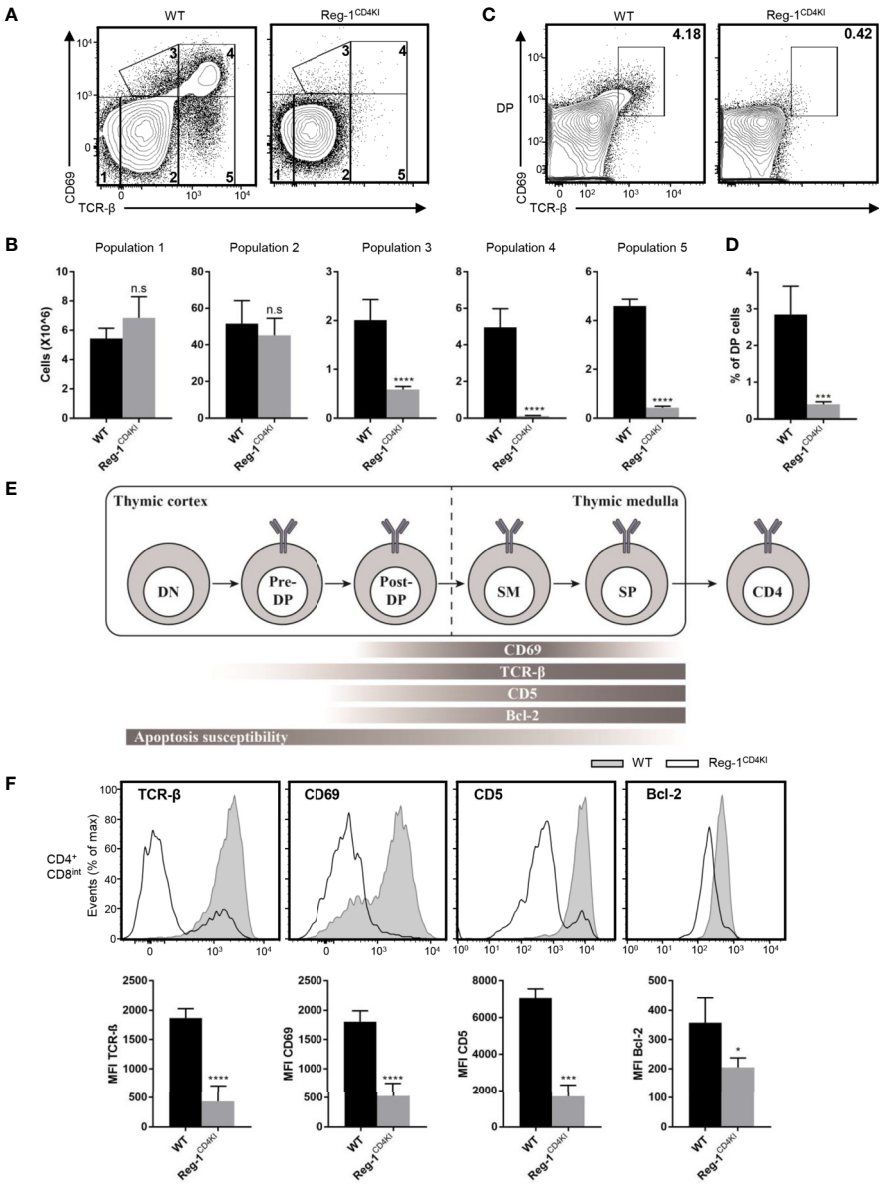

FIGURE 6 | Mutant Regnase-1 impairs thymocyte positive selection *in vivo*. **(A)** Flow cytometry analysis of the surface expression of CD69 and TCR-β on WT or Reg-1^{CD4KI} thymocytes, gated on $CD45^+$ alive cells. The number in outlined area indicates five subpopulations during thymocytes development. **(B)** Bar graphs representing the number of five subpopulations at different stage during T cell development in WT or Reg-1^{CD4KI} thymus. **(C)** Flow cytometric analysis of DP thymocytes through thymic positive selection based on the expression of CD69 and TCR-β, gated on $CD45^+$ DP thymocytes. **(D)** Bar graph indicates the percentage of post-selection DP ($CD69^+TCR\text{-}\beta^{hi}$) thymocytes. **(E)** Schematic graph of $CD4^+$ T cell development in thymus. T cells undergo differentiation from DN to DP to CD4 or CD8 single positive cell, accompanied with changes of several surface and intercellular markers (Pre-DP: pre-selection double positive cells; Post-DP: post-selection double positive cells; SM: susceptible to apoptosis mature cells). **(F)** Flow cytometric analysis and mean fluorescence intensity of TCR-β, CD69, CD5 and Bcl-2 in gated $CD4^+CD8^{int}$ thymocytes from WT or Reg-1^{CD4KI} mice. Data are mean ± SD (n=5) from one experiment, representative of three independent experiments. n.s not significant; $^*P < 0.05$; $^{***}P < 0.001$; $^{****}P < 0.0001$ (unpaired two-tailed Student's *t* test).

between the R111A mutant Regnase-1 and UPF1 was not affected. A number of studies have shown that Regnase-1 is uniquely important in controlling T cell activation. For example, in Regnase-1 deficient mice, T cells are spontaneously activated, resulting in fetal autoimmune disease (8). In our transgenic mice, the mutant Regnase-1 led to fewer peripheral $CD4^+$ and $CD8^+$ T cells and Tregs in spleen, supporting an inhibitory role of Regnase-1 in T cell activities. In lymphopenic mice, residual T cells usually undergo robust homeostatic expansion, thus giving rise to population of T cells with an effector memory phenotype ($CD44^{hi}CD62L^{lo}$) (23). We observed that in the Reg-1^{CD4KI} mice, such effector memory T cells are functionally impaired, as they

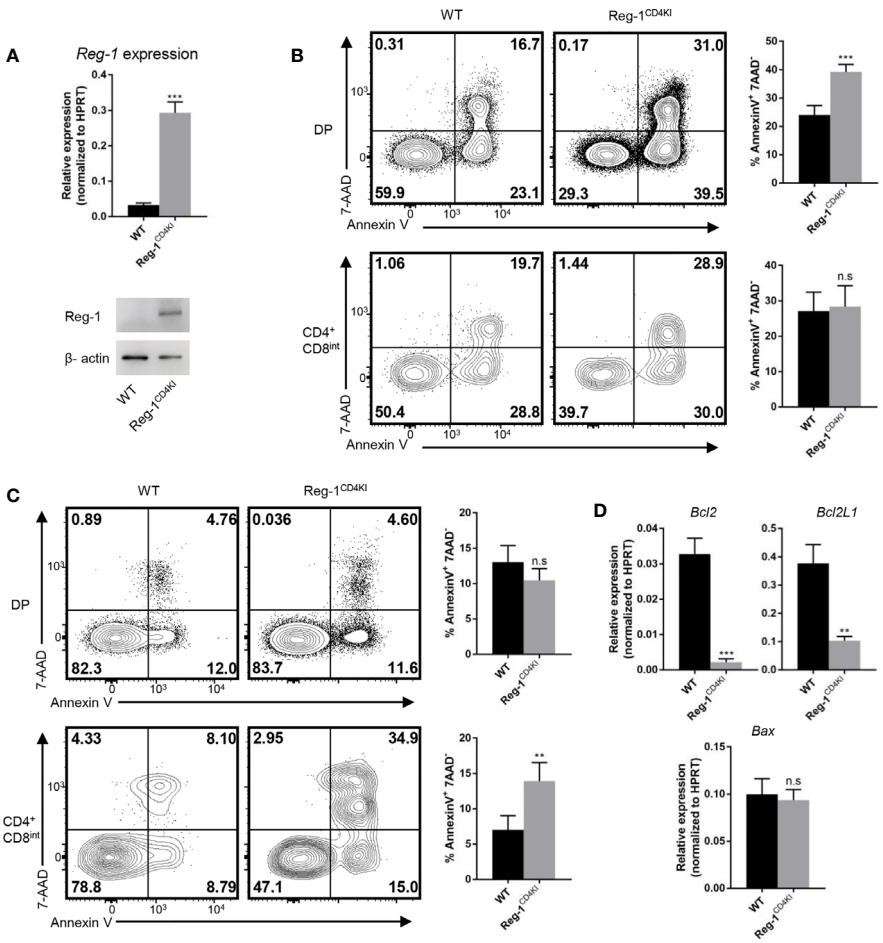

FIGURE 7 | Thymocytes that express mutant Regnase-1 are more susceptible to apoptosis. **(A)** Immunoblot analysis of Regnase-1 expression and RT-qPCR analysis of Regnase-1 mRNA in sorted DP thymocytes. **(B)** Flow cytometric analysis of the apoptosis of DP and $CD4^+CD8^{int}$ thymocytes with anti-CD3 (5 μg/ml) and anti-CD28 (1 μg/ml) for 24h. Bar graph indicates Annexin V^+7-AAD^- fraction of DP and $CD4^+CD8^{int}$ thymocytes. **(C)** Flow cytometric analysis of the apoptosis of DP and $CD4^+CD8^{int}$ thymocytes with PMA (50 ng/ml) and ionomycin (500 ng/ml) stimulation for 4h. Bar graph indicates Annexin V^+7-AAD^- fraction of DP and $CD4^+CD8^{int}$ thymocytes. **(D)** RT-qPCR analysis of apoptotic genes expression. Data are mean ± SD (n=3) from one experiment, representative of three independent experiments. n.s not significant; $^{**}P < 0.01$; $^{***}P < 0.001$ (unpaired two-tailed Student's *t* test).

completely failed to expand and reject the skin allografts. The rejection to allografts depends on the number and function of alloreactive T cells (26, 27). Although with the stimulation of alloantigens, the peripheral T cells in transgenic mice could express activation markers (ICOS, OX40, PD-1, KLRG1), but expressed reduced levels of cytokines (IFN-γ, TNF-α). The expansion of alloreactive effect T cells was inhibited in transgenic mice. Thus, the reduced number of T cells in the periphery and the impaired functions of peripheral T cells may both contribute to the acceptance of skin allografts. In this study, at 10 days post skin transplantation, fewer effect T cells infiltrated into the skin allograft in transgenic recipients, indicating the reduced anti-graft T cells. Although the peripheral T cells showed reduced effector functions, approaches to selectively overexpress mutant Regnase-1 in mature T cell may yield additional insights into the regulatory role of Regnase-1 in T effector cell activities.

Our finding that in the thymus of transgenic mice, the inhibition of TCR expression and/or signaling at the DP stage by mutant Regnase-1 was highly interesting. Previous studies showed that the deletion of Regnase-1 or expression of protease-dead Malt1 in T cells has no obvious effects on T cell development (8, 28), which is very different from our findings, highlighting the complexity of Regnase-1 in regulating T cell biology. Clearly, the interaction of TCR with self-peptide/MHC complex is vital for the fate of developing T cells. For example, in *Rag*$^{-/-}$ mice, the lack of antigen receptor leads to the loss of all mature lymphocytes (29, 30). In our transgenic mice, the mutant Regnase-1 was initially expressed in DP thymocytes under the control of CAG promoter, enabled by *Cd4*-Cre mediated excision of the stop cassette. Although the exact mechanisms remain to be defined, a lower frequency of post-selection thymocytes ($TCR^{hi}CD69^{hi}$) indicated a defect of thymic positive selection in Regnase-1 mutant mice, most likely due to an

impaired TCR-β expression and signaling. Also, CD69 can directly compete with S1P1, a chemokine receptor that is required for mature thymocyte egression from thymus (31–33). Developing thymocytes with high level of CD69 can be retained in the thymus until their maturation (34). Thus, the DP thymocytes from Reg-1^{CD4KI} mice might egress from thymus, and the lack of interaction with thymic stromal cells could impair thymic selection. In addition, the upregulation of CD5 during thymocytes development may be blocked by mutant Regnase-1, indicating its profound suppressive effect on TCR signaling. Overexpression of Regnase-1 affected translocation of TCR-β from cytoplasm to membrane, which can also contribute to the insufficient of TCR signal during T cell development. TCR signaling provides survival signal for T cells during thymic selection, and Regnase-1 has been reported to enhance mRNA decay of several anti-apoptotic genes including *Bcl2L1, Bcl2A1, RelB, Birc3*, and *Bcl3* (24). We provided evidence that the mRNA of anti-apoptotic genes (*Bcl-2*, *Bcl2L1*) were significantly lower in Regnase-1 knock-in DP T cells, and these T cells were more susceptible to apoptosis with TCR stimulation.

Our study suggests that the ribonuclease Regnase-1 could be therapeutically targeted to modulate T cell activities under various circumstances, including transplant survival. But the clinical implications of our approach remain uncertain. Clearly, the induction of profound lymphopenia is not ideal and would expose patients to greater risks of immunodeficiency. However, the finding that the Regnase-1 mutant exhibits an inhibitory effect in TCR signaling and effector activities may open the door in the development of novel therapeutic approaches in treatment of multiple T cell-mediated immune diseases.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Houston Methodist Animal Care Committee.

AUTHOR CONTRIBUTIONS

GK, YD, XX, and XL were involved in the design of the study. GK, YD, and YW performed the experiments and analysed the data. GK, YM, and XL contributed to the writing and editing of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.682220/full#supplementary-material

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Immune Monitoring Assay for Extracorporeal Photopheresis Treatment Optimization After Heart Transplantation

Maja-Theresa Dieterlen[1], Kristin Klaeske[1], Alexander A. Bernhardt[2], Michael A. Borger[1], Sara Klein[1], Jens Garbade[1], Sven Lehmann[1], Francis Ayuketang Ayuk[2], Herrmann Reichenspurner[3] and Markus J. Barten[3]*

[1] Heart Center, HELIOS Clinic, Department of Cardiac Surgery, University Hospital Leipzig, Leipzig, Germany, [2] Department of Stem Cell Transplantation, University Medical Center Hamburg-Eppendorf, Hamburg, Germany, [3] Department of Cardiovascular Surgery, University Heart and Vascular Center Hamburg, Hamburg, Germany

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***Correspondence:**
Maja-Theresa Dieterlen
mdieterlen@web.de

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Background: Extracorporeal photopheresis (ECP) induces immunological changes that lead to a reduced risk of transplant rejection. The aim of the present study was to determine optimum conditions for ECP treatment by analyzing a variety of tolerance-inducing immune cells to optimize the treatment.

Methods: Ten ECP treatments were applied to each of 17 heart-transplant patients from month 3 to month 9 post-HTx. Blood samples were taken at baseline, three times during treatment, and four months after the last ECP treatment. The abundance of subsets of tolerance-inducing regulatory T cells (T_{regs}) and dendritic cells (DCs) in the samples was determined by flow cytometry. A multivariate statistical model describing the immunological status of rejection-free heart transplanted patients was used to visualize the patient-specific immunological improvement induced by ECP.

Results: All $BDCA^+$ DC subsets ($BDCA1^+$ DCs: $p < 0.01$, $BDCA2^+$ DCs: $p < 0.01$, $BDCA3^+$ DCs: $p < 0.01$, $BDCA4^+$ DCs: $p < 0.01$) as well as total T_{regs} ($p < 0.01$) and $CD39^+$ T_{regs} ($p < 0.01$) increased during ECP treatment, while $CD62L^+$ T_{regs} decreased ($p < 0.01$). The cell surface expression level of BDCA1 ($p < 0.01$) and BDCA4 ($p < 0.01$) on DCs as well as of CD120b ($p < 0.01$) on T_{regs} increased during the study period, while CD62L expression on T_{regs} decreased significantly ($p = 0.04$). The cell surface expression level of BDCA2 ($p = 0.47$) and BDCA3 ($p = 0.22$) on DCs as well as of CD39 ($p = 0.14$) and CD147 ($p = 0.08$) on T_{regs} remained constant during the study period. A cluster analysis showed that ECP treatment led to a sustained immunological improvement.

Conclusions: We developed an immune monitoring assay for ECP treatment after heart transplantation by analyzing changes in tolerance-inducing immune cells. This assay allowed differentiation of patients who did and did not show immunological improvement.

Based on these results, we propose classification criteria that may allow optimization of the duration of ECP treatment.

Keywords: extracorporeal photopheresis, heart transplantation, regulatory T cells, dendritic cells, immune tolerance

INTRODUCTION

Since the first report in 1991, the American Society of Apharesis recommends extracorporeal photopheresis (ECP) for the treatment of acute cellular and recurrent rejection (ACR) as well as for rejection prophylaxis after heart transplantation (HTx) (1). Additionally, experts in the field of transplantation medicine recommend chronic ECP treatment of HTx patients with donor specific antibodies (DSA) (2). Although ECP has been used to treat an increasing number of patients in recent years, there is still no consensus about the optimal ECP therapy for any individual patient. For example, questions remain about the best time point to initiate or reintroduce ECP therapy as well as about the optimal number of ECP treatments that are required for different indications, such as ACR or antibody-mediated rejection (AMR). Thus, a reliable monitoring tool for optimizing ECP therapy is required (3).

Based on the results of our previous ECP studies, we proposed that monitoring specific immune cells during ECP treatment might provide information that could be used to optimize ECP treatments (3, 4), which has been mentioned in the updated European Dermatology Forum on the use of extracorporeal photopheresis (5). Currently, two different mechanisms of action are discussed for ECP therapy. One hypothesizes that the return of apoptotic T cells activates dendritic cells (DCs), which leads to cytokine alterations and results in an increase in regulatory T cells (T_{regs}) (6). The other hypothesizes that ECP presents an apoptotic stimulus that affects activated alloreactive T cells, which are preferentially processed and presented by DCs resulting in suppression of alloantigen-responding T cells (3). In particular, the effect of ECP on an increase of T_{regs} in HTx was studied by different research groups (7–11).

In previous studies, we showed that T_{reg} and DC subsets in HTx patients with different indications for ECP treatment, such as prophylactic treatment, ACR, or cardiac allograft rejection (CAV) responded differently to ECP (3, 4).

Thus, for the current study we proposed that analysis of the expression of DCs and T_{regs} in peripheral blood could be helpful in designing a monitoring tool for ECP. We validated our immune cell assays to differentiate between patients with and without immunological effects after ECP therapy. Ideally, such a monitoring tool should allow optimization of individual ECP treatment schedules and reduce or prolong ECP treatments depending on the immunological effects.

MATERIALS AND METHODS

Patient Cohorts

The study cohort included 17 patients aged over 18 years who received HTx between May 2016 and January 2018 at the Department of Cardiovascular Surgery of the University Heart and Vascular Center in Hamburg, Germany. Four patients were excluded because they refused to receive ECP. In accordance with the recommendations of the ECP guidelines of both, the American Apharesis Society and the European Dermatology Forum, the patients were classified into two study groups (5, 12). The first group consists of patients who had no rejection before ECP start (prophylactic treatment) and the second group had an AMR or ACR before ECP start (rejection treatment). Therefore, patients of both study groups received ECP as chronic treatment to avoid rejection. Written informed consent was obtained from each participant before initiation of ECP (vote no. PV7246, Ärztekammer Hamburg, Germany) in accordance with the Declaration of Helsinki and the local ethical regulations. Patient demographics, disease and treatment parameters as well as their immunosuppressive regimens were documented.

Extracorporeal Photopheresis

ECP was performed using the closed inline THERAKOS CELLEX photopheresis system (Therakos Inc., West Chester, PA, USA) with a total of ten ECP treatments that were grouped to five ECP cycles. ECP treatments were conducted on two consecutive days, and ECP cycles were performed every 4-6 weeks (**Figure 1**). The daily ECP procedure included separation of peripheral blood mononuclear cells (PBMCs) by centrifugation of the patient's whole blood. Following centrifugation, the remaining blood components were reinfused immediately. PBMCs were sequentially exposed to 8-methoxypsoralen (20 µg/mL) and ultraviolet A light (~1.5 J/cm^2). Photoactivation time and the entire cell volume were automatically calculated using the patient's hematocrit in the buffy coat by integrated software in the Cellex© ECP machine. After completion of the photoactivation process, PBMCs were immediately re-transfused to the patient. Blood count analysis after reinfusion of the ECP product was performed by documenting platelet and erythrocyte count, and the hemoglobin and hematocrit content.

Flow Cytometric Assessment

Phlebotomy was performed before each ECP cycle and a follow-up blood analysis was conducted four months after the last ECP cycle. Peripheral blood samples obtained from the patients were treated as described previously (3). T_{regs} were defined as $CD3^+$/$CD4^+$/$CD25^{high}$/$CD127^{low}$ cells; from this population, the T_{reg} subsets expressing CD39, CD62L, CD120b or CD147 were analyzed. Subsets of DCs were quantified by stainings using lineage cocktail-1, HLA-DR, and blood dendritic cell antigen (BDCA) 1, 2, 3 or 4. Antibodies were obtained from Becton Dickinson (BD, Heidelberg, Germany) or BioLegend (Fell, Germany). For each staining, 200 µl (T_{reg} analysis) or 300 µl

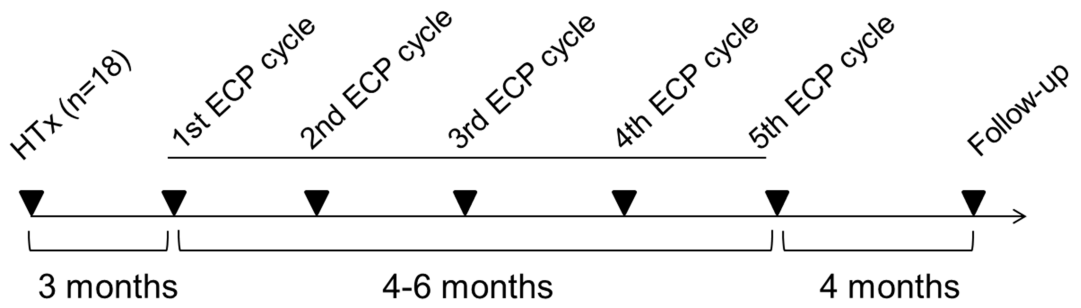

FIGURE 1 | Overview about the treatment regimen for extracorporeal photopheresis. ECP, extracorporeal photopheresis; HTx, heart transplantation.

(DC analysis) of human heparinized whole blood were mixed with the appropriate antibody cocktail and incubated for 20 min at room temperature in the dark. Next, 2 ml of FACS lysing solution (BD) were added and samples were incubated for 10 min. After centrifugation at 300x g for 5 min, the supernatant was discarded and samples were washed with 4 ml phosphate-buffered saline (PBS). The supernatant was discarded after washing, and the cells were fixed with 500 µl 1% formaldehyde-PBS solution. Samples were analyzed directly using a *BD LSR II Flow Cytometer* and *BD FACSDiva version 6.1.3* software (both BD); 10,000 events of $CD3^+CD4^+$ cells (for T_{reg} analysis) and 500,000 vital cells (for DC analysis) were analyzed per sample. Mean fluorescence intensities (MFIs) were documented for BDCA1-4 on DCs as well as for CD39, CD62L, CD120b, and CD147 on T_{reg} subsets.

Statistics

The patient cohort was characterized by mean (± standard deviation) for continuous and by number (percent) for categorical variables. Time-dependent changes of cellular parameters were analyzed by the generalized linear model for repeated measurements. A simple contrast was used, and the first measurement (pre ECP) was set as the reference. Tests were performed two-sided at 5% significance level. All analyses were done using Intel SPSS Statistics version 23 (IBM Corp. 1989, 2011).

We combined immune markers that are involved in tolerance induction after ECP to describe the patient's immune transplant tolerance phenotype. The immune phenotype is defined as the percentage of tolerance-inducing immune cells and is called immunological profile. A valid statistical tool to perform this systemic analysis of immune profiles is the hierarchical clustering which has been performed in previous clinical studies for comparable analyses (12, 13). Hierarchical cluster analysis using the ClustVis software (Bioinformatics, Algorithms and Data Mining Group, University of Tartu, Estonia) was performed for every ECP-treated patient in combination with the dataset described in the recent work of Klaeske et al. (13) Five flow cytometric parameters of DCs (% total DCs/PBMCs, % $BDCA1^+$ DCs/total DCs, % $BDCA2^+$ DCs/total DCs, % $BDCA3^+$ DCs/total DCs and % $BDCA4^+$ DCs/total DCs) and six parameters of T_{regs} (% $CD4^+$ T cells/total T cells, % $T_{regs}/CD4^+$ T cells, % $CD39^+$ T_{regs}/total T_{regs}, % $CD62L^+$ T_{regs}/total T_{regs}, % $CD120b^+$ T_{regs}/total T_{regs} and % $CD147^+$ T_{regs}/total T_{regs}) were included in the cluster analysis. The hierarchical cluster analysis leads to the pattern recognition of a tolerance-inducing phenotype and displays the distance connectivity of the immunological profile for every measurement of an ECP-treated patient. As a result, it is possible to monitor whether an ECP-treated HTx patient develops a tolerance-promoting immunological phenotype. This tool could be helpful for clinicians to monitor, to shorten or prolong the ECP schedule for patients depending on the immunological profile. Patient-specific results could be available 4-5 hours following blood withdrawal.

Classification of Immunological Effects Induced by ECP

A classification system for the objective evaluation of immunological effects induced by ECP was established. The hierarchical cluster analysis of the dataset reported by Klaeske et al. (14) formed two clusters. The first cluster included 75% long-term HTx patients and the second cluster included 67% pre-HTx patients (**Supplementary Figure 1**). It can be assumed that stable long-term transplanted patients who never suffered from transplant rejection received an optimal immunosuppression and have an immune phenotype promoting transplant tolerance. Klaeske et al. used hierarchical clustering and principle component analyses to show that this immune phenotype of long-term HTx patients differed from that of pre-HTx patients (14). A hierarchical cluster analysis including the dataset of the previous study from Klaeske et al. and measurements of an ECP-treated patient will allow to evaluate if the immune phenotype of the ECP-treated patients changes to the transplant tolerance immune phenotype during ECP treatment by changes of the position in the heat map of the cluster analysis towards the cluster consisting of long-term HTx patients. The patient-specific reference point in the heat map was the measurement prior to ECP treatment. Every subsequent immunological measurement during and after ECP produced a new point in the heat map. An immunological improvement existed if the measurement shifted toward the cluster containing the majority of long-term HTx-patients. Thus,

patients were classified into five categories according to the time point of immunological improvement during ECP (category A: improvement after the 1st ECP cycle, category B: improvement after the 3rd ECP cycle, category C: improvement after the 5th ECP cycle, category D: improvement after the 5th ECP cycle, but declining in the follow-up period, category E: no improvement).

RESULTS

ECP Performance and Blood Monitoring

The study cohort consisted of n = 17 HTX patients (11 male, 6 female) with a mean age of 48.8 ± 10.8 years and a mean body mass index of 25.7 ± 5.8 kg/m^2). The etiology for HTx was dilated cardiomyopathy (n = 11), ischemic cardiomyopathy (n = 4) or had other reasons (n = 2). All patients received a triple-drug immunosuppressive regimen at study begin, whereas n = 10 patients received tacrolimus/everolimus/steroids, n = 4 patients received tacrolimus/mycophenolic acid/steroids and n = 3 patients received everolimus/mycophenolic acid/steroids. The indication for ECP treatment was an existing ACR or AMR (n = 6), and a prophylactic treatment (n = 11). ECP treatment was performed according to the manufacturer's instruction and was accompanied by blood cell counts of erythrocytes and platelets as well as the hematocrit and hemoglobin content as quality control metrics. Platelet count ($p = 0.24$), erythrocyte count ($p = 0.57$), hemoglobin content ($p = 0.92$), and hematocrit ($p = 0.81$) did not change significantly during ECP or the follow-up period (**Table 1**). However, the hemoglobin content of the ECP-treated patients was below the hemoglobin reference value (men: 13.5 ± 17.5 g/dL, women: 12.0 ± 15.5 g/dL). In two patients with prophylactic ECP treatment, ACR episodes occurred during ECP treatment. One patient (female, 34 years old) had a higher immunological risk due to two pregnancies and chronic left ventricular assist device therapy before HTx. She got an ACR of a histological grade 3R (ISHLT 2004). The other patient (male, 63 years old) suffered from an early cytomegalovirus infection in the first month post-HTx and got an ACR of histological grade 2R (ISHLT 2004). The ACRs were without hemodynamic compromise, and, therefore, both patients were treated with methylprednisolone (total of 3000 mg) in addition to ECP as well as with an increase of both the tacrolimus and everolimus exposure at time of diagnosis of rejection (month 2 and month 3 after ECP start, respectively). Both patients completed the scheduled ECP treatments. At the end of the study the female patient had a grade 1R ACR and the male patient had no ACR.

Dendritic Cell Analysis

While the percentage of total DCs on PBMCs did not change significantly during ECP ($p = 0.24$; **Figure 2A**), differential consideration showed that all BDCA$^+$ DC subsets increased during ECP treatment (BDCA1$^+$ DCs: $p < 0.01$, BDCA2$^+$ DCs: $p < 0.01$, BDCA3$^+$ DCs: $p < 0.01$, BDCA4$^+$ DCs: $p < 0.01$), but decreased to values observed prior to ECP in the follow-up period (BDCA1$^+$: pre-ECP 43.0 ± 12.6%, ECP follow-up 43.7 ± 9.1%; BDCA2$^+$: pre-ECP 20.5 ± 8.6%, ECP follow-up 23.9 ± 6.3%; BDCA3$^+$: pre-ECP 76.2 ± 7.1%, ECP follow-up 75.6 ± 13.1%; BDCA4$^+$: pre-ECP 21.9 ± 9.3%, ECP follow-up 21.3 ± 6.3%) (**Figures 2B–E**).

An increase of the surface expression level estimated by mean fluorescence intensity, was detected in the follow-up period for BDCA1 (2028 ± 389 U, $p < 0.01$) as well as after the third ECP cycle (22079 ± 4265 U, $p < 0.01$) and in the follow-up period (24212 ± 5172 U, $p < 0.01$) for BDCA4 (**Figures 3A, D**). The surface expression levels of BDCA2 ($p = 0.47$) and BDCA3 ($p = 0.22$) were unaffected (**Figures 3B, C**).

An overview about the DC analysis for ECP-treated patients with ACR or AMR as well as for patients treated prophylactically with ECP was presented in **Supplementary Table 1**.

Regulatory T Cell Analysis

The percentage of CD4$^+$ T cells among total T cells decreased from 22.8 ± 7.2% prior to ECP to 16.2 ± 7.8% in the follow-up period ($p < 0.01$, **Figure 4A**), while the percentage of T_{regs} in the CD4$^+$ T cell population increased from 9.9 ± 2.5% to 17.7 ± 4.2% ($p < 0.01$, **Figure 4B**). The T_{reg} subset expressing CD39 increased within the T_{reg} population during ECP (pre-ECP: 38.5 ± 17.4%, third ECP cycle: 54.6 ± 21.6%) and throughout the follow-up period (54.9 ± 22.9%, $p < 0.01$; **Figure 4C**). The CD62L$^+$ T_{regs} decreased during ECP from 77.2 ± 12.5% prior to ECP to 56.0 ± 14.4% after the fifth ECP cycle ($p < 0.01$), while CD120b$^+$ ($p = 0.56$) and CD147$^+$ T_{regs} ($p = 0.48$) remained constant (**Figures 4D–F**). The expression of CD39 ($p = 0.14$) and CD147 ($p = 0.08$) on the surface of T_{regs} was unchanged during ECP treatment (**Figure 5**). While the surface expression of CD62L decreased during ECP (pre-ECP: 8606 ± 2617 U, third ECP cycle: 5979 ± 1452 U, fifth ECP cycle: 5459 ± 1843 U, $p = 0.04$), CD120b expression increased significantly at the end of the ECP treatment (pre-ECP: 1199 ± 319 U, fifth ECP cycle: 1496 ± 31 U, $p < 0.01$).

TABLE 1 | Blood parameters of patients treated with extracorporeal photopheresis.

	Extracorporeal photophoresis					p
	Pre-ECP	1st cycle	3rd cycle	5th cycle	ECP FU	
Platelets [10^9/L]	191 ± 56	238 ± 88	226 ± 67	222 ± 91	217 ± 93	0.24
Erythrocytes [10^9/L]	3.9 ± 0.7	3.8 ± 0.6	4.3 ± 0.6	4.3 ± 0.5	4.3 ± 0.7	0.57
Hemoglobin [g/dL]	10.4 ± 1.5	10.2 ± 1.2	11.6 ± 1.4	11.6 ± 1.3	11.6 ± 1.9	0.92
Hematocrit [%]	31.9 ± 4.6	31.7 ± 3.3	35.4 ± 4.1	35.9 ± 3.5	34.8 ± 5.4	0.81

pre-ECP, prior extracorporeal photopheresis; ECP FU, follow-up of extracorporeal photopheresis.

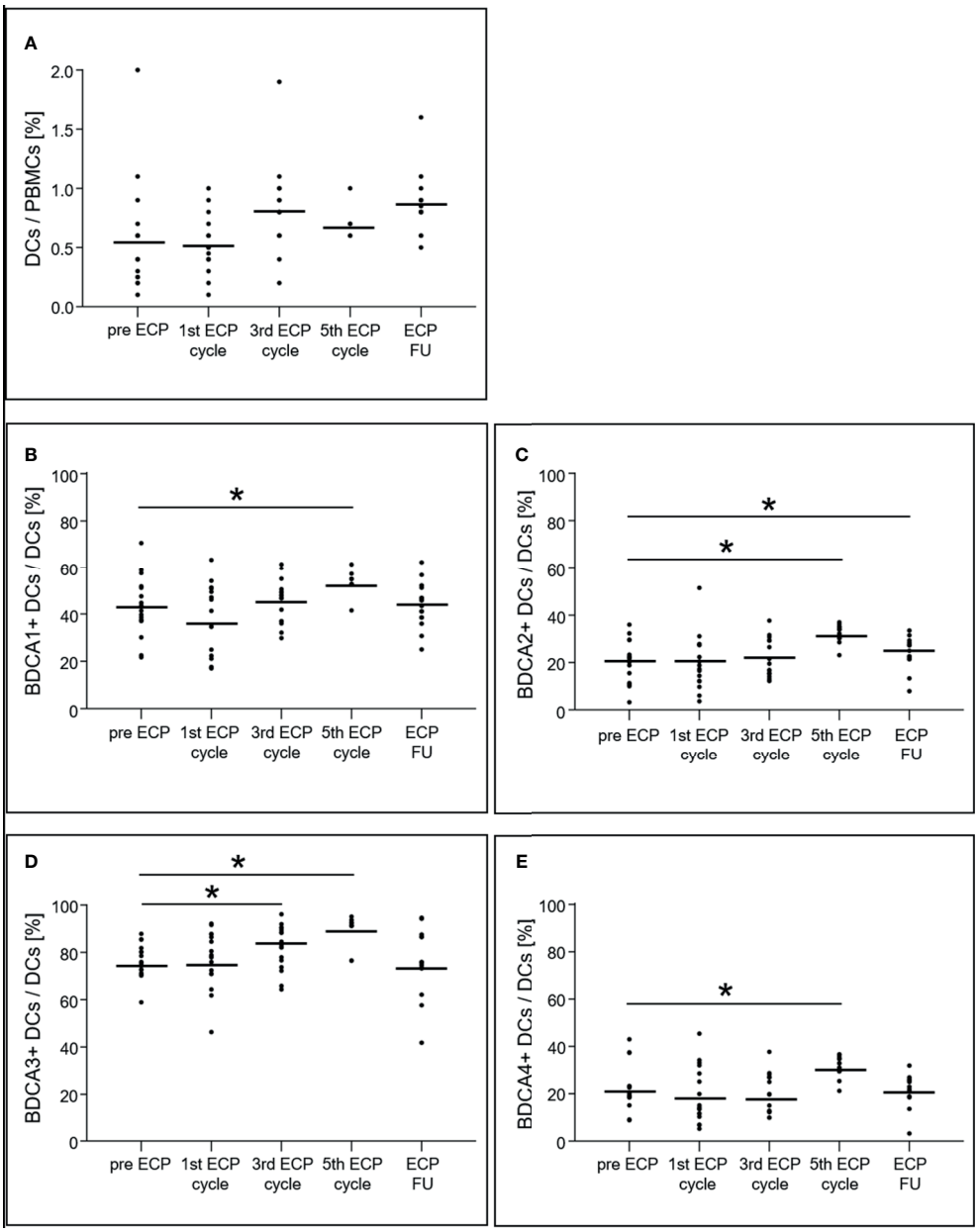

FIGURE 2 | Expression of dendritic cells **(A)** and their subsets **(B–E)** in heart-transplanted patients receiving extracorporeal photopheresis. * marks significant differences ($p \leq 0.05$); BDCA1/2/3/4, blood dendritic cell antigen 1/2/3/4; DCs, dendritic cells; ECP, extracorporeal photopheresis; FU, follow-up; PBMCs, peripheral blood mononuclear cells.

An overview about the T_{reg} cell analysis for ECP-treated patients with ACR or AMR as well as for patients treated prophylactically with ECP was presented in **Supplementary Table 2**.

Grouping According to Immunological Profiles

To monitor the patient-specific success of ECP treatment, monitoring data from each ECP-treated patient were combined with a dataset generated by a previous study comprising pre-HTx and long-term HTx patients and were evaluated by cluster analysis. The individual immunological improvement of the ECP treatment was classified according to the time-point of immunological upgrade towards the long-term HTx configuration of tolerance-inducing cell subsets. Exemplary classifications are shown in **Supplementary Figures 1–3**.

The patient-specific cluster analyses identified immunological improvement for six patients in the category A, four patients in the category B, and three patients in the category C. For these 13

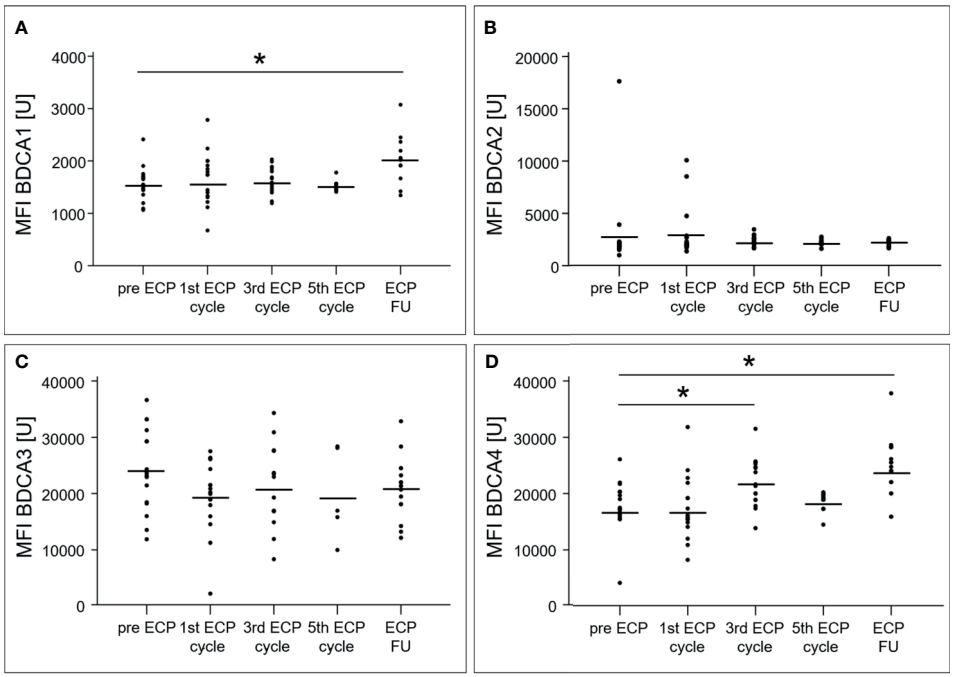

FIGURE 3 | Mean fluorescence intensities of blood dendritic cell antigens 1 **(A)**, 2 **(B)**, 3 **(C)** and 4 **(D)** of dendritic cells in heart-transplanted patients receiving extracorporeal photopheresis. * marks significant differences ($p \leq 0.05$); BDCA1/2/3/4, blood dendritic cell antigen 1/2/3/4; ECP, extracorporeal photopheresis; FU, follow-up; MFI, mean fluorescence intensity; U, unit.

HTx patients (72%), ECP treatment led to an immunological improvement during ECP and throughout the follow-up period. Ten of the 17 ECP-treated patients (56%) were classified into category A or B and showed an immunological improvement in the latest after three ECP cycles. For the patients in category D (n = 4) and E (n = 1), the immunological efficacy of ECP treatment is questionable. However, clinical outcome measurements were not included in the current study.

DISCUSSION

The Guidelines on the Use of Therapeutic Apheresis in Clinical Practice stated that ECP treatments after HTx should be continued until stabilization of symptoms or improvement of cardiac function, biopsy findings or donor-specific antibody levels (15). Although these goals are of paramount interest, the duration of ECP therapy required differs from individual to individual and from indication to indication. Thus, the purpose of our study was to develop a classification system based on the immunological effects of ECP to support clinical decisions regarding the optimal number of ECP treatments for HTx patients. However, a proof-of-concept was not part of the present study.

Overall, the clinical efficacy of ECP therapy in this study was high and in line with published data from the landmark trial of Barr et al. who showed a significant reduction of rejection episodes in patients treated with ECP as compared to the control patients (16). Our results show that monitoring DC and T_{regs} expression in peripheral blood might qualify to analyze patient-specific ECP effects. Furthermore, we combined this immune cell monitoring with a multivariate analysis of ECP-induced effects. The basis of this analysis was a multiparametric setting of immune cell subsets involved in tolerance induction. To present the multidimensionality of the immune system, we analyzed eleven parameters and, amended the statistical model accordingly; we also performed cluster analysis with a hierarchical clustering algorithm; data preprocessing and modification was avoided. This statistical method is useful for unusual similarity measures and extracts useful information from larger datasets with many groups (17).

The ECP-induced increase of T_{regs} (4, 7–10, 18) and pDCs (4, 19) has been demonstrated in several studies of heart and lung transplant patients as well as of patients suffering from graft-versus-host disease. Previous work from our group showed that it is possible to differentiate between ECP-treated patients with a "positive ECP immunological effect" and "no ECP effect" (4). The present study refined those observations, including a more detailed cell subset analysis and a more eligible statistical methodology to handle a multivariate dataset.

All $BDCA^+$ subsets of DCs increased during ECP, but only the percentage of $BDCA2^+$ DCs remained high after ECP. Furthermore, an increased surface expression for $BDCA1^+$ and $BDCA4^+$ DCs was induced by ECP and was detected by analysis of the MFIs. BDCA2 is a pDC-specific transmembrane lectin that inhibits induction of interferon-α/β, thereby preventing a Th1-

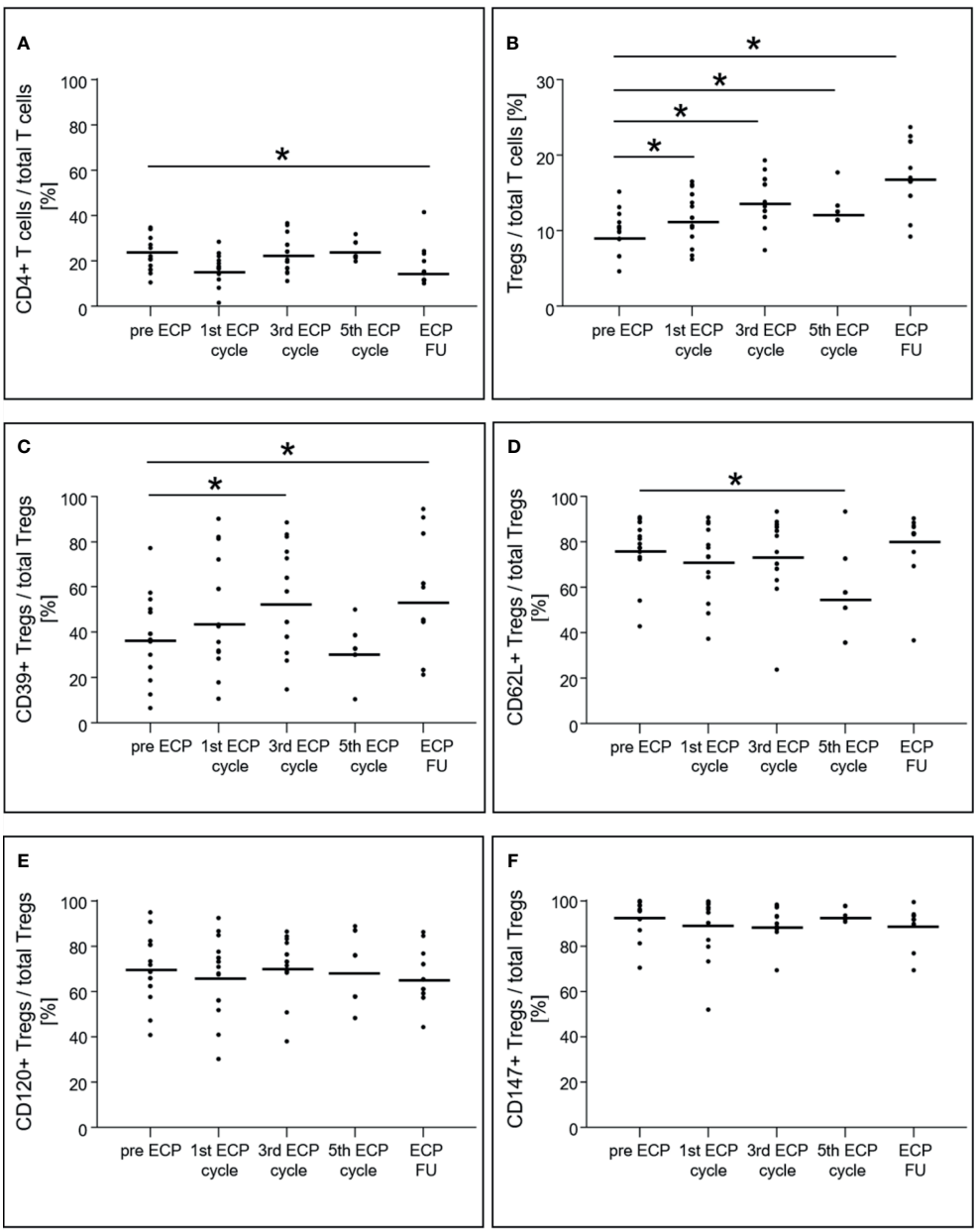

FIGURE 4 | Expression of $CD4^+$ T cells **(A)**, regulatory T cells **(B)** and their subsets **(C–F)** in heart-transplanted patients receiving extracorporeal photopheresis. * marks significant differences ($p \leq 0.05$); CD, cluster of differentiation; ECP, extracorporeal photopheresis; FU, follow-up; T_{regs}, regulatory T cells.

type immune response (20). Thus, it can be hypothesized that one mechanism of action of ECP treatment is the suppression of Th1-type immune responses *via* inhibition of interferon-α/β by pDCs. This example clearly demonstrates that immunological monitoring can help to further clarify the mechanism of action of ECP and could uncover unknown cellular effects.

In contrast to difficulties in interpreting increased surface expression of BDCA1 in the context of tolerance induction, the increase of BDCA4 expression in DCs is an observation of great interest. It has been reported that BDCA4, also known as neuropilin-1, can be transferred from DCs to T cells *via* trogocytosis (21), and, therefore, could be detected in natural T_{regs} with a proven suppressive function (22). Thus, the increase of BDCA4 expression during and after ECP treatment detected in our study could be associated with the induction of tolerance in ECP treated patients. This hypothesis is reinforced by the findings of a murine transplantation study that indicated a suppressive role of $CD4^+$/$BDCA4^+$ T cells (23). Furthermore, a reduction of $BDCA4^+$ cells in kidney transplant biopsies was observed during acute rejection compared to those in non-rejecting individuals (24).

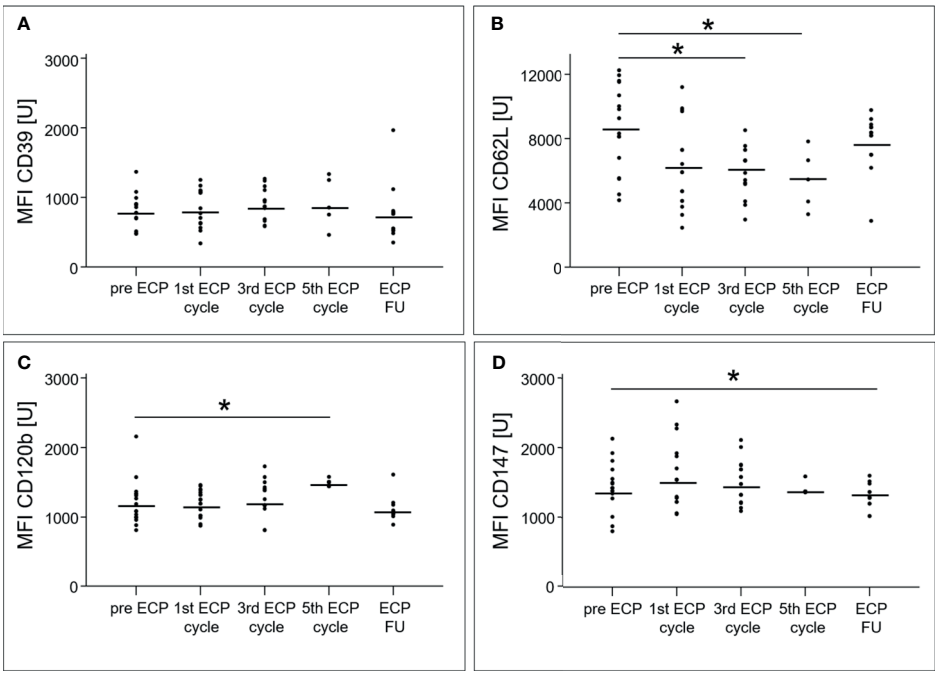

FIGURE 5 | Mean fluorescence intensities of the surface molecules CD39 **(A)**, CD62L **(B)**, CD120b **(C)** and CD147 **(D)** of regulatory T cell subsets in heart-transplanted patients receiving extracorporeal photopheresis. * marks significant differences ($p \leq 0.05$); CD, cluster of differentiation; ECP, extracorporeal photopheresis; FU, follow-up; MFI, mean fluorescence intensity; U, unit.

Besides the ECP-induced changes in DC subsets, the composition of T_{reg} subsets exhibited substantial modifications during and after ECP treatment. The measured effects documented in earlier reports of ECP treatment were limited to the increase of the total T_{reg} population and the highly suppressive $CD39^+$ T_{reg} subset (4, 25). Our data are consistent with these reports, and once again showed that ECP induced an increase of T_{regs} and the $CD39^+$ T_{reg} subsets within the first three ECP cycles (= six ECP treatments) of our ECP treatment schedule. We also showed that additional ECP treatment, up to ten cycles, reduced the percentage of $CD62L^+$ T_{regs} compared to those observed in our previous results in which three ECP cycles (= six ECP treatments) did not show a reduction of $CD62L^+$ T_{regs} (4). The CD62L expression of T_{regs} has been associated with optimal suppressive function of these cells (26, 27). Although $CD62L^+$ and $CD62L^-$ T_{regs} have been shown to be equally anergic and suppressive upon *in vitro* stimulation, only the $CD62L^+$ T_{regs} protect against lethal acute graft-versus-host disease after bone marrow transplantation (27, 28). The reduction in the fraction of $CD62L^+$ T_{regs} after the fifth ECP cycle and the reduced CD62L surface expression indicates that T_{regs} shifted from central memory to an activation state (26, 28). Several animal studies have documented that the loss of CD62L expression leads to a reduction in the protective properties of T_{regs} (26, 27, 29). Therefore, we concluded that the number of ECP cycles could be reduced to six treatments, because the loss of CD62L only appears after six treatments.

To evaluate the patient-specific benefit of ECP treatment, we defined classification criteria to calculate the individual immunological improvement. In our study cohort, 72% of the patients responded to five ECP cycles (= ten ECP treatments) according to our immunological profiling of the stimulation of tolerance-inducing cell subsets. However, ECP-induced effects were not detectable with our immunological profile in 28% of our patients. We hypothesized that the immunological changes would not be substantial enough to induce a clinical benefit in these patients. Furthermore, 56% of the ECP-treated patients showed an immunological improvement after no more than three ECP cycles. For these patients a less intense ECP regimen seems to be adequate.

There are several limitations to the current study. First, this is a descriptive study design that bears to risk for biases and a lack of variability of statistical results. Second, the ECP product was not investigated. About 30% of the centers who treat HTx patients with ECP perform quality controls such as measurement of hematocrit, lymphocyte count, monocyte count etc. (information received from Therakos Inc.). Further analyses are not recommended or performed in centers that treat HTx patients with ECP, but identification of laboratory parameters to qualify the ECP product is recommended (30). Third, we investigated ECP effects using the closed, inline THERAKOS CELLEX photopheresis system. Several closed and open offline systems exist, that may have different technical capacities (31). Therefore, ECP products may differ

with regard to cellular composition, total cell numbers, apoptotic cell content, the presence of psoralen photoadducts and excipients (32, 33).

In summary, our study described the changes of tolerance-inducing cell subsets during and after ECP treatment of HTx-patients. Compared to that in previous studies, the DC subsets were analyzed in detail, which revealed an important role in tolerance induction following HTx. The established monitoring tool can distinguish between patients who developed an immunological effect to ECP and patients that did not. Furthermore, we developed classification criteria that may allow identifying patients that would benefit from a reduction or an extension of the number of ECP cycles. Monitoring results including analysis could be available within 4-5 hours following blood withdrawal.

This tool could be helpful for clinicians to monitor ECP treatment for shortening or prolonging the ECP schedule for patients depending on their immunological profile. It is recommended to create a center-specific non-ECP-treated control group dataset consisting of long-term, rejection-free HTx patients and pre-HTx patients to work with a center-specific database for hierarchical clustering. Further, a multicenter study for ECP treatment in HTx patients will be helpful to prove our monitoring tool in the clinical routine.

DATA AVAILABILITY STATEMENT

The raw data of the flow cytometric measurements and immunological profile grouping supporting the conclusions of this article will be made available by the authors. Patient-specific data can not be transferred to the journal due to reasons of protection of personal data.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ärztekammer Hamburg KdÖR, Hamburg, Germany. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization: M-TD, MJB, and HR. Methodology: M-TD, KK, FA, MJB, and SK. Validation: MJB, AB, and FA. Formal analysis: M-TD, KK, SK, and SL. Investigation: M-TD and KK. Resources: MJB, FA, and MAB. Data curation: MJB, AB, KK, and SK. Writing—original draft preparation: M-TD and KK. Writing—review and editing: MJB, AB, FA, HR, MAB, SK, and SL. Visualization: M-TD and KK. Supervision: M-TD and MJB. Project administration: M-TD and MJB. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.676175/full#supplementary-material

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Impact of Graft-Resident Leucocytes on Treg Mediated Skin Graft Survival

Romy Steiner[1], *Anna M. Weijler*[1], *Thomas Wekerle*[1], *Jonathan Sprent*[2,3] *and Nina Pilat*[1,2*]

[1] Department of General Surgery, Medical University of Vienna, Vienna, Austria, [2] Immunology Division, Garvan Institute of Medical Research, Sydney, NSW, Australia, [3] St Vincent's Clinical School, University of New South Wales, Sydney, NSW, Australia

The importance and exact role of graft-resident leucocytes (also referred to as passenger leucocytes) in transplantation is controversial as these cells have been reported to either initiate or retard graft rejection. T cell activation to allografts is mediated *via* recognition of intact or processed donor MHC molecules on antigen-presenting cells (APC) as well as through interaction with donor-derived extracellular vesicles. Reduction of graft-resident leucocytes before transplantation is a well-known approach for prolonging organ survival without interfering with the recipient's immune system. As previously shown by our group, injecting mice with IL-2/anti-IL-2 complexes (IL-2cplx) to augment expansion of CD4 T regulatory cells (Tregs) induces tolerance towards islet allografts, and also to skin allografts when IL-2cplx treatment is supplemented with rapamycin and a short-term treatment of anti-IL-6. In this study, we investigated the mechanisms by which graft-resident leucocytes impact graft survival by studying the combined effects of IL-2cplx-mediated Treg expansion and passenger leucocyte depletion. For the latter, effective depletion of APC and T cells within the graft was induced by prior total body irradiation (TBI) of the graft donor. Surprisingly, substantial depletion of donor-derived leucocytes by TBI did not prolong graft survival in naïve mice, although it did result in augmented recipient leucocyte graft infiltration, presumably through irradiation-induced nonspecific inflammation. Notably, treatment with the IL-2cplx protocol prevented early inflammation of irradiated grafts, which correlated with an influx of Tregs into the grafts. This finding suggested there might be a synergistic effect of Treg expansion and graft-resident leucocyte depletion. In support of this idea, significant prolongation of skin graft survival was achieved if we combined graft-resident leucocyte depletion with the IL-2cplx protocol; this finding correlated along with a progressive shift in the composition of T cells subsets in the grafts towards a more tolerogenic environment. Donor-specific humoral responses remained unchanged, indicating minor importance of graft-resident leucocytes in anti-donor antibody development. These results demonstrate the importance of donor-derived leucocytes as well as Tregs in allograft survival, which might give rise to new clinical approaches.

Keywords: transplantation, allo-recognition, Regulatory T cells (Tregs), tolerance, IL-2 complexes, passenger leucocytes, graft-resident leucocytes

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Luis Graca,
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Reg Gorczynski,
University of Toronto, Canada
Alain Le Moine,
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***Correspondence:**
Nina Pilat
nina.pilat@meduniwien.ac.at

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INTRODUCTION

T cell reactions to organ allografts are directed largely to major histocompatibility (MHC) antigens expressed on donor cells, both within the graft and on graft-resident leucocytes; these "passenger leucocytes" migrate from the graft to the periphery to activate host T cells (1). In part, these reactions reflect "direct" contact of T cells with intact donor MHC antigens on graft-derived antigen-presenting cells (APCs), mostly dendritic cells (DC) (2). In parallel, T cells also make "indirect" contact with donor antigens as the result of degradation of their MHC molecules into specific donor peptides that bind to host MHC molecules displayed on host APC. Although both direct and indirect pathways of allorecognition are involved in graft rejection, the relative importance of these two pathways is still controversial (3).

The theory of "passenger leucocytes" as a target for allorecognition was first suggested in 1957 by G. D. Snell who described a highly immunogeneic cell population within the parenchyma and stroma of a solid organ allograft in mice (4). Thereafter, it was shown that graft immunogenicity can be attenuated by reduction of passenger leucocytes, *via* either prior organ culture *in vitro* or pre-transplantation on host-type mice. With both approaches, depletion of passenger leucocytes retarded rejection and thus prolonged survival of skin allografts in rodent models (5, 6). Likewise, studies of heart allograft transplantation in rats showed prolongation of survival if donor and/or organ were pretreated with a combination of photochemicals, total body irradiation (TBI) and injection of antilymphocyte globulin (7, 8).

Notably, Barker and Billingham demonstrated in 1968 that allograft survival was prolonged if donor cell trafficking through lymphatic vessels was inhibited (9). This finding suggested that sensitization of recipient T cells depended crucially on graft-resident leucocytes leaving the organ shortly after transplantation. In this respect, recovery of lymphatic vessels severed during skin transplantation surgery takes 5 to 7 days, thus impeding migration of donor cells to the draining lymph nodes (10). More recently, Marino et al. (11) suggested a different mechanism independent of intact donor APC, namely responses to donor-derived extracellular vesicles (EVs). Here, it was demonstrated that trafficking of EVs from the graft into recipient lymph nodes resulted in host cells expressing donor intact (rather than processed) MHC molecules alongside self MHC as early as 12 hours after transplantation. These allo-MHC "cross-dressed" recipient APCs efficiently activated alloreactive T cells in a skin transplantation setting. In addition, it was recently shown that donor-derived dendritic cells (DCs) are a major source of exosomes capable of promoting allograft rejection in a murine heart transplant model (12). This mechanism was originally suggested by Herrera et al. in 2004 where cross-dressing of DCs with donor MHC was described as a third pathway of allo-recognition, termed the "semi-direct" pathway (13). This pathway of acquired absorption of intact MHC-bearing membrane vesicles is now termed trogocytosis (14) and dates back to studies on cell-membrane exchange in the 1970s (15).

Despite the well-established migration of graft-derived APC and EVs to the draining lymph nodes, it is important to emphasize that shedding of EVs is not an exclusive property of APC and so may also arise from parenchymal cells in the graft. For this reason, the precise role of donor-derived leucocytes in transplantation continues to be controversial (16, 17). Here we have investigated the graft-resident leucocytes in a skin allograft transplantation setting largely devoid of passenger leucocytes, including T cells. We utilized our prior finding that injecting mice with IL-2/antibody complexes (IL-2cplx) led to Treg expansion and prolonged survival of skin allografts. Notably, a proportion of the expanded Tregs entered the grafts, raising the question whether these immigrant Tregs contributed to graft survival. To investigate this question, we examined the effects of irradiating skin allografts to deplete APC and T cells before transplantation (18), followed by IL-2cplx treatment of the host to promote host Treg entry into the grafts. In brief, the results show that, in combination, these procedures significantly enhance graft survival.

RESULTS

Depletion of Skin-Resident Leucocytes After TBI

To investigate the effect of passenger leucocytes on skin allograft survival, we established a fully-mismatched, clinically-relevant murine transplantation model (BALB/c on C57BL/6) largely depleted of graft-resident bone-marrow (BM)-derived cells. Donor mice (BALB/c) were subjected to lethal TBI (8.5 Gy) at day 8 prior to skin graft donation and were reconstituted with bone marrow (BM) cells from isogenic donors at day -7 (**Figure 1A**) (18). When irradiated (IR) donor skin is compared to non-irradiated (non-IR) skin at the time point of skin donation, percentages of leucocytes (identified as CD45+ cells amongst total viable skin cells) were significantly reduced (**Figure 1B**). There was comparable 80-90% depletion of CD4+ and CD8+ T cell frequencies (**Figure 1C**) and ~70% depletion of (dermal) DCs (CD45+ MHCII+ CD11c+), with a parallel reduction of LCs (CD45+ MHCII+ CD11b+) though this difference was not significant (**Figure 1D**). These data confirm the effective depletion of skin-resident leucocytes in the tail skin of donor mice subjected to IR (day 8 post IR).

IR Exposure Leads to Elevated Early Graft Infiltration of Recipient Leucocytes in Naïve but Not IL-2cplx Treated Host Mice

To study the immunological mechanisms leading to graft rejection, graft-infiltrating leucocytes (GILs) were investigated starting at day 6 after skin transplantation. Analysis of GILs in untreated recipients (wild type C57BL/6 grafted with BALB/c skin) revealed ~2.5-fold higher CD45+ cell frequencies in grafts previously exposed to IR in comparison to naïve (non-IR) grafts (mean 80% IR *vs* 32% non-IR; $p = 0.02$; **Figures 2A, B**). Based on staining for donor H-2Dd, nearly all (~98%) of the cells in the grafts at day 6 were of recipient origin (**Supplementary Figure 1**). Hence, prior IR of the donor grafts potentiated an early influx of host-derived inflammatory cells into the grafts (19). The GILs at day 6 included host CD4+ and CD8+ T cells (**Figure 2C**) which were enriched for cells with an effector phenotype (CD44+ CD62-) (**Figure 2D**). Within the CD4+

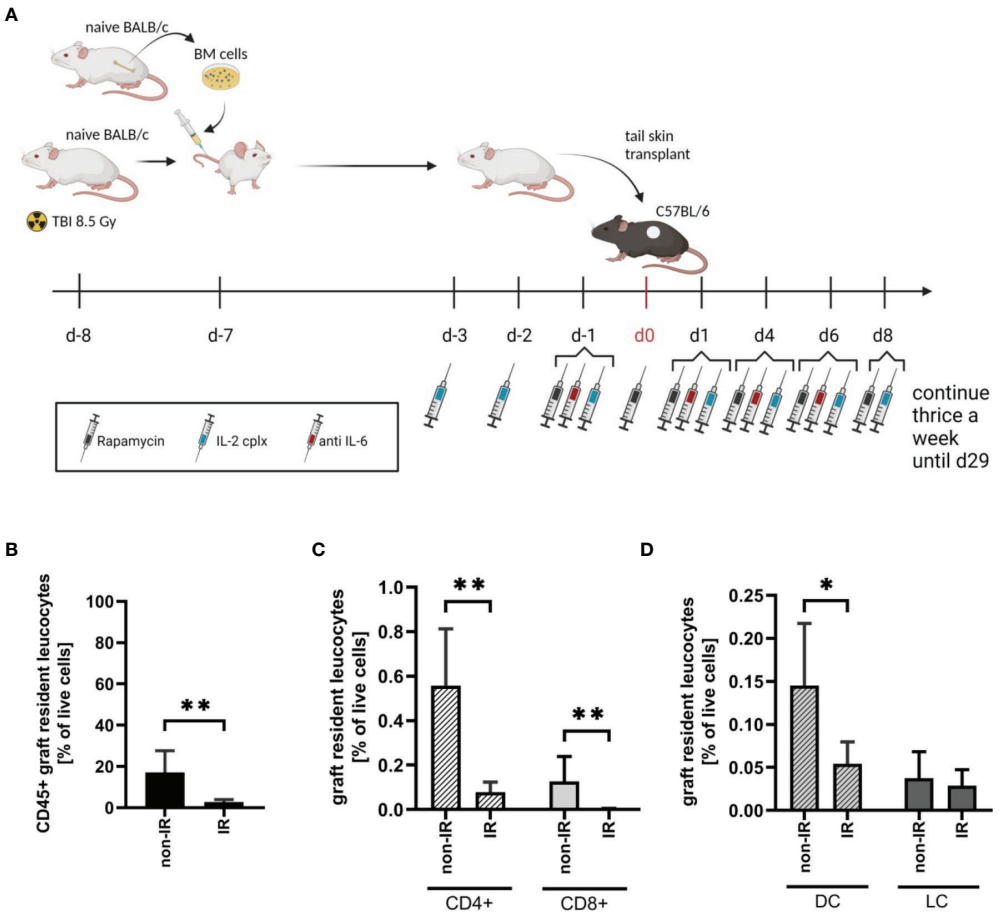

FIGURE 1 | Effective reduction of donor tail skin resident leucocytes 8 days after exposure to lethal total body irradiation. **(A)** Design of an allograft transplantation setting with reduced passenger leucocytes and IL-2 complex protocol treatment schema for indicated groups of skin allograft recipients. Donor mice were lethally irradiated (8.5Gy) d-8 and reconstituted with bone marrow cells of a naïve BALB/c mouse d-7. Donor tail skin was transplanted d0 onto recipient C57BL/6 mice. Indicated recipient mice in addition received a combination of IL-2 complexes, Rapamycin and a short term treatment of anti-IL-6. **(B)** Frequency of graft-resident CD45+ leucocytes within irradiated (IR; n = 7) and non-irradiated (non-IR; n = 4) skin grafts at time point of transplantation (d0) (mean 3% IR *vs* 17% non-IR; p = 0.006). **(C)** Graft-resident T-cell subsets within irradiated (n = 7) and non-irradiated (n = 4) skin grafts d0. Frequency of CD4+ (mean 0.079% IR *vs* 0.56% non-IR; p = 0.006) and CD8+ (mean 0.004% IR *vs* 0.13% non-IR; p = 0.006) T-cells within total graft-resident cells. **(D)** Frequency of graft-resident dendritic (CD45+ MHCII+ CD11c+; DC; mean 0.05% IR *vs* 0.15% non-IR; p = 0.03) and Langerhans cells (CD45+ MHCII+ CD11b+; LC; mean 0.03% IR *vs* 0.04% non-IR; NS) within IR and non-IR skin grafts d0 (n = 5 each). Analysis **(B–D)** was performed using flow cytometry and mean percentages are shown. Error bars indicate SD. (*P < 0.05; **P < 0.01; two-tailed t test with unequal variances).

and CD8+ T cell compartment, however, IR grafts show an increase in naïve T cells, although it was significant only in CD4+ cells (**Supplementary Figure 2A**). However, IR did not lead to an influx of Tregs (**Figure 2E**). Notably, the results were substantially different when the hosts were treated with our tolerogeneic IL-2cplx protocol (**Figure 1A**) (20). Here, IL-2cplx treatment considerably reduced the influx of recipient leucocytes into the grafts and led to comparable low levels of GILs for both IR and normal (non-IR) grafts. This finding applied to CD45+ cells and conventional T cell subsets but, notably, not to Tregs. In fact, for Tregs there was a marked increase in migration of host Tregs into the grafts, both for normal and IR grafts (mean 38% IL-2cplx non-IR *vs*. 42% IL-2cplx IR; **Figure 2E**). This finding confirmed our previous observation that IL-2cplx-expanded Tregs can traffic rapidly into skin allografts (20). In addition, however, the data indicated that the influx of Tregs prevented the selective influx of other leukocytes into the IR grafts, consistent with the well-documented anti-inflammatory function of Tregs.

Passenger Leucocyte Depletion Leads to Reduced Infiltration of Effector T Cells

As discussed below, prolonged survival of skin allografts was seen only in IL-2cplx-treated mice. Hence, examination of GILs at late stages of engraftment was restricted to the two groups of IL-2cplx-treated mice. Data on cells recovered from the grafts on day 20 are shown in **Figure 3**. At this time point, no macroscopic differences of skin grafts were visible. However, the percentage of CD45+ leukocytes was significantly (~20%) higher in normal

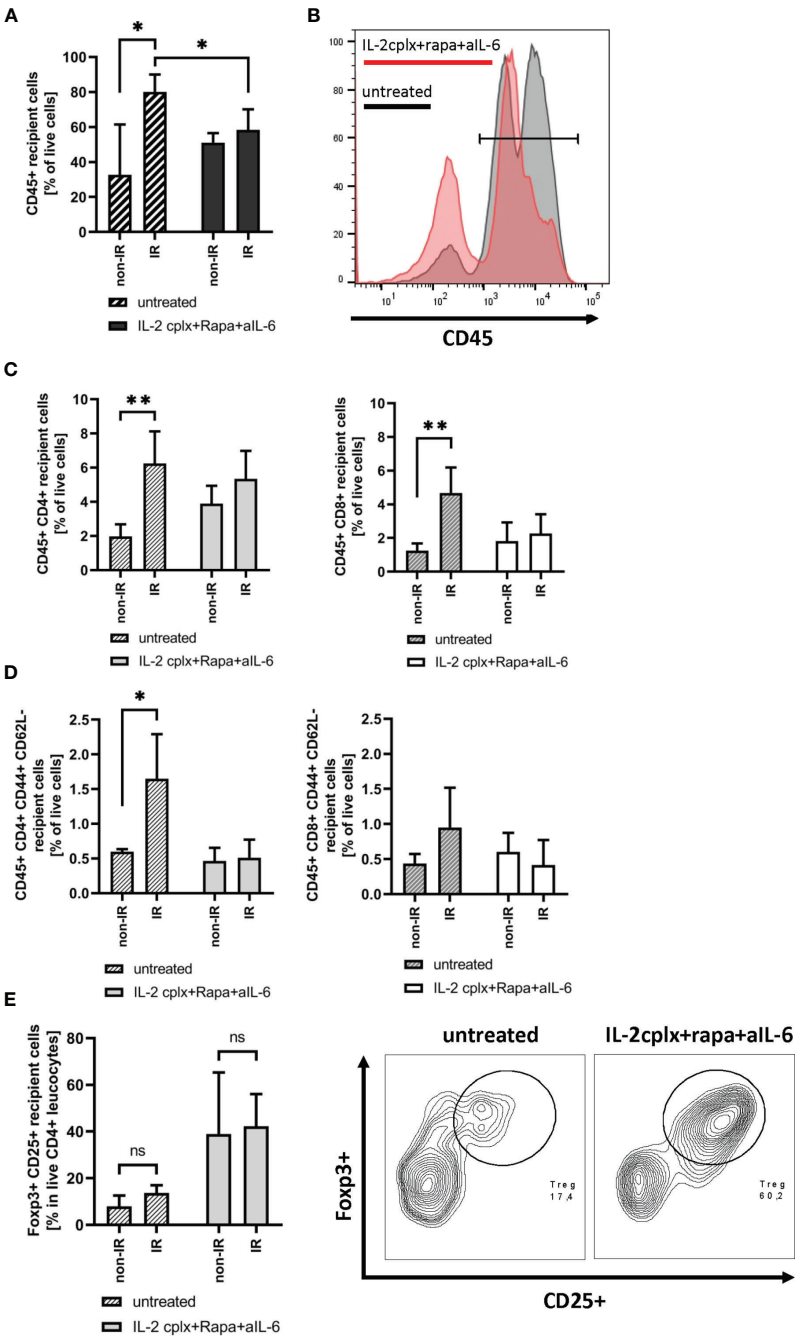

FIGURE 2 | Analysis of graft infiltrating leucocytes (recipient) 6 days post transplantation. **(A)** Frequency of CD45+ graft infiltrating leucocytes within skin grafts that were irradiated (IR) *vs*. non-irradiated (non-IR) before transplantation on mice receiving IL-2 complexes in combination with rapamycin and a short course of anti-IL-6 (IR: n = 5; non-IR: n = 6) or left untreated (IR: n = 5; non-IR: n = 5). **(B)** Representative histogram showing frequency of CD45+ graft infiltrating leucocytes in previously irradiated skin allografts. Results for recipients treated with IL-2cplx protocol are indicated in red, untreated are shown in grey. **(C)** Frequency of CD4+ (left) and CD8+ T cells (right) infiltrating the graft d6 post transplantation. **(D)** Frequency of CD4+ (left) and CD8+ (right) effector memory T cells (CD44+ CD62L-) found in skin allografts day 6 post transplantation. **(E)** Proportion of regulatory T cells (CD45+ CD4+ Foxp3+ CD25+) in the CD45+ CD4+ T cell population found within transplanted skin allografts day 6 after transplantation (left). Representative contour plot for regulatory T cell frequency within CD45+ CD4+ graft infiltrating cells (data from IR skin allograft transplanted mice; right). Analysis **(A–E)** was performed using flow cytometry and mean percentages of two independent experiments of untreated (IR: n = 5; non-IR: n = 5) and IL-2cplx protocol treated (IR: n = 5; non-IR: n = 6) recipients transplanted with irradiated or non-irradiated skin grafts are shown. Error bars indicate SD. (ns, not significant $P > 0.05$; *$P < 0.05$; **$P < 0.01$; two-tailed t test with unequal variances).

than in IR grafts (mean 62% IR *vs* 73% non-IR; p = 0.01; **Figure 3A**). For CD4+ and CD8+ cells, there was also a shift towards a higher percentage of T effector cells in the IR grafts (**Figures 3B, C**; **Supplementary Figure 2B**). Comparably high Treg frequencies (~50% of CD4+ T cells) were found within IR and non-IR skin grafts, suggesting active regulation in both groups (**Figure 3D**).

Reduction of Passenger Leucocytes Leads to Prolonged Skin Allograft Survival in IL-2cplx Protocol Treated Mice

Since the IR grafts were largely depleted of APC at the time of grafting, we expected survival of these grafts to be significantly prolonged. For the groups that were not IL-2cplx treated, however, this was not the case. Thus, both the control non-IR and IR grafts were rejected rapidly at the same time, 8 days after transplantation (**Figure 4A**). With the IL-2cplx-treated groups, the results were different. Here, as expected, IL-2cplx treatment led to prolonged survival of both the control and IR grafts. Notably, however, the IR grafts survived for around twice as long as the control grafts (MST IR = 53 days; non-IR = 30 days; p = 0.02), (**Figure 4A**).

These data indicate therefore that the capacity of IL-2cplx treatment to enhance allograft survival could be further improved by prior irradiation of the grafts. With regard to the mechanisms involved, for the control non-IR grafts the

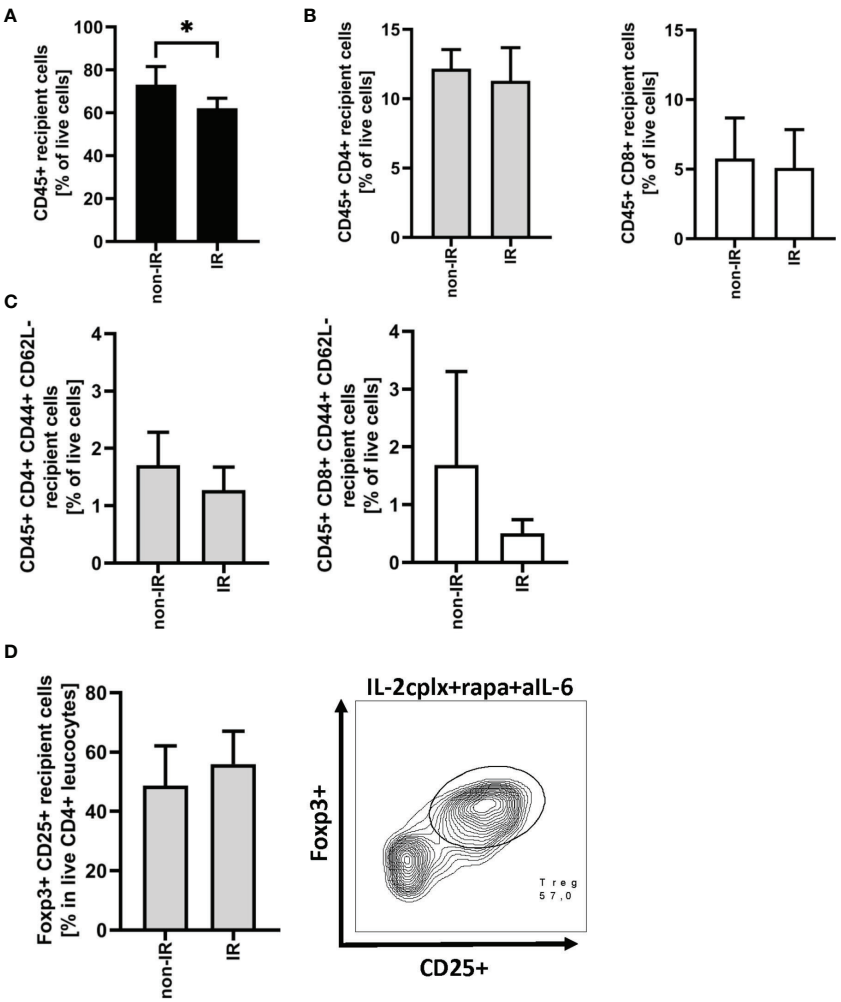

FIGURE 3 | Graft infiltrating leucocytes (recipient) 20 days after transplantation. **(A)** Frequency of CD45+ graft infiltrating leucocytes within BALB/c skin grafts that were irradiated (IR) or non-irradiated (non-IR) before transplantation onto IL-2 complex protocol treated C57BL/6 recipients (IR: n = 6; non-IR: n = 7). **(B)** Frequency of CD4+ (left) and CD8+ (right) allograft infiltrating leucocytes. **(C)** Proportion of CD4+ (left) and CD8+ (right) effector memory T cells found within previously irradiated or non-irradiated skin allografts 20 days after transplantation. **(D)** Percentage of regulatory T cells (CD45+ CD4+ Foxp3+ CD25+) in the CD45+ CD4+ T cell population present within the skin graft 20 days after transplantation (left). Representative contour plot of regulatory T cell frequency within CD45+ CD4+ graft infiltrating cells if recipient was treated with IL-2cplx protocol (right). Analysis **(A–D)** was performed using flow cytometry and mean percentages of two independent experiments of IL-2cplx protocol treated recipients transplanted with irradiated (n = 6) or non-irradiated (n = 7) skin graft are shown. Error bars indicate SD. (*P < 0.05; two-tailed t test with unequal variances).

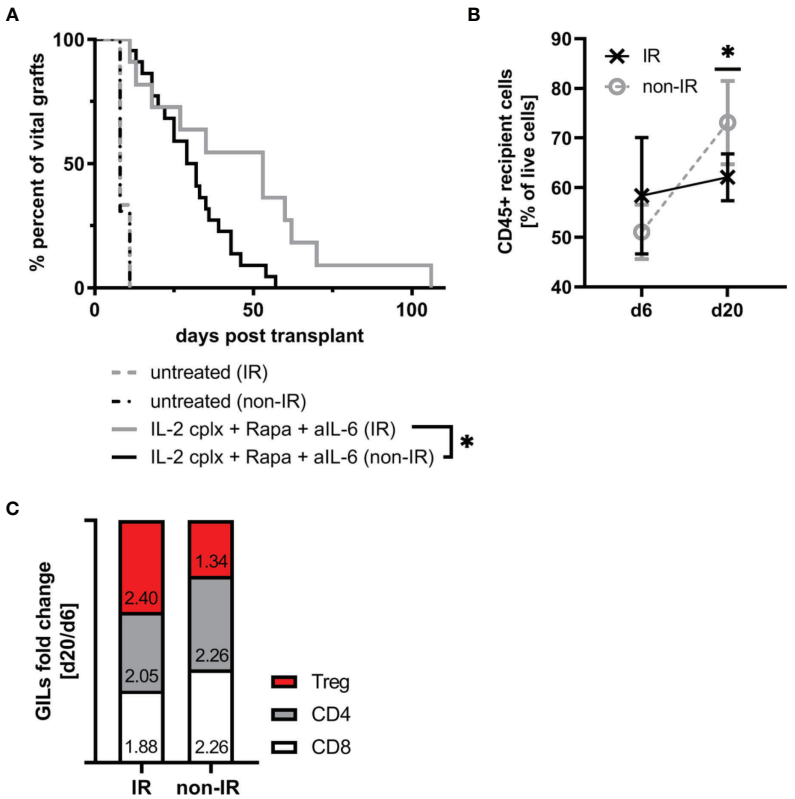

FIGURE 4 | Irradiation of donor skin graft prior to transplantation leads to significant prolongation of skin allograft survival in combination with IL-2cplx protocol treatment (MST: IR=53 days; non-IR=30.5 days; p = 0.0192, log-rank test). **(A)** Survival of irradiated (IR) and non-irradiated (non-IR) skin grafts on mice treated with (IR: n = 8; non-IR: n = 8) or without (IR: n = 11; non-IR: n = 22) a combination of IL-2 complexes, Rapamycin and a short term treatment of anti-IL-6. Survival proportions of at least two independent experiments are shown. **(B)** Mean frequency of CD45+ graft infiltrating leucocytes on day 6 and day 20 after transplantation in irradiated (d6: n = 3; d20: n = 6) and non-irradiated (d6: n = 6; d20: n = 7) skin allografts transplanted on IL-2cplx protocol treated recipient mice. **(C)** Differences in fold change between d6 and d20 post transplantation in non-irradiated (left; d6: n = 6; d20: n = 7) and irradiated (right; d6: n = 3; d20: n = 6) skin allografts on IL-2cplx protocol treated recipient mice. Calculation: Fold change was obtained by dividing the mean value of two independent experiments for d20 by the mean value of d6. Fold changes were normalized to values obtained for non-irradiated skin grafts (shown as baseline with value 0). Analysis **(B, C)** was performed using flow cytometry and mean percentages of two independent experiments are shown. (*P < 0.05; two-tailed t test with unequal variances).

frequency of CD45+ cells infiltrating the grafts increased significantly between day 6 and day 20 day after transplantation (mean 51% d6 *vs* 73% d20; p = 0.001). For the IR grafts, by contrast, levels of CD45+ cells did not increase between day 6 and day 20 (mean 58% d6 *vs* 62% d20) (**Figure 4B**). Nevertheless, during this time there was a subtle change in the proportions of T cell subsets in the grafts. Thus, when fold changes of graft-infiltrating T cell subsets between days 6 and 20 were compared, the IR grafts showed a selective enrichment of Tregs relative to normal CD4 and CD8 T cells (**Figure 4C**). Hence, based on the GILs examined at these time points, the enhanced survival of the IR grafts correlated with a shift from conventional T cell subsets to Tregs in the grafts.

Reduction of Passenger Leucocytes Does Not Affect Donor-Specific Humoral Response

To determine whether the absence of passenger leucocytes also affects humoral responses, serum of mice grafted with IR or non-IR skin was investigated day 14 post rejection of skin allografts. Donor H2k-d (MHC I) and IA-d (MHC II) specific ELISA showed that recipient mice receiving IL-2cplx do not develop donor specific IgG responses (**Figures 5A, B**). This was seen for MHC I as well as MHC II and was independent of whether IR or non-IR skin allografts were applied, suggesting that the absence of passenger leucocytes has no direct influence on the development of anti-donor antibodies.

MATERIAL AND METHODS

Mice

Female C57BL/6 (recipient; H-2^b), BALB/c (donor; H-2^d) and C3H (H-2^k) mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and housed in ventilated HEPA-filtered cages at the Medical University of Vienna under specific pathogen-free like conditions. Mice were used for experiments between 6 and 8 weeks of age with an average

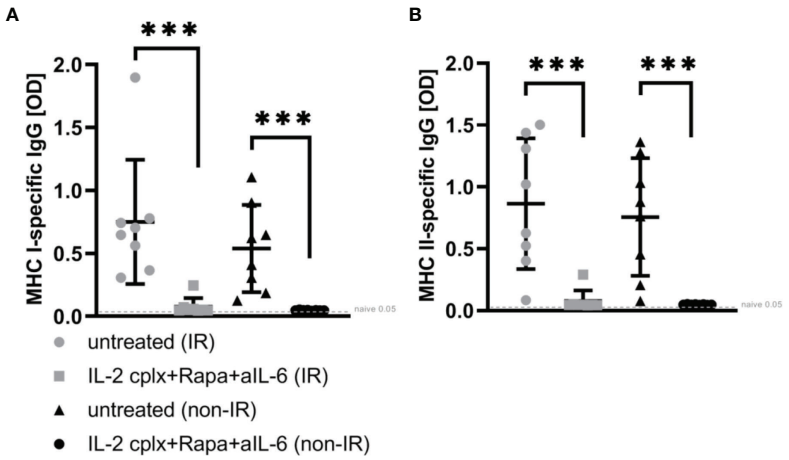

FIGURE 5 | ELISA for donor-specific antibodies within blood sera 14 days post skin graft rejection. Irradiated (IR) or non-irradiated (non-IR) skin allograft recipients (C57BL/6) were either left untreated or IL-2 complexes in combination with rapamycin and anti-IL-6 (short term) were administered. Blood sera isolation was performed d14 post rejection of BALB/c skin allograft and assessment of donor specific IgG (IgG1, IgG 2a/b, IgG3) against **(A)** MHC class I (H2k-d) and **(B)** MHC class II (IA-d) was done utilizing ELISA. Mean percentages of at least two independent experiments are shown. Error bars indicate SD. (***$p < 0.001$; two-tailed t test with unequal variances) (untreated (IR): n = 8; untreated (non-IR): n = 8; IL-2cplx protocol (IR): n = 8; IL-2cplx protocol (non-IR): n = 8).

weight of 18 to 21 g. All experiments were approved by the ethics votum of the Austrian Federal Ministry of Science, Research and Economy (Permission number GZ: BMWFW-66.009/0292-V/3b/2018).

Preparation of IL-2/anti-IL-2 mab Complexes (IL-2cplx)

IL-2 complexes were prepared as previously described (20, 21). Briefly, IL-2/anti-IL-2 complexes were prepared by mixing recombinant mouse IL-2 (PeproTech) with anti-IL-2 mab (clone JES6-1A12, BioXCell) in a 1:5 ratio and incubating at 37°C for 30 min. Mice received a final volume of 300 µl intraperitoneally (i.p.).

Generation of Skin Resident Leucocyte Reduced Donor Mice

Donor mice (BALB/c) were subjected to lethal total body irradiation (TBI) of 8.5 Gy at day -8 and were reconstituted with 10 x 10^6 bone marrow cells of naïve BALB/c mice at day -7. Skin graft donation followed 8 days post TBI.

IL-2 Complex Protocol

The IL-2 complex protocol was administered as previously described (20). Briefly, indicated groups of age-matched C57BL/6 mice received IL-2 complexes (1 µg IL-2/5 µg JES6-1A12 i.p.) on 3 consecutive days starting on day 3 before transplantation, which was continued to day 1 after transplantation with injections thrice a week until day 30. In addition, rapamycin (1 mg/kg i.p., LC Laboratories) was administered day -1/0/1 followed by injections thrice a week until day 30. Furthermore, mice received a short-term treatment with anti-IL-6 mab (clone MP5-20F3, BioXCell) at days -1/1/4/6 (300 µg i.v.).

Skin Grafting

Full thickness tail skin of naïve BALB/c (donor) mice or BALB/c mice previously subjected to lethal TBI and reconstituted with isogenic BM were grafted on the flank (lateral thorax wall) of naïve C57BL/6 (recipient) mice. After transplantation skin grafts were secured with band aids for 6 days, followed by visual inspections at short intervals. Skin grafts were considered rejected if less than 10% remained viable. During transplantation mice were anesthetized with Ketanest (Ketamin, 100 mg/kg) and Rompun (Xylazine, 5 mg/kg). Postoperatively mice received Temgesic (Buprenophin, day 0; 0,01 – 0,05 mg/kg i.p.) and Dipidolor (Piritramide, 15 mg in 250 ml 0,4% glucose water) in drinking water ad libitum for 1 week.

Flow Cytometric Analysis and Antibodies

For characterization of leucocyte subtypes antibodies against mouse CD45.2 (104), $H2D^d$ (34-2-12), CD3 (17A2), CD4 (RM4-5 and GK1.5), CD8 (53-6.7), CD44 (IM7), CD62L (MEL-14), CD25 (PC61.5), CD80 (16-10A1), CD86 (PO3), CD11b (M1/70), CD11c (N418), MHC II (M5/114.15.2), 7AAD (viability staining solution, purchased from BioLegend), Foxp3 (FJK-16s) and fixable viability dye (Fixable Viability Dye eFlour 450 and 506 purchased from eBioscience) were used. Cell suspensions were stained for surface markers for 30 min., 4°C in the dark. Intracellular Foxp3 staining was done using a Fixation/Permeabilization kit (eBioscience) according to the manufacturer's instructions. Erythrocyte lysis in spleen cells was performed utilizing red blood cell lysis buffer (Sigma). Analysis was done with BD FACS Canto II and FlowJo software.

Isolation of Graft-Resident/Graft Infiltrating Leucocytes (GILs)

Skin grafts were cut into small pieces in RPMI medium before digestion using a murine Tumor Dissociation Kit (Miltenyi

Biotec) according to the manufacturer`s instructions. Cells were resuspended in cold PBS and the number of viable cells was determined using a CASY cell counter (Innovatis) before flow cytometrical staining. GILs were defined as viable single cells expressing CD45.

Enzyme-linked Immunosorbent Assay (ELISA)

To detect IgG and IgM against donor MHC I (H2k-d) and MHC II (IA-d), a donor MHC-specific ELISA was performed as previously described (22). Briefly, a 96-well plate was coated with 5µg/mL of $H2K^d$ or $I\text{-}A^d$ monomers at 4°C, overnight. Serum samples obtained 14 days post rejection of skin allografts were diluted 1:100 before incubation with monomers at 4°C, overnight. Donor-specific antibodies bound to monomers were detected utilizing monoclonal rat anti-mouse IgG1, IgG2a/b, IgG3 in combination with an HRP-coupled goat anti-rat serum (1:2000). ABTS was used as substrate for HRP and absorption was measured using a Victor microplate reader (405nm). The biotinylated MHC monomers were kindly provided by the National Institutes of Health's Tetramer Core Facility (https://www.niaid.nih.gov/research/nih-tetramer-core-facility).

Statistics

The statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Error bars indicate standard deviation (SD) and differences between groups were compared using a 2-tailed Student´s t-test with unequal variances. Survival of skin allografts was calculated based on Kaplan-Meier product limit method and compared between groups using the log-rank test. Fold change in GILs was calculated dividing the mean value of two independent experiments for d20 by the mean value of d6. A p-value < 0.05 was considered statistically significant.

DISCUSSION

The role of passenger leucocytes and its impact on graft survival after solid organ transplantation is complex. Although several groups demonstrated increased survival of mismatched organs depleted of graft-resident cells in rodent models (5-8), there is also contrary evidence suggesting beneficial immunomodulatory effects of passenger leucocytes in transplantation (17). Studies on the long-term tolerance of allogeneic liver in rats, for example, revealed an essential function of donor-derived intrahepatic leucocytes on graft survival (23). Furthermore, using a rodent model for fully MHC-mismatched heart transplantation, tolerance induction was prevented if donor leucocytes were depleted on the day of transplantation using a monoclonal antibody against donor-specific CD45-expressing cells (24). In the study presented herein, we used a tolerogeneic treatment regimen based on IL-2cplx injection to demonstrate that the survival of fully MHC-mismatched skin grafts is significantly prolonged if donor-derived leucocytes are reduced. These data thus support the hypothesis that passenger leucocytes promote the rejection of allogeneic skin grafts.

Contact with DCs is crucial for immune responses after solid organ transplantation with DCs being 100 times more potent in activating T cells than MHC II-bearing macrophages or B cells (25). Within the skin, two types of resident DCs are well characterized: Dermal DCs and epidermal Langerhans cells (LC) (26). Besides being professional and efficient APCs, LCs of the epidermis are suggested to be effective in stimulating effector responses resulting in graft rejection, i.e. inducing naïve CD4+ T cell differentiation into effector phenotypes (27–29) or priming of naïve CD8+ T cells (30). We could show that irradiation of donor mice (8 days prior to tail skin donation) leads to substantial reduction of these graft-resident APCs, in accordance to what has been previously shown for dermal DCs and epidermal LCs in murine ear skin (18). In addition, we were able to demonstrate IR-related effective reduction of CD4+ as well as CD8+ T cells within the donor tail skin. The reduction and depletion of donor leucocytes not only decreases donor antigen-dependent recipient (direct) T cell allo-recognition (31), but also reduces donor-derived CD4+ T cell-related increase of recipient immune responses leading to early graft failure (32). Besides, using this approach we were able to deplete graft-resident leucocytes without altering the host´s immune system, creating an eligible model for analysis of allo-recognition mechanisms.

At face value, the finding that the IR grafts were rapidly rejected on normal hosts would seem to argue against the concept that removal of passenger leukocytes improves graft survival. However, as demonstrated here the IR grafts were rapidly infiltrated with host T cells. Thus, at day 6 after transplantation the frequencies of recipient CD4+ and CD8+ effector T cells were exceptionally high in skin grafts previously subjected to IR, suggestive of active inflammation. This finding is in line with literature showing that IR leads to the release of pro-inflammatory cytokines and chemokines as well as oxidative stress (19). Hence, the enhanced influx of host T cells into the IR grafts would be expected to augment the rejection process. Our suggestion therefore is the failure of passenger leukocyte depletion to delay rejection of the IR grafts was largely due to irradiation-induced inflammation causing an enhanced host-*versus*-graft response. This notion is in line with the report that early infiltration of donor-reactive CD8+ T cells promoted fast allograft rejection *via* IFNγ production in a cardiac transplant model (33). However, we cannot exclude the possibility that the fully-mismatched IR grafts were less immunogenic in terms of long-term sensitization. Relative to non-IR grafts, the IR grafts elicited lower levels of memory-phenotype T cells after rejection, though whether these cells were antigen-specific was unclear (34). Studies on the kinetics of second-set rejection are in progress. Importantly, for both grafted groups Treg numbers soon returned to the normal levels seen in naïve C57BL/6 mice, confirming that there are no unspecific long-term immunosuppressive effects due to IL-2cplx treatment (unpublished data). Overall, the data provide further support for the view that passenger leukocytes play a significant though not obligatory role in allograft rejection. Definitive information on which particular subsets of skin APC are needed for sensitization will require studies on the selective depletion of DC, LC and related cells (35).

Here, we could show that in mice receiving tolerogeneic IL-2cplx treatment, the reduction of graft-resident leucocytes leads to significant increase of skin allograft survival, illustrating the effect of graft-resident cells on long-term transplant acceptance. As already mentioned, irradiation induces tissue damage and inflammation independent from allo-specific responses. Treg expansion induced by IL-2cplx treatment leads to an anti-inflammatory, suppressive environment, both within the graft and in the host lymphoid tissues. We suggest that under tolerogeneic conditions the decreased immunogenicity of leucocyte depleted grafts favors improved long-term survival.

We also analyzed donor-specific antibody (DSA) formation since DSAs are known to be one major issue in graft injury and chronic rejection (36). As previously demonstrated by our group DSA IgG development is impaired if recipients are treated with IL-2cplx protocol (20). In line with this, we could show that IgG-related humoral immunity was almost absent in the recipients depleted of graft-resident leucocytes suggesting that passenger leucocytes seem to be important in T cell priming but do not influence GC formation or antibody production.

Although this study is restricted to skin allograft transplantation, it clearly demonstrates that the absence of graft-resident cells (in this study achieved by IR pre-treatment of the donor) is beneficial for long-term transplant survival if early IR-related pro-inflammatory processes are overcome. As already mentioned IR is accompanied by a variety of side effects (19), discouraging clinical translation. Alternative approaches for reduction/depletion of passenger leucocytes are based on graft-specific modifications using i.e. RNA interference for prevention of donor cell trafficking (CCL7) or suppression of donor-derived EV release may have superior potential for future translation to the clinical setting (12, 37). Since the discovery of RNA interference (38) promising data have been obtained in preclinical studies of rodent heart and kidney models utilizing small interference RNA (siRNA) targeting complement system or blocking co-stimulation with successful prolongation of graft survival (39–41). More importantly, there are ongoing phase 3 clinical trials involving siRNA in kidney transplantation with evidence of successful prevention of acute kidney injury and delayed graft function (42–44). By use of perfusion machines, donor leucocytes are mobilized into the perfusate, which allows their removal with a leucocyte filter before transplantation (45). Normothermic machine perfusion including a leucocyte filter was shown to reduce acute rejection and T cell priming in a porcine lung transplantation model (46). Future approaches could include *ex vivo* organ perfusion with depleting antibodies or anti-thymocyte globulin (ATG) to reduce the number of specific graft-resident leucocyte subsets within the graft.

In this proof-of-principle study we could demonstrate the importance of donor-derived leucocytes in recipient sensitization and allograft rejection, which may lay the ground to new translational approaches. Graft survival and long-term outcome could be improved by targeting early allogeneic responses and acute rejection episodes due to rapid recognition of allogeneic MHC in direct T cell allorecognition. Studies investigating the effect on long-term survival and chronic rejection in primarily vascularized grafts are clearly warranted.

More importantly, these data suggest that treatment with the IL-2cplx protocol ameliorates (non-allogeneic) inflammatory processes caused by e.g. IR and therefore counteracts the early augmented infiltration of leucocytes into the graft, which could trigger acute rejection and early graft loss.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Federal Ministry Republic of Austria Education, Science and Research.

AUTHOR CONTRIBUTIONS

All authors have actively contributed to this project. RS and NP designed all experiments and analyzed data. RS, NP, and AW performed experiments. RS, NP, JS, and TW interpreted data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.801595/full#supplementary-material

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Early Myeloid Derived Suppressor Cells (eMDSCs) Are Associated With High Donor Myeloid Chimerism Following Haploidentical HSCT for Sickle Cell Disease

Deepali K. Bhat[1†], Purevdorj B. Olkhanud[1†], Arunakumar Gangaplara[1], Fayaz Seifuddin[2], Mehdi Pirooznia[2], Angélique Biancotto[3], Giovanna Fantoni[3], Corinne Pittman[1], Berline Francis[1], Pradeep K. Dagur[4], Ankit Saxena[4], J. Philip McCoy[4], Ruth M. Pfeiffer[5] and Courtney D. Fitzhugh[1]*

[1] Cellular and Molecular Therapeutics Branch, National Heart, Lung, and Blood Institute (NHLBI), National Institutes of Health (NIH), Bethesda, MD, United States, [2] Bioinformatics and Computational Biology Core Facility, National Heart, Lung, and Blood Institute (NHLBI), National Institutes of Health (NIH), Bethesda, MD, United States, [3] Center for Human Immunology, Autoimmunity, and Inflammation, National Institutes of Health (NIH), Bethesda, MD, United States, [4] Flow Cytometry Core, National Heart, Lung, and Blood Institute (NHLBI), National Institutes of Health (NIH), Bethesda MD, United States, [5] Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health (NIH), Bethesda, MD, United States

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***Correspondence:**
Courtney D. Fitzhugh
fitzhughc@nhlbi.nih.gov

[†]These authors have contributed equally to this work

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Haploidentical hematopoietic stem cell transplantation (haplo-HSCT) is a widely available curative option for patients with sickle cell disease (SCD). Our original non-myeloablative haplo-HSCT trial employing post-transplant (PT) cyclophosphamide had a low incidence of GVHD but had high rejection rates. Here, we aimed to evaluate immune reconstitution following haplo-HSCT and identify cytokines and cells associated with graft rejection/engraftment. 50 cytokines and 10 immune cell subsets were screened using multiplex-ELISA and flow cytometry, respectively, at baseline and PT-Days 30, 60, 100, and 180. We observed the most significant differences in cytokine levels between the engrafted and rejected groups at PT-Day 60, corresponding with clinical findings of secondary graft rejection. Of the 44 cytokines evaluated, plasma concentrations of 19 cytokines were different between the two groups at PT-Day 60. Factor analysis suggested two independent factors. The first factor (IL-17A, IL-10, IL-7, G-CSF, IL-2, MIP-1a, VEGF, and TGFb1 contributed significantly) was strongly associated with engraftment with OR = 2.7 (95%CI of 1.4 to 5.4), whereas the second factor (GROa and IL-18 contributed significantly) was not significantly associated with engraftment. Sufficient donor myeloid chimerism (DMC) is critical for the success of HSCT; here, we evaluated immune cells among high (H) DMC (DMC≥20%) and low (L) DMC (DMC<20%) groups along with engrafted and rejected groups. We found that early myeloid-derived suppressor cell (eMDSC) frequencies were elevated in engrafted patients and patients with HDMC at PT-Day 30 ($P< 0.04$ & $P< 0.003$, respectively). 9 of 20 patients were evaluated for the source of eMDSCs. The HDMC group had high mixed chimeric eMDSCs as compared to the LDMC group ($P< 0.00001$). We found a positive correlation between the frequencies of

eMDSCs and Tregs at PT-Day 100 (r=0.72, P <0.0007); eMDSCs at BSL and Tregs at PT-Day 100 (r=0.63, P <0.004). Of 10 immune regulatory cells and 50 cytokines, we observed mixed chimeric eMDSCs and IL-17A, IL-10, IL-7, G-CSF, IL-2, MIP-1a, VEGF, TGFb1 as potential hits which could serve as prognostic markers in predicting allograft outcome towards engraftment following haploidentical HSCT employing post-transplant cyclophosphamide. The current findings need to be replicated and further explored in a larger cohort.

Keywords: donor myeloid chimerism, haploidentical HSCT, Tregs, IL-10, sickle cell disease, early myeloid derived suppressor cells

1 INTRODUCTION

Sickle cell disease (SCD) is a debilitating monogenic disorder that affects over 5 million people worldwide (1) and approximately 90,000 people in the United States (2). A substitution of valine for glutamic acid at the sixth position of the beta-globin chain in hemoglobin (Hb) leads to abnormal Hb polymerization in areas of low oxygen tension, causing recurrent vaso-occlusion. SCD is associated with early mortality and severe morbidity, including recurrent painful crises, chronic renal injury often progressing to end-stage renal disease (3, 4), avascular necrosis, stroke (5), acute chest syndrome, and cardiopulmonary complications (6). Hematopoietic stem cell transplantation (HSCT) offers a potentially curative option for SCD and can improve morbidity and overall quality of life in severely affected patients (7, 8). While human leukocyte antigen (HLA)-matched donor HSCT has high efficacy (7), this option is limited by the availability of such donors and is further complicated by the inheritance pattern of SCD (9). HLA-haploidentical (haplo) donors expand the donor pool with approximately 90% of patients having a haplo-donor (10).

Unlike hematological malignancies where complete replacement of the diseased marrow with healthy donor marrow is required, SCD does not require full donor chimerism. Using mathematical modeling, we reported that 20% donor myeloid chimerism (DMC) is sufficient to reverse SCD due to the short half-life of the sickle red blood cells (RBCs) compared to the healthy donor RBCs (11–14). We developed a non-myeloablative haplo-protocol for patients with SCD intending to maintain mixed chimerism by employing escalating doses of post-transplant cyclophosphamide (PT-Cy) (15). Graft success rate was increased with an increasing dose of PT-Cy (83% engraftment rate and 50% event-free survival rate with 100 mg/kg). The major limitation of the study, however, was the high rate of allograft rejection.

Our study therefore aimed to evaluate non-invasive prognostic cytokines and cells associated with graft rejection/engraftment in the recipients before and at defined PT time points. Understanding the transplanted patients' immune milieu may provide cues for subsequent allograft outcome (16), either successful engraftment or allograft rejection. Here, we sought to evaluate the circulatory cytokines and immune regulatory and effector cells in peripheral blood and their intracellular cytokine-producing abilities in association with allograft outcome.

2 MATERIALS AND METHODS

2.1 Patients and Samples

A total of 23 adults underwent non-myeloablative haplo-HSCT at the National Institutes of Health (NIH) from March 2010 through September 2015 for SCD (21/23) and beta-thalassemia (2/23). One patient with SCD died <6 months post-HSCT and was not included in the study. 20 patients with SCD were evaluated for cytokines and immune cell subsets (**Supplementary Table S1**). Patients were conditioned with alemtuzumab, 400 cGy total body irradiation, PT-Cy doses ranging from 0-100 mg/kg body weight in three dose dependent cohorts, (cohort 1: 0mg/kg body weight, cohort 2: 50mg/kg body weight and cohort 3: 100 mg/kg body weight). Sirolimus was loaded 1 day before transplant in cohort 1 and in the first 6 patients who received a transplant in cohort 2 and 1 day after PT-Cy in the remaining cohort 2 patients (day 4) and in all cohort 3 patients (day 5). A trough level of 10 to 15 ng/mL was targeted until 3 to 4 months posttransplant, and then the level was decreased to 10 to 12 ng/mL until 1 year posttransplant and then 5 to 10 ng/mL thereafter in engrafted patients (15). Donor engraftment was defined as sufficient donor chimerism (DMC≥20%) at PT-Day 180 and reversal of acute SCD complications. Immunophenotyping of the peripheral blood mononuclear cells (PBMCs) was performed in all available patient samples. The study was approved by the Institutional Review Board of the National Heart, Lung, and Blood Institute (NHLBI, ClinicalTrials.gov Identifier NCT00977691). All patients gave written informed consent. The study was monitored by an independent data and safety monitoring board.

Peripheral blood samples were collected at baseline (BSL) and serially at PT-Day 30, 60, 100, and 180. Blood samples were collected in EDTA tubes (Becton Dickinson, San Jose, CA, USA) and plasma stored at -80° C and PBMCs at -140° C until analysis. PBMCs were isolated using the Ficoll density gradient protocol. patients were grouped at each PT-time point based on their engraftment status [engrafted or rejected (**Supplementary Table S1**)] and DMC level [high DMC (HDMC) with ≥ 20% or low DMC (LDMC) with < 20%] (**Supplementary Table S2**).

2.2 Cytokine Analysis

A multiplexed magnetic bead assay was employed to analyze 48 cytokines in plasma (Bio-Rad, Hercules, CA, USA). Two

cytokines [transforming growth factor-b1 (TGF-b1) and B-cell-activating factor (BAFF)] were measured using an enzyme-linked immunosorbent assay (ELISA) based DuoSet kit (R&D, Minneapolis, MN, USA). All assays were performed according to the manufacturer's instructions. Four cytokines [interleukin (IL)-1a, IL-12p40, monocyte-chemotactic protein (MCP)-3, and tumor necrosis factor-b (TNF-b)] had more than 75% of values below the lowest limit of detection (LLOD) and two cytokines [cutaneous T-cell-attracting chemokine (CTACK), stromal cell-derived factor-1a (SDF-1a)] failed standard curves. Therefore, we excluded these cytokines from the analysis (**Supplementary Table S3**). Abbreviations for all the cytokines that are evaluated in this study are listed in **Supplementary Data**.

2.3 Immunophenotyping of Immune Regulatory Cells

Based on the cytokine results, two panels (**Supplementary Tables S4A, B**) were designed to evaluate various regulatory and effector immune cell subsets (**Supplementary Table S5**) by flow cytometry. Cell surface staining of PBMCs was performed as described with some modification (17). After thawing frozen vials, cells were suspended in a sterile complete medium. For surface staining, cells were stained in flow cytometry staining buffer (PBS, 2% heat-inactivated FBS), and prior to surface human antibody conjugates staining samples were treated with human FC block antibody. The immunophenotyping analysis was performed in two ways. The first analysis involved a comprehensive phenotyping of the following eight major immune cell subsets: (i) B cells: $CD19^+$, (ii) $CD8^+$ T cells: $CD3^+CD8^+$, (iii) regulatory T cells (Tregs): $CD4^+FoxP3^+$, (iv) effector $CD4^+$ T cells: $CD4^+FoxP3^-$, (v) natural killer (NK) cells: $CD3^-CD56^+$, (vi) Monocytes: $CD14^+$, (vii) dendritic cell (DC) subsets, plasmacytoid DCs (pDCs): lineage (CD3, CD19, CD56) $(lin)^-$ $HLA\text{-}DR^+CD123^+CD11c^-$ (18) and myeloid DCs (mDCs): $lin^-HLA\text{-}DR^+CD123^-CD11c^+$ (19), and (viii) myeloid-derived suppressor cell (MDSC) subsets (20, 21), early MDSCs (eMDSCs): $lin^-HLA\text{-}DR^-CD11b^+CD33^+$, monocytic MDSCs (mMDSCs): $lin^-HLA\text{-}DR^{-/low}CD14^+CD15^-$, polymorphonuclear MDSCs (PMN-MDSCs): $lin^-HLA\text{-}DR^{-/low}CD14^-CD15^+CD11b^+$. Later more detailed analysis was performed to evaluate the following immune regulatory/effector cell types (**Supplementary Table S5**): (i) Tregs: $CD4^+CD25^+FoxP3^+$ (22); (ii) type 1 regulatory (Tr1) cells: $CD4^+FoxP3^-CD45RA^-LAG3^+CD49b^+$ (23) (iii-v) eMDSCSs, mMDSCs, and PMN-MDSCs; (vi-vii) pDCs and mDCs; (viii) regulatory B cells (Bregs): $CD19^+CD24^{hi}CD38^{hi}$ (24) (ix) T helper (Th)1 cells: $CD3^+CD4^+CD45RO^+CXCR3^+$ (19), and (x) Th17 cells: $CD3^+CD4^+CD45RO^+CCR6^+$ (19). The gating strategies for these 10 subsets are described in **Supplementary Figures S1–S4**. The gating strategy was adapted from the referenced articles indicating each cell type and validated by the NHLBI Flow Cytometry Core. The PBMCs were first stained with cell surface markers. Then FoxP3, LAG3, TGF-b1, IL-10, and IL-7 were stained intracellularly.

2.3.1 Intracellular Cytokine Staining

TGF-b1, IL-10, and IL-7 were stained intracellularly after stimulating the PBMCs with cell stimulation cocktail (phorbol 12-myristate 13-acetate, ionomycin, brefeldin A and monensin; ThermoFisher Scientific, Waltham, MA, USA) in culture medium and incubated for 5-6 hours at 37°C (25). Cells were stained with surface markers as described in the *Material and Methods* section. Then the cells were fixed using fixation and permeabilization buffer (ThermoFisher Scientific) for 30 minutes at 4°C. Fixed cells were incubated in permeabilization buffer overnight with antibodies for FoxP3 and IL-7, IL-10, and TGF-b1 cytokines at 4°C. The stained cells were acquired using multiparameter FACSymphony flow cytometer (Broomfield, CO) and analyzed by FlowJo software version10.6.2 (Tree Star, Ashland, OR, USA).

2.3.2 Flow Cytometric Sample Acquisition

Samples were acquired on a Becton Dickinson Symphony flow cytometer equipped with Seven lasers (355, 407, 445, 488, 532, 633, and 785 nm wavelengths) and 35 PMT detectors, optimized as described by Perfetto et al. (26). Between 100,000 and $1x10^6$ events were collected per FCS file for each tube, depending on the number of cells available, to have sufficient events for statistical analysis of rare subsets defined by multiple markers. Data were acquired using DIVA 6.1.2 software (BD, San Jose, CA) and the analysis was performed using FlowJo™ Software (for Mac) Version 9.9.6. (Ashland, OR: Becton Dickinson and Company; 2019).

2.4 Statistical Methods

Mean, median, standard deviation (SD), minimum and maximum values of cytokine concentrations were calculated (**Supplementary Table S3**). LLOD categories and logistic regression model details are described in **Supplementary Data**. Additionally, we used linear regression models to compare continuous cytokine concentrations between the engrafted and rejected groups at each time point. Spearman's rank correlations were employed to examine the correlation between the different cytokines at each time point. Factor analysis was used to examine the relationships between the selected cytokines. The factors computed based on the BSL time point for all patients were categorized into quartiles and used as predictors in logistic regression models fit to all time points for all subjects, accounting for repeated measures for the same person over time in the variance computation. Random forests using continuous cytokine levels were implemented as additional sensitivity analysis. Missing values were excluded from the analyses.

The cellular flow cytometric data highlighting the immune reconstitution were analyzed by comparing the log10-transformed frequencies. Log10-transformed frequencies were used to compare differences between the engrafted versus rejected groups and HDMC versus LDMC groups using pairwise multiple t-tests at each time point. We calculated Spearman's rank correlations between phenotypic frequencies of immune cell subsets at each time point. All tests were two-sided, and $P < 0.05$ was considered statistically significant. Bonferroni corrections were applied to adjust for multiple testing. Analyses were performed using STATA software (version 14.2, StataCorp LLC., College Station, TX, USA), and

graphs were generated using GraphPad Prism software (version 7 and 8).

3 RESULTS

3.1 Patient Characteristics

The characteristics of the patients in the engrafted and rejected groups and their donors are described in **Table 1A**. The engrafted group comprised of an equal number of males and females (5), whereas the rejected group consisted of 7 males and 3 females. The mean age in the engrafted group was 34.4 ± 6.8 years and in the rejected group 34.2 ± 12.21 years. More donors were female in both engrafted and rejected groups, 7/10 (70%) and 8/10 (80%), respectively. There were no significant differences between the recipient's or donor's age, race, gender, and cell numbers infused between the two groups (**Table 1B**).

3.2 Associations of Cytokine Levels With Engraftment

Among 44 cytokines evaluated, 23 with values over LLOD were further categorized into two groups: above or below the overall median of each cytokine. The remaining 21 cytokines were categorized into three groups: <LLOD, below the median, and above the median of detectable values (**Supplementary Table S3**). We first assessed the association with engraftment for all 44 cytokines (Fisher's exact P-values given in **Table 2**). The sample at PT-Day 60 revealed the lowest P-value difference between the engrafted and rejected groups. Fibroblast growth factor (FGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-12p70, IL-9, and macrophage inflammatory protein (MIP)-1a were associated with engraftment (Bonferroni-corrected P <0.001), whereas granulocyte colony-stimulating factor (G-CSF), interferon (IFN)-g, IL-10, IL-13, IL-17, IL-1b, IL-1RA, IL-4, macrophage migration inhibitory factor (MIF), TGF-b1, TNF-a, and vascular endothelial growth factor (VEGF) were associated with P <0.01, and growth-regulated protein (GRO-a), platelet-derived growth factor (PDGF)-BB with P =0.05 at PT-Day 60. At PT-Day 100, IL-7 was associated with engraftment (P <0.01), as were IL-2 and VEGF (P <0.05). MIF was associated with P <0.01, and IL-7 and TGF-b1 with P <0.05 at PT-Day 180. In contrast, IL-18 was associated with rejection at PT-Day 100 with P <0.005. Notably, IL-6 was associated with engraftment at BSL (P <0.017). The remaining markers did not show any associations.

Odds ratios (ORs) from logistic models using the categories of cytokine concentrations as ordinal variables and P values for all time points are given in **Supplementary Table S6**. ORs of PDGF-BB, TGF-b1, and TNF-a were associated with engraftment with a P <0.05 at PT-Day 30. At PT-Day 60, ORs of FGF, GM-CSF, IL-9, and MIP-1a were associated with P <0.001, and G-CSF, IFN-g, IL-10, IL-17A, IL-1RA, IL-4, TGF-b1, and VEGF were associated with P <0.01; and GRO-a, IL-12p70, IL-7, MIF, and PDGF-BB with P <0.05. OR of IL-7 was associated with engraftment at PT-Day 100 with P <0.01, and IL-12p70, IL-13, IL-17, IL-2, IL-9, PDGF-BB, TNF-related apoptosis-inducing ligand (TRAIL), and VEGF were associated with P <0.05. At PT-Day 180, OR of MIF was associated with engraftment with P <0.01, whereas G-CSF, GRO-a, IL-10, IL-17A, IL-7, MIP-1a, PDGF-BB, TGF-b1, and TNF-a were associated with P <0.05. In contrast, the OR of IL-18 was associated with rejection at PT-Day 100 with P <0.01 and at PT-Day 180 with P <0.05. There were no significant differences between the two groups at BSL for any cytokines.

Results from linear regression models are presented in **Supplementary Table S7**. As expected, all cytokine

TABLE 1A | Characteristics of the study population by transplant outcome.

Patient ID	E/R	Age	Sex	Disease	Donor Age	Donor Sex	Relation	HLA-match	CD34 x10^6/kg	CD3 x10^8/kg
225-03	E	37	F	HbSS	66	F	Mother	8/10	10.2	3.78
225-07	E	31	F	HbSS	60	F	Mother	7/10	13	8.07
225-19	E	36	M	HbSS	28	M	Brother	7/10	28	5.01
225-23	E	24	M	HbSS	20	M	Brother	7/10	13.4	2.59
225-33	E	37	M	HbSS	61	F	Mother	8/10	25.6	4.73
225-34	E	41	M	HbSS	45	F	Sister	8/10	15.9	5.08
225-38	E	31	F	HbSS	30	F	Sister	7/10	15.1	4.00
225-44	E	26	M	HbSS	51	F	Mother	6/10	16.8	3.95
225-43	E	47	F	HbSS	23	F	Sister	5/10	16.6	2.95
225-51	E	34	F	HbS β^0-thal	30	M	Brother	8/10	9.70	5.28
225-10	R	36	F	HbSS	46	F	Sister	7/10	9.76	2.83
225-11	R	20	M	HbSS	47	F	Mother	6/10	15	2.65
225-16	R	47	M	HbSS	60	F	Sister	6/10	11.9	7.93
225-29	R	21	M	HbSS	51	F	Mother	5/10	12.2	3.51
225-36	R	37	M	HbSS	56	F	Mother	7/10	10.2	2.98
225-40	R	56	F	HbSC	31	M	Son	8/10	29.7	3.78
225-47	R	20	F	HbSS	51	F	Mother	6/10	10.2	6.14
225-52	R	27	M	HbSS	52	F	Mother	5/10	11.5	3.65
225-55	R	36	M	HbSS	64	M	Father	5/10	12.2	2.42
225-56	R	42	M	HbSS	23	F	Sister	7/10	10.1	6.12

E, engrafted; R, rejected; M, male; F, female; HbSS, homozygous sickle cell disease; HbSC, compound heterozygous HbS and HbC disease; HbS β^0-thal, Compound heterozyzous HbS and β^0 thalassemia disease.

TABLE 1B | Descriptive statistics and comparative demographics of the study population by transplant outcome.

		Engrafted N = 10 (50)	Rejected N = 10 (50)	Total N = 20 (100)	P value
Recipient	Age, Average years (SD)	34.4 (6.8)	34.2 (12.21)	34.8 (9.6)	0.96
	Sex, Male N (%)	5 (50.0)	7 (70.0)	12 (60)	0.99
	BMI, Average (SD)	23.3 (3.1)	23.0 (5.4)	23.1 (4.5)	0.89
	Race, N (%)	9 (90.0)	10 (100)	19 (95.0)	0.99
	o African American	1 (10.0)	-	1 (5.0)	
	o Caucasian				
Donor	Age, Average years (SD)	41.4 (17.24)	48.1 (12.55)	44.75 (15.0)	0.33
	Sex, Male N (%)	3 (30.0)	2 (20.0)	5 (25.0)	0.99
	Relation, N (%)	-	1 (10.0)	1 (5.0)	0.99
	o Father	4 (40.0)	5 (50.0)	9 (45.0)	0.99
	o Mother	3 (30.0)	-	3 (15.0)	0.99
	o Brother	3 (30.0)	3 (30.0)	6 (30.0)	0.99
	o Sister	-	1 (10.0)	1 (5.0)	0.500
	o Son	2 (20.0)	2 (20.0)	4 (20.0)	-
	Gender match	4 (40.0)	1 (10.0)	5 (25.0)	0.99
	o Same sex, parent	-	1 (10.0)	1 (5.0)	
	o Same sex, sibling				
	o Different sex, child				
Cell number	$CD34^+$ (SD) in 10^6	16.5 (6.5)	13.7 (5.4)	14.9 (5.9)	0.168
	$CD3^+$ (SD) in 10^8	4.5 (0.9)	4.3 (2.1)	4.4 (1.6)	0.557

N, Number; SD, standard deviation; BMI, body mass index

concentrations in plasma substantially dropped from their BSL levels after the HSCT (**Figure 1**). Further, 18 cytokine concentrations were higher in the engrafted group from PT-Day 60 to 100. Only the concentration of IL-18 was higher in the rejected group. The remaining cytokines did not show statistically significant differences in concentrations between the two groups at any time. We thus found the most significant differences in cytokine levels between the engrafted and rejected groups at PT-Day 60, the time point around which secondary graft failure typically occurs.

We used factor analysis to describe the variability among the correlated cytokines in terms of a lower number of unobserved variables called "factors" that are linear combinations of the original cytokines. After removing highly correlated cytokines, we included the following ten cytokines in a factor analysis: GROa, G-CSF, IL-10, IL-17A, IL-18, IL-2, MIP-1a, PDGF-BB, TGFb1, and VEGF. We identified two factors as important, estimated factor loadings (i.e. the coefficients in the linear combination) based on the BSL levels, and computed factors for all time points. We then categorized the factors into quartiles and used them as predictors in logistic regression models. The first factor (IL-17A, IL-10, IL-7, G-CSF, IL-2, MIP-1a, VEGF, and TGFb1 contributed significantly) was strongly associated with engraftment with OR = 2.75 (95% CI of 1.40 to 5.38) whereas the second factor (GROa, and IL-18 contributed significantly) was not statistically significant (**Supplementary Table S8**).

3.3 Immune Reconstitution Following Haplo-HSCT

Immunophenotypic analysis of the patients' immune cell repertoire comprising of B cells, $CD8^+$ T cells, Tregs, effector $CD4^+$ T cells, NK cells, monocytes, DCs (pDCs and mDCs), and MDSCs (eMDSCs, mMDSCs, and PMN-MDSCs) at BSL, PT-Days 30, 60, 100, and 180 were performed. The cellular frequencies of these cells are plotted (**Figures 2A–H**). A non-significant trend in the frequency of MDSCs was observed at PT-Day 60 ($P > 0.06$). Since DMC is a critical factor in promoting allograft acceptance and treating SCD, we grouped the patients into HDMC (≥20%) and LDMC (<20%) at each time point and observed consistent PT-time point visual differences in DCs, and MDSCs between the engrafted and rejected patients between HDMC and LDMC patients (**Supplementary Figure S5 A–E**).

3.4 Early Myeloid-Derived Suppressor Cells Associate With Successful Graft Outcome

We evaluated the percentages of three different types of MDSCs (20, 21) in our patients and compared the frequencies of each type between the engrafted and rejected groups and HDMC and LDMC groups at each time point. We observed higher frequencies of eMDSCs in the HDMC group ($P < 0.003$; **Figure 3A** and **Supplementary Figure S6A**) and engrafted group ($P < 0.04$; **Figure 3B**) at PT-Day 30. We used distinguishable HLA to determine the source of eMDSCs in 9/20 patients (**Table 3**). These nine patients had a total of 16 HDMC time points and 14 LDMC time points. The source of eMDSCs revealed the following patterns: in engrafted patient 225-19 up to 99% eMDSCs were donor-derived at all time points, wherein with rejected patients 225-10, 225-52, and 225-55, all eMDSCs were 100% recipient-derived in origin. Interestingly, in rejected patient 225-40, at day 30-PT, eMDSCs were 100% recipient-derived. At later time points, however, eMDSCs were 100% from the donor. Other patients maintained more mixed donor and recipient origins until at least day 180-PT. eMDSCs from both donor and recipient (mixed chimeric state) origins were observed at 15/16 HDMC time points as compared to only two LDMC time points (**Table 3**; $P < 0.00001$ and **Supplementary Figures S6B, C**).

TABLE 2 | Fisher's exact test of cytokines for association with engraftment between the engrafted and rejected groups, P values (*P < 0.05, **P < 0.01, ***P < 0.001).

Cytokines	BSL	PT-Day 30	PT-Day 60	PT-Day 100	PT-Day 180
BAFF	0.350	1.000	0.620	0.650	0.622
bNGF	1.000	0.604	0.589	0.195	0.827
Eotaxin	0.170	1.000	0.153	0.650	0.622
FGF	1.000	0.303	0.000***	0.170	0.153
G-CSF	0.474	0.141	0.002**	0.370	0.050
GM-CSF	1.000	0.141	0.000***	0.170	0.335
GROa	1.000	1.000	0.024*	0.188	0.069
HGF	0.656	0.628	0.637	0.179	0.762
IFN-a2	0.656	0.170	1.000	0.484	1.000
IFN-g	1.000	0.582	0.003**	0.243	0.335
IL-10	0.211	0.170	0.002**	0.350	0.050
IL-12p70	1.000	0.139	0.001***	0.106	0.134
IL-13	0.582	0.340	0.004**	0.106	0.234
IL-15	0.408	0.232	0.718	0.777	0.485
IL-16	0.777	1.000	0.352	0.459	0.647
IL-17	1.000	0.303	0.009**	0.070	0.058
IL-18 #	1.000	0.656	0.637	0.005**	0.058
IL-1b	1.000	0.141	0.006**	0.478	0.153
IL-1RA	1.000	1.000	0.002**	0.245	0.335
IL-2	0.628	0.459	0.263	0.048*	0.350
IL-2RA	0.650	1.000	0.637	0.628	1.000
IL-3	1.000	0.187	1.000	0.714	0.377
IL-4	1.000	0.141	0.002**	0.170	0.153
IL-5	0.700	0.361	0.073	1.000	0.473
IL-6	0.017*	0.500	0.352	0.286	0.377
IL-7	1.000	0.389	0.090	0.004**	0.032*
IL-8	1.000	0.350	0.637	1.000	0.423
IL-9	1.000	0.303	0.000***	0.070	0.153
IP10	0.628	0.087	1.000	0.170	0.644
LIF	0.714	0.125	1.000	1.000	1.000
MCP-1	1.000	0.628	0.153	0.370	0.304
MCSF	1.000	1.000	0.793	0.800	0.377
MIF	1.000	0.293	0.008**	0.577	0.002**
MIG	0.350	0.087	1.000	1.000	1.000
MIP-1a	1.000	0.350	0.000***	0.170	0.050
MIP-1b	0.303	0.650	1.000	0.170	0.134
PDGF-BB	1.000	0.057	0.029*	0.070	0.058
RANTES	1.000	0.303	1.000	1.000	0.622
SCF	0.714	1.000	1.000	0.607	0.219
SCGFb	0.656	0.628	1.000	0.350	1.000
TGF-b1	1.000	0.057	0.009**	0.370	0.015*
TNF-a	1.000	0.057	0.002**	0.170	0.050
TRAIL	0.650	1.000	1.000	0.070	1.000
VEGF	1.000	0.179	0.002**	0.020*	0.335

BSL, baseline; PT, post transplantation; #, cytokine associated with rejection.

3.5 Evidence of High Frequencies of Tregs in Engrafted and HDMC Patients

Tregs are the most commonly observed cellular population in patients with immune tolerance (27), and they prevent acute graft versus host disease (GVHD) (28) following HSCT. We compared frequencies of Tregs between the engrafted and rejected patients and among HDMC and LDMC groups. While we did not find any significant differences after multiple testing correction, we noticed a trend towards increased frequencies of Tregs in the engrafted group at PT-Day 100, (P <0.04; **Figures 4A, B**) and in the HDMC group at PT-Day 100 (P <0.09; **Figure 4C**). The elevated frequencies of Tregs agree with our cytokine results, where we observed elevated plasma levels of IL-10 at PT-Day 60 (P <0.05), PT-Day 100 (P <0.01), and PT-Day 180 (P <0.05) in engrafted patients. We tracked the source of Tregs using distinguishable HLA in 9/20 patients. We did not observe statistically significant differences between mixed chimeric or non-chimeric Tregs in HDMC and LDMC groups (**Table 4**).

We further calculated the percent change in the frequencies of Tregs from the BSL for each patient and compared the percent change between HDMC versus LDMC groups. We observed a higher Treg change in HDMC patients (**Figure 4D**). Although we observed no significant correlation between the frequencies of Tregs and percentages of DMC, frequencies of Tregs mirrored the DMC dynamics. This was observed in two of the patients who engrafted initially before they rejected their grafts at PT-Days 60 and 100 respectively. The frequencies of Tregs at these time points decreased close to BSL as opposed to one patient who maintained engraftment and high frequency of Tregs persisted

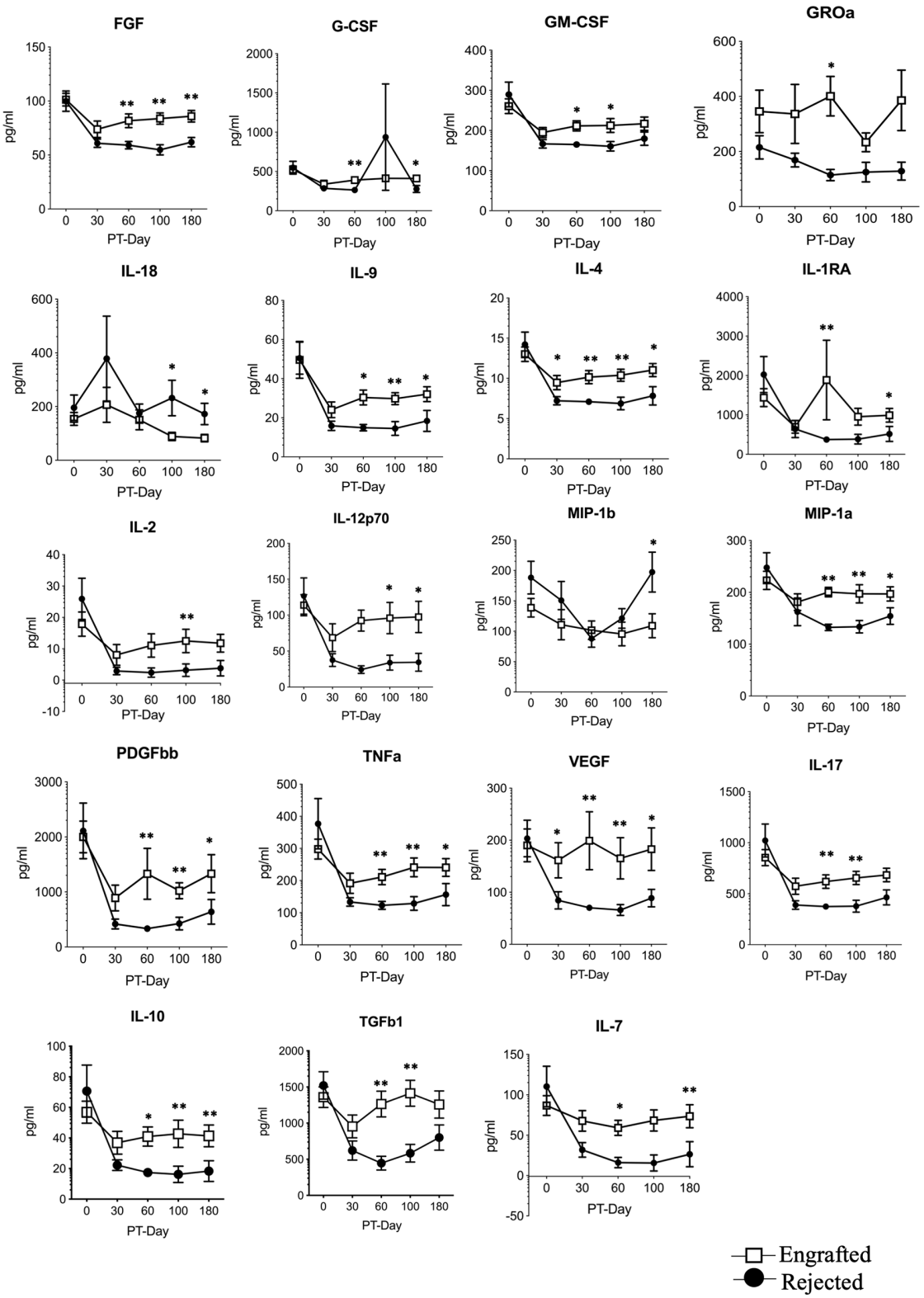

FIGURE 1 | The selected differential cytokines between the engrafted and rejected groups at BSL and PT time points. Multiplex magnetic-bead based assay or ELISA for all indicated cytokines except TGF-b1was performed. Graphs shown here represent 19 differential cytokines between engrafted and rejected patients at BSL, PT-Days 30, 60, 100, and 180. Data represent the mean ± standard error, *P < 0.05, **P < 0.01.

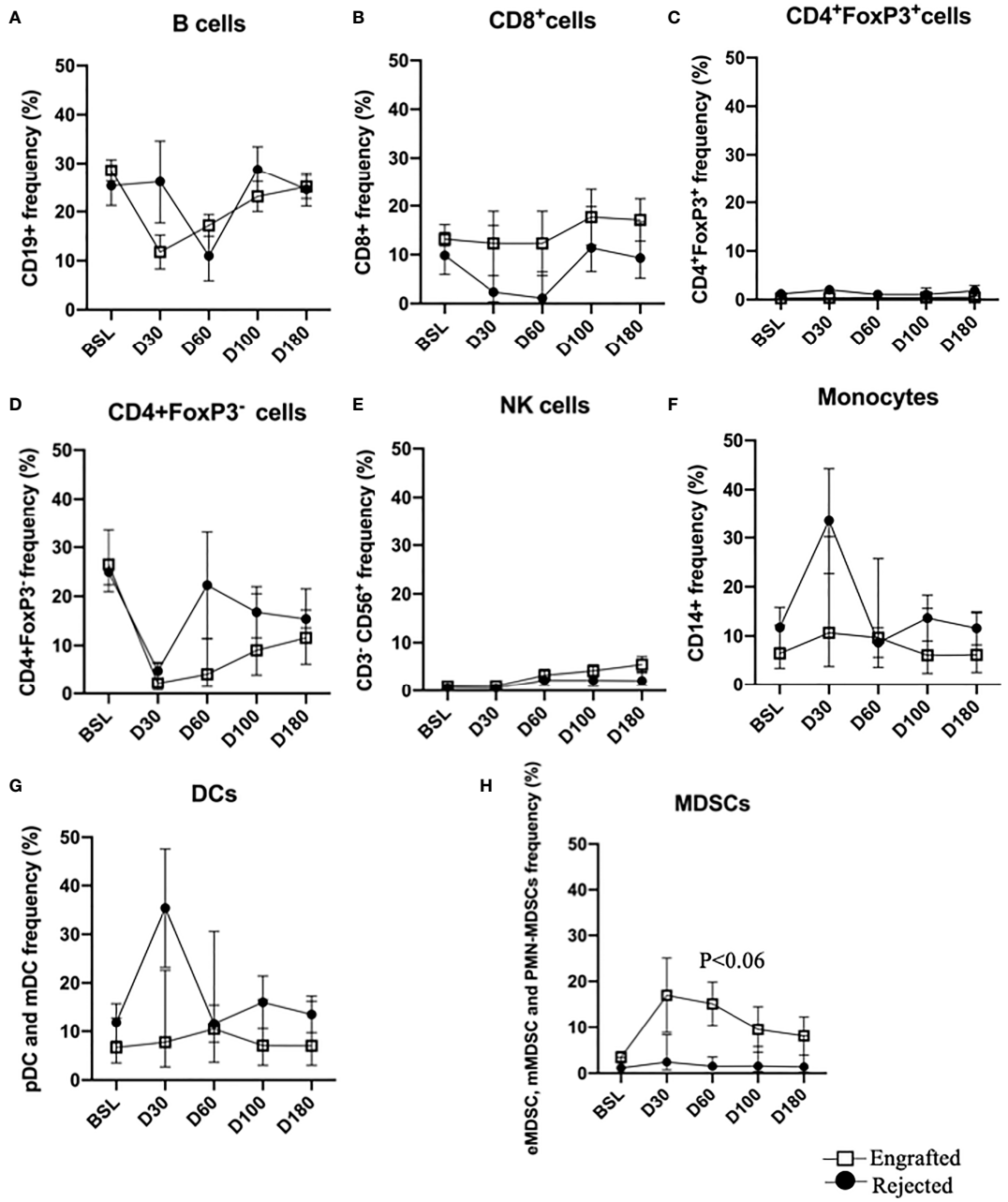

FIGURE 2 | Immune reconstitution following haplo-HSCT at all time points. **(A–H)** Percent frequencies of major immune cell subsets: B cells, $CD8^+$ T cells, $CD4^+FoxP3^+$ (Tregs), $CD4^+Foxp3^-$ (effector T cells), NK cells, monocytes, DCs and MDSCs at specified time points. Mean frequencies of specified immune cells are provided in the engrafted and rejected patients at BSL, PT-Days 30, 60, 100, and 180. A trend of increased MDSCs in engrafted patients is observed at PT-Day 60 ($P < 0.06$).

(**Supplementary Figure S7A–C**). Further, we evaluated IL-10 and TGF-b1 producing Tregs and found a trend of higher IL-10 producing Tregs in the HDMC group at PT-Day 30 ($P < 0.02$; **Figure 4E**), however, TGF-b1 producing Tregs did not show any difference between groups (data are not shown).

Plasma cytokine data revealed higher levels of IL-17 in the engrafted patients at PT-Days 60 and 100 (**Figure 1**). However, we did not observe any statistically significant difference in the frequencies of Th17 cells between engrafted and rejected patients and HDMC and LDMC groups (data are not shown). We also did not find a statistically significant difference in TGF-b1 and IL-10 producing Th17 cells between the HDMC and LDMC groups nor the frequencies of Bregs, pDCs, mDCs, mMDSCs, PMN-MDSCs, and Tr1 cells either

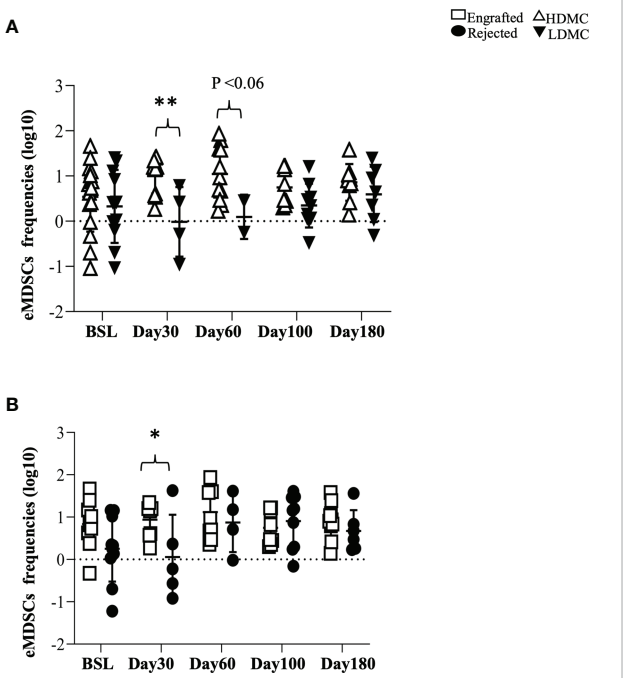

FIGURE 3 | Early myeloid-derived suppressor cells associate with successful graft outcome. **(A, B)** PBMC samples were stained for eMDSCs at defined time points. **(A)** eMDSCs from HDMC and LDMC patients are plotted (PT-Day 30, ** P < 0.003) and **(B)** eMDSCs from engrafted and rejected patients are plotted (PT-Day 30, *P < 0.04).

between the engrafted and rejected or the HDMC and LDMC groups (data not shown).

3.6 Early Myeloid-Derived Suppressor Cells and Tregs Correlate Positively With Each Other

Since Tregs have been associated with tolerance, we next performed the correlation analysis between eMDSCs and Tregs and observed a positive correlation between the frequencies of eMDSCs and Tregs at PT-Day 100 (r=0.72, P <0.0007; **Figure 5A**). Importantly, Tregs at PT-Day 100 correlated positively with the eMDSCs at BSL (r=0.63, P <0.004; **Figure 5B**). Tregs at PT-Day 60 tend to show positive correlation with eMDSCs at PT-Day 180 but the association was not significant after applying correction for multiple testing (**Supplementary Figure S8A**). We next tested the correlation of the frequencies of eMDSCs with percentages of DMC at all PT time points. We observed a trend towards a positive correlation between frequencies of eMDSCs at PT-Day 60 with the percentage of DMC at PT-Day 180, but the association could not stand the correction applied for multiple testing (**Supplementary Figure S8B**).

We next evaluated the number of patients at each post-transplantation time point who experienced graft failure with donor myeloid chimerism (DMC) levels below 20% as an indicator of graft failure based on Kaplan Meier estimates (**Supplementary Table S9**). Of the total 20 patients, DMC levels decreased below 20% at PT-Day 30 in 3 patients. At PT-Day 60, 4 additional patients had their DMC below 20% and at PT-Day 100, 5 additional patients. Finally, DMC levels decreased below 20% in 6 additional patients at PT-Day 180, adding up to 11 patients with graft failure in total. The time to graft failure (DMC below 20%) is also plotted in **Supplementary Figure S9**, that also shows numbers of subjects at risk for graft failure at each time point.

4 DISCUSSION

Allograft rejection is a complex process involving an interplay between different cells and multiple cellular mediators. Although several promising molecular targets for early detection of GVHD and response to its treatment are known (29, 30), reliable biological markers to identify graft rejection in HSCT still do not exist (31, 32). In this study, we evaluated the plasma levels of 44 cytokines and 10 immune regulatory and effector cells with an aim to get target cell populations and cytokines for our future studies. Since adequate donor myeloid chimerism is critical and predictive of positive allograft status in terms of resolution of SCD related symptoms (15, 33),we evaluated the cellular data between patients with high (≥20%) and low (<20%) donor myeloid chimerism levels.

Since HSCT conditioning regimens usually lead to the potent induction and release of pro-inflammatory cytokines as a reflection of severe systemic inflammation (34), we identified several pro-inflammatory and regulatory cytokines, chemokines, and growth factors that were associated with successful engraftment at PT-Day 60. We observed increased expression of G-CSF, GM-CSF, IFN-g, IL-1b, IL-2, IL-4, IL-7, IL-10, IL-12p70, IL-17A, MIP-1a, TNF-a, TGF-b1, and VEGF in successfully engrafted patients. These cytokines reflect hematopoiesis of engrafted cells (35), activation of T (36), B (37), and macrophage differentiation (38) and induction of tolerance (39). Notably, we identified only one marker, IL-18, which was downregulated in engrafted patients and stayed at a low level through PT-Day 180. An important role of IL-18 in allograft rejection has been postulated in a recent study using a rat model of liver transplantation, which showed that specific suppression of IL-18 was associated with significantly decreased serum alanine aminotransferase levels, diminished histologic hepatic injury early after transplantation, and prolonged allograft survival (40).

MDSCs have gained attention for their potential role in allograft tolerance following heart and islet transplantation in mice (41, 42) along with renal transplantation in rats (43). The pro-inflammatory environment, which induces the development of MDSCs in cancer and infection, mimics the anti-donor response following transplantation (44–47). Our data revealed an increased and consistent presence of MDSCs in engrafted patients starting at PT-Day 30 and onwards. Notably, we observed elevated G-CSF, GM-CSF, IL2, VEGF, IL-1b, FGF, TNF-a, TGF-b1, and IL-10 levels, which are reported to be the

TABLE 3 | Source of early myeloid-derived suppressor cells at specified post-transplantation time points.

Patient ID	E/R	HDMC/LDMC	Distinguishable HLA	PT time point	eMDSCs Recipient (%)	eMDSCs Donor (%)	Mixed Chimerism present
225-19	E	HDMC	Recipient is A3+	PT-Day30	1	99	Yes
		HDMC		PT-Day60	1	99	Yes
		HDMC		PT-Day180	1	99	Yes
225-43	E	HDMC	Donor is A2+	PT-Day30	76	24	Yes
		HDMC		PT-Day100	85	15	Yes
		HDMC		PT-Day180	82	18	Yes
225-51	E	HDMC	Donor is A2+	PT-Day60	96	4	Yes
		HDMC		PT-Day100	98	2	Yes
		HDMC		PT-Day180	94	6	Yes
225-44	E	HDMC	Donor is A2+	PT-Day30	50	50	Yes
		HDMC		PT-Day60	99	1	Yes
		HDMC		PT-Day100	91	9	Yes
		HDMC		PT-Day180	84	16	Yes
225-10	R	LDMC	Donor is A3+	PT-Day30	100	0	No
		LDMC		PT-Day60	100	0	No
		LDMC		PT-Day100	100	0	No
225-52	R	LDMC	Donor is A3+	PT-Day30	100	0	No
		LDMC		PT-Day60	100	0	No
		LDMC		PT-Day100	100	0	No
		LDMC		PT-Day180	100	0	No
225-55	R	LDMC	Donor is A2+	PT-Day60	100	0	No
		LDMC		PT-Day100	100	0	No
		LDMC		PT-Day180	100	0	No
225-36	R	HDMC	Donor is A2+	PT-Day30	56	44	Yes
		HDMC		PT-Day60	98	2	Yes
		LDMC		PT-Day100	99	1	Yes
		LDMC		PT-Day180	99	1	Yes
225-40	R	HDMC	Donor is A2+	PT-Day30	0	100	No
		LDMC		PT-Day100	100	0	No
		LDMC		PT-Day180	100	0	No

Table showing the source of eMDSCs at HDMC and LDMC time points. High chimerism in eMDSCs observed in HDMC as compared to LDMC groups (chi-square =17.099; P< 0.00001, Yates correction applied).

E, engrafted; R, Rejected; HDMC, high donor myeloid chimerism; LDMC, low donor myeloid chimerism; PT, post-transplant; eMDSCs, early myeloid-derived suppressor cells.

drivers of MDSC activation (48, 49), sustenance (50), and suppressive activity (51, 52). MDSC subpopulations are hypothesized to be highly plastic, and little is known about their relevance in transplantation. A renal transplantation study in humans revealed mMDSCs to be present in the peripheral blood of tolerant patients (53). A recent study demonstrated the significance of eMDSCs in controlling acute GVHD following allo-HSCT in humanized mice (54). Here, we observed that the frequencies of eMDSCs are elevated at early time point PT-Day 30 in HDMC patients.

MDSCs favor mixed chimerism in a combined murine bone marrow-cardiac transplantation model and control anti-donor T cell response *in vitro* (55). The DMC level at PT-Day 180 correlated positively with the frequencies of eMDSCs at PT-Day 60 (r=0.45, P <0.04), which suggests they have a role in maintaining high levels of DMC. More than 90% (93.8%) of chimeric eMDSCs in the HDMC group compared to less than 10% (6.6%) in the LDMC group bolsters the relevance of the promotion of chimerism in promoting graft acceptance. We observed that the presence of donor MDSCs promoted allograft acceptance as all the engrafted patients and all HDMC timepoints had them. Although it could be presumed that the high chimerism status at these time points account for their donor derived origin but a recent murine study revealed that donor MDSCs promote cardiac allograft tolerance *via* induction of recipient derived MDSCs (56).

MDSCs suppressive activity is based on their ability to directly suppress proliferation of effector T, B, and NK cells by expressing inducible nitric oxide synthase and arginase (57) and by modifying IFN-g and IL10 dependent T cell differentiation pathways, which promote Treg differentiation (58). Ample evidence indicates robust crosstalk between MDSCs and Tregs favoring immunosuppression (59–61). Indeed, we observed a positive correlation between eMDSCs at PT-Day 100 and Tregs at PT-Day 100 (r=0.72, P <0.0007). Notably, a positive correlation was also observed between eMDSCs at BSL and Tregs at PT-Day100 (r=0.63, P <0.004), which suggests a possible synergistic association between eMDSCs at BSL in promoting graft tolerance by increasing Tregs.

We observed evidence of increased Tregs at PT-Day 100 in engrafted patients. However, the association was not significant after Bonferroni correction was applied for correction of multiple testing, possibly due to limited sample size which further is reduced when comparisons are made at specific PT time points. We also observed the change in the frequencies of Tregs in the HDMC group from BSL following HSCT was higher than the LDMC group. Interestingly, with seven time points where chimeric Tregs were observed, six belonged to the HDMC time

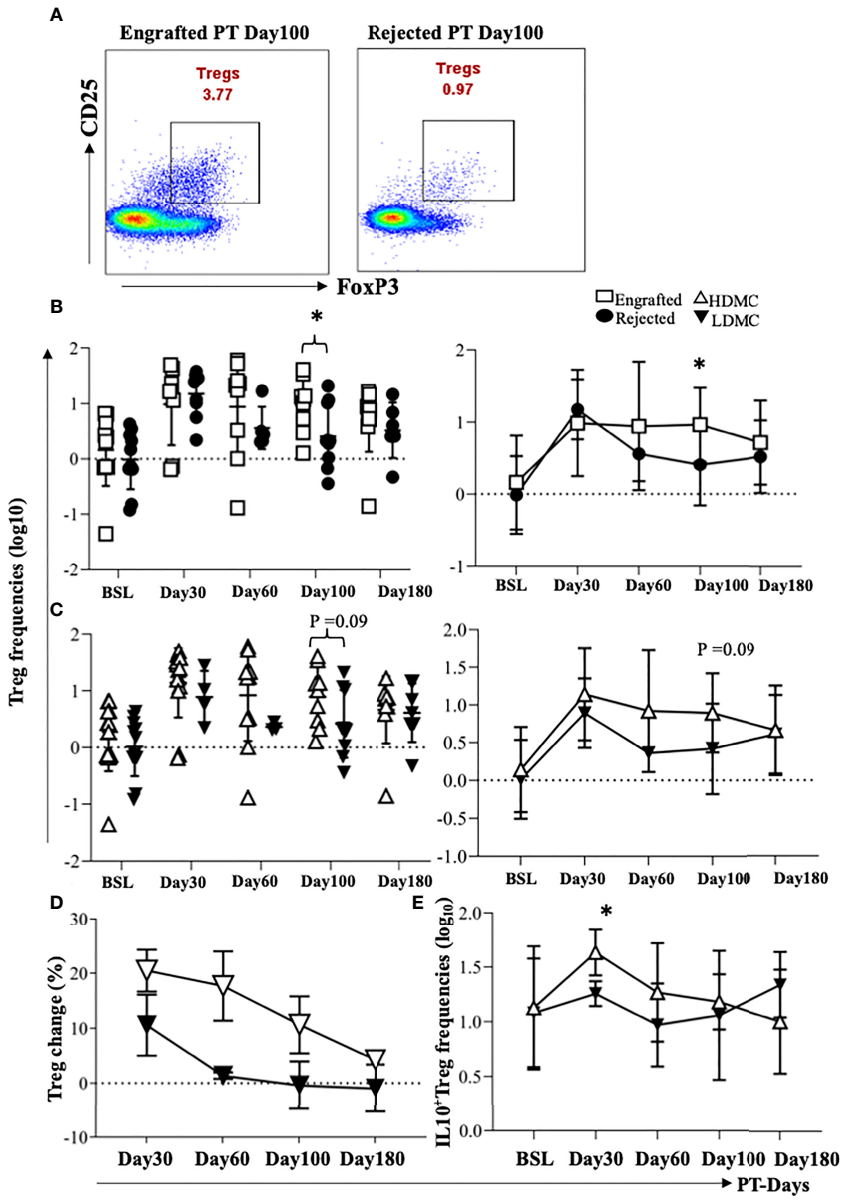

FIGURE 4 | Evidence of high frequencies of Tregs in engrafted and HDMC patients. **(A)** PBMC samples were stained for Tregs, and representative plots of Tregs from an engrafted and rejected patient at PT Day 100 are shown. **(B)** Individual and mean Treg frequencies from engrafted and rejected groups are plotted (PT-Day 100, *P < 0.04). **(C)** Individual and mean Treg frequencies from each sample in HDMC and LDMC groups are plotted. **(D)** Percent change in the frequencies of Tregs at different time PT with respect to BSL was plotted in HDMC and LDMC groups. **(E)** Tregs were intracellularly stained for IL-10 and mean IL-10 producing Tregs were plotted within HDMC and LDMC groups (PT-Day 30, *P < 0.02).

point group and only 1 to the LDMC group, which supports chimerism favoring tolerance. Further, Tregs showed a trend towards increased IL-10 at PT-Day 30 in the HDMC group, suggesting their active presence in allograft acceptance. Tregs comprise the major arm of immunosuppression (22) and their presence in the engrafted patients is therefore not surprising. Tregs mediate their suppressive function through a variety of different mechanisms (62, 63) including the production of the anti-inflammatory cytokine, IL-10 (64). A significant elevation of IL-10 in engrafted patients at various times post-HSCT validates their immune-suppressive activity and functionality. IL-10 serves to directly or indirectly inhibit effector T-cell responses by inhibiting cytokine production, suppressing Th1 and Th2 cell proliferation, and downregulating major histocompatibility complex class II on monocytes (65–70). We observed statistically significantly elevated plasma levels of IL-17 in engrafted patients at PT-Day 60 and 100. However, the frequencies of Th17 cells were not significantly different

TABLE 4 | Source of Tregs at specified post-transplantation time points.

Patient ID	E/R	HDMC/LDMC	Distinguishable HLA	PT time point	TregsRecipient (%)	TregsDonor(%)	Mixed Chimerism present
225-19	E	HDMC	Recipient is A3+	PT-Day30	100	0	No
		HDMC		PT-Day60	100	0	No
		HDMC		PT-Day180	100	0	No
225-43	E	HDMC	Donor is A2+	PT-Day30	99	1	Yes
		HDMC		PT-Day60	99	1	Yes
		HDMC		PT-Day180	98	2	Yes
225-51	E	HDMC	Donor is A2+	PT-Day60	100	0	No
		HDMC		PT-Day100	100	0	No
		HDMC		PT-Day180	100	0	No
225-44	E	HDMC	Donor is A2+	PT-Day30	93	7	Yes
		HDMC		PT-Day60	99	1	Yes
		HDMC		PT-Day100	100	0	No
		HDMC		PT-Day180	96	4	Yes
225-10	R	LDMC	Donor is A3+	PT-Day30	100	0	No
		LDMC		PT-Day60	100	0	No
		LDMC		PT-Day100	100	0	No
225-52	R	LDMC	Donor is A3+	PT-Day30	100	0	No
		LDMC		PT-Day60	100	0	No
		LDMC		PT-Day100	100	0	No
		LDMC		PT-Day180	100	0	No
225-55	R	LDMC	Donor is A2+	PT-Day60	100	0	No
		LDMC		PT-Day100	100	0	No
		LDMC		PT-Day180	100	0	No
225-36	R	HDMC	Donor is A2+	PT-Day30	100	0	No
		HDMC		PT-Day60	100	0	No
		LDMC		PT-Day100	100	0	No
		LDMC		PT-Day180	100	0	No
225-40	R	HDMC	Donor is A2+	PT-Day30	100	0	No
		LDMC		PT-Day60	100	0	No
		LDMC		PT-Day100	100	0	No
		LDMC		PT-Day100	95	5	Yes

Table showing the presence or absence of mixed chimerism in Tregs at HDMC and LDMC time points (chi-square =2.63; P< 0.10, Yates correction applied). No differences were observed between the two groups.

E, engrafted; R, Rejected; HDMC, high donor myeloid chimerism; LDMC, low donor myeloid chimerism; PT, post-transplant.

between engrafted versus rejected patients and HDMC versus LDMC groups. Growing evidence suggests that Tregs are highly plastic with the potential to convert into pro-inflammatory Th17 cells (71, 72). However, we did not examine the plasticity of either Treg or Th subsets in our study.

Based on our observations, the allograft outcome may be determined by the complex molecular network of pro-inflammatory and anti-inflammatory cytokines along with the relative presence of effector and suppressive cells. In accordance, we observed that the increased presence of mixed chimeric eMDSCs and Tregs could be associated with tolerance in our study.

There were several limitations to this study. First, the small sample size limited statistical power and the data came from a single institution. Because samples at all PT-time points were limited and only as early as day 30 PT, we do not know whether our findings represent a pre-rejection trend or a post-rejection phenomenon. More frequent sampling, especially at early time points, may also help to assess real-time characterization of immunological tolerance. We could not evaluate the source of eMDSCs and Tregs in all the samples due to unavailability of distinguishable HLA antibodies. Further, due to limited cells, we were unable to perform *in vitro* suppression studies to evaluate whether eMDSCs and Tregs from engrafted patients could mediate better immune suppression of effector T cells. Although we evaluated the data concerning the three sub cohorts, no cyclophosphamide, low dose cyclophosphamide, and high dose cyclophosphamide, and observed no statistical differences in the frequency of various cellular fractions, the numbers were also too small for sufficient statistical power. We did not evaluate the variability that might have occurred due to variations in sirolimus dosages. In addition, post-transplant CMV reactivation or other common infections could be confounding factors too which could not be adequately assessed due to the small sample size.

In summary, among the evaluated cells, mixed chimeric eMDSCs were present differentially in the two groups with varied outcomes. Our future trials will also focus on evaluating the presence of eMDSCs and their origin early after transplantation and their *in-vitro* suppressive abilities. In addition, Tregs and IL-10 producing Tregs showed higher trends in the HDMC group which will be evaluated in a subsequent cohort. Here, our data demonstrate that the presence of mixed chimeric eMDSCs at early time points, elevated plasma levels of IL-10 and TGF-b1, and IL-10 producing Tregs could serve as potential prognostic markers in predicting the allograft outcome following haploidentical HSCT

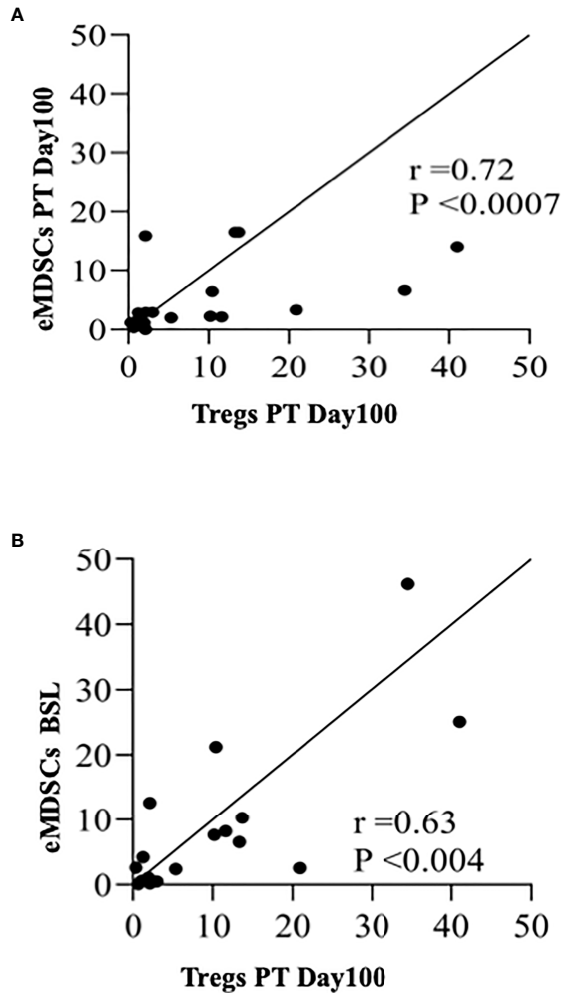

FIGURE 5 | Early myeloid derived suppressor cells and Tregs correlate positively with each other. **(A)** Spearman rank correlation between Tregs at PT-Day100 and eMDSCs at PT-Day 100 (r=0.72; P < 0.0007). **(B)** Spearman rank correlation between Tregs at PT-Day 100 and eMDSCs at BSL (r = 0.63; P < 0.004) are shown.

employing similar pre and post-transplant conditioning for SCD. eMDSCs and the associated cytokines (G-CSF, GM-CSF, IL2, VEGF, IL-1b, FGF, TNF-a, TGF-b1, IL-10), and the rest of first-factor plasma cytokines (IL-17A, IL-7, MIP-1a) will be further validated in a larger cohort with frequent post-transplant time points.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The study was approved by the Institutional Review Board of the National Heart, Lung, and Blood Institute (NHLBI, ClinicalTrials.gov Identifier NCT00977691). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DB designed, performed, and analyzed the flow cytometry data and wrote the manuscript. PO designed, performed, and analyzed the multiplex experiments and wrote the manuscript. AG designed and performed flow experiments and reviewed the manuscript. PD assisted with flow cytometry panel designing and calibration and reviewed the manuscript. AS assisted with flow cytometry experiments and reviewed the manuscript. RP helped with statistical analysis and reviewed the manuscript. JM helped to analyze flow cytometry data and reviewed the manuscript. FS and MP helped with cytokine data analysis and reviewed the manuscript. CP, AB, GF, and BF participated in performing the experiments and reviewed the manuscript. CF conceived the study, designed, and analyzed experiments and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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frontiers in Immunology

ORIGINAL RESEARCH
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Donor NK and T Cells in the Periphery of Lung Transplant Recipients Contain High Frequencies of Killer Cell Immunoglobulin-Like Receptor-Positive Subsets

Anna-Maria Hitz[1†], Kim-Alina Bläsing[1†], Bettina Wiegmann[2,3], Ramon Bellmàs-Sanz[1], Evgeny Chichelnitskiy[1], Franziska Wandrer[1], Lisa-Marie Horn[1], Christine Neudörfl[1], Jana Keil[1], Kerstin Beushausen[1], Fabio Ius[2], Wiebke Sommer[4], Murat Avsar[2], Christian Kühn[2], Igor Tudorache[5], Jawad Salman[2], Thierry Siemeni[6], Axel Haverich[2], Gregor Warnecke[4], Christine S. Falk[1,3,7‡] and Jenny F. Kühne[1‡]*

[1] Institute of Transplant Immunology, Hannover Medical School, Hannover, Germany, [2] Department of Cardiothoracic, Transplantation and Vascular Surgery, Hannover Medical School, Hannover, Germany, [3] German Center for Lung Research, DZL, BREATH Site, Hannover, Germany, [4] Department of Cardiac Surgery, Heidelberg University Hospital, Heidelberg, Germany, [5] Department of Cardiac Surgery, University Hospital of Duesseldorf, Duesseldorf, Germany, [6] Department of Cardiothoracic Surgery, University Hospital Jena, Jena, Germany, [7] German Center for Infection Research, DZIF, TTU-IICH, Hannover-Braunschweig, Germany

Introduction: For end-stage lung diseases, double lung transplantation (DLTx) is the ultimate curative treatment option. However, acute and chronic rejection and chronic dysfunction are major limitations in thoracic transplantation medicine. Thus, a better understanding of the contribution of immune responses early after DLTx is urgently needed. Passenger cells, derived from donor lungs and migrating into the recipient periphery, are comprised primarily by NK and T cells. Here, we aimed at characterizing the expression of killer cell immunoglobulin-like receptors (KIR) on donor and recipient NK and T cells in recipient blood after DLTx. Furthermore, we investigated the functional status and capacity of donor *vs*. recipient NK cells.

Methods: Peripheral blood samples of 51 DLTx recipients were analyzed pre Tx and at T0, T24 and 3wk post Tx for the presence of HLA-mismatched donor NK and T cells, their KIR repertoire as well as activation status using flow cytometry.

Results: Within the first 3 weeks after DLTx, donor NK and T cells were detected in all patients with a peak at T0. An increase of the KIR2DL/S1-positive subset was found within the donor NK cell repertoire. Moreover, donor NK cells showed significantly higher frequencies of KIR2DL/S1-positive cells ($p<0.01$) 3wk post DLTx compared to recipient NK cells. This effect was also observed in donor KIR^+ T cells 3wk after DLTx with higher proportions of KIR2DL/S1 ($p<0.05$) and KIR3DL/S1 ($p<0.01$) positive T cells. Higher activation levels of donor NK and T cells ($p<0.001$) were detected compared to recipient cells *via* CD25 expression as well as a higher degranulation capacity upon activation by K562 target cells.

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Colin C. Anderson, University of Alberta, Canada

***Correspondence:**
Jenny F. Kühne
kuehne.jenny@mh-hannover.de

[†]These authors have contributed equally to this work

[‡]These authors share senior authorship

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Conclusion: Higher frequencies of donor NK and T cells expressing KIR compared to recipient NK and T cells argue for their origin in the lung as a part of a highly specialized immunocompetent compartment. Despite KIR expression, higher activation levels of donor NK and T cells in the periphery of recipients suggest their pre-activation during the *ex situ* phase. Taken together, donor NK and T cells are likely to have a regulatory effect in the balance between tolerance and rejection and, hence, graft survival after DLTx.

Keywords: lung transplantation, passenger leukocytes, NK cells, T cells, killer cell immunoglobulin-like receptor, primary graft dysfunction, cold ischemic time

INTRODUCTION

Double lung transplantation (DLTx) remains the only curative treatment option for end-stage lung diseases (1). Despite continuous progress in the optimization of the transplantation procedure, clinical outcome of patients undergoing DLTx still is poorer compared to other solid organ transplantations (2). Survival rates after DLTx are limited early due to primary graft dysfunction (PGD), occurring in the first 72 hours after transplantation. Later, survival is impaired by chronic lung allograft dysfunction (CLAD), comprising bronchiolitis obliterans syndrome (BOS) and restrictive allograft syndrome (RAS), which develop typically later than two years after DLTx. PGD grade 2 and 3 have been associated with significantly higher short-term as well as a negative effect on long-term outcome. While PGD has been extensively examined in terms of risk factors, epidemiology and treatment options (3), the underlying immunological mechanisms are still not completely understood. Since T cells represent the main effector cells of the adaptive immunity, they are the preferred subset in studies addressing allograft including lung rejection. In contrast, the role of natural killer (NK) cells in the context of solid organ transplantation is still discussed controversially as they have been shown to be involved in both graft rejection and tolerance induction in different models (4). Recently, the missing-self genetics of KIR-ligand mismatches has been discussed to contribute to microvascular rejection (5, 6). NK cells act as first defense line of the innate immune system against pathogens, capable of producing cytokines and possessing cytotoxic activity. Moreover, they have the ability to discriminate between cells of self and non-self origin, using an inhibitory recognition system of self-human leukocyte antigen (HLA) class I molecules (7, 8). The 'missing self-hypothesis' proposes that recognition of self HLA class I molecules inhibits their lytic activity, while the absence or downregulation of self HLA class I, i.e. missing self, leads to direct recognition and lysis of target cells (8, 9). The lytic activity of NK cells is thoroughly regulated by a multidirectional interaction of inhibitory and activating receptors, such as killer cell immunoglobulin-like receptors (KIR), C-type lectin and natural cytotoxicity receptors (NCR) (10). Activating KIR-S and inhibitory KIR-L genes encode surface receptors that recognize primarily two groups of HLA-C alleles and HLA-B supratypes, respectively. In addition to NK cell regulation, KIR can also be expressed by certain $CD8^+$ T cell subsets and act as inhibitors of TCR signaling. Hence, both NK and T cell subsets expressing these KIR receptors may play a crucial role in the field of organ, especially lung transplantation due to its unique tissue-resident immune repertoire (11). In genetic analyses, a negative association of inhibitory KIR genes within haplotype A was shown for long-term outcome after DLTx suggesting that the KIR-ligand mismatch system may have clinical relevance for lung Tx (12).

Due to this capacity of NK and T cells for allorecognition, these regulatory mechanisms are of critical importance especially in the context of HLA mismatched settings like lung transplantation. Although the appearance of donor passenger leukocytes migrating from the implanted lung into the periphery of the recipient has been described decades ago, the respective NK cell subsets have not been characterized so far. This transient lymphocyte chimerism in the blood of lung recipients has first been described in the 1990s and especially donor NK and T cells were detected for up to four weeks after transplantation (13). The clinical impact of donor cells on lung allograft survival has been discussed ever since, with recent findings suggesting that special subsets of donor-derived T cells, i.e. tissue-resident memory T (TRM) cells, in bronchioalveolar lavage may be associated with lower incidence of primary graft dysfunction (14). Since NK and T cells were shown to account for the major passenger leukocyte populations (13) comprising important subsets of the innate and adaptive immune system, we focused on these two major effector cell populations.

In our study, 51 lung transplant recipients were analyzed for the kinetics of donor passenger NK and T cells in recipient blood directly after lung transplantation and at three weeks post Tx. We could demonstrate the existence of high frequencies of donor NK and T cells directly after transplantation in all recipients, which generated a transient lymphocyte chimerism within the first three weeks after DLTx. Unexpectedly, NK cells represented higher relative proportions of donor cells compared to T cells. In addition, the characterization of donor and recipient NK and T cell subsets revealed high frequencies of KIR-positive NK and T cell subsets, particularly with respect to $KIR2DL/S1^+$ subsets. Moreover, donor NK cells displayed higher cytotoxic activity at

Abbreviations: ACR, acute cellular rejection; BOS, bronchiolitis obliterans syndrome; CIT, cold ischemic time; CLAD, chronic lung allograft dysfunction; DC, dendritic cells; DLTx; double-lung transplantation; HLA, human leukocyte antigen; KIR, killer cell immunoglobulin-like receptor; mAb, monoclonal antibody; NK cells, natural killer cells; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PGD, primary graft dysfunction; RAS, restrictive allograft syndrome; SOC, standard of care.

early stages compared to recipient NK cells. Correlation analyses were performed with clinical parameters but did not show a significant impact on primary graft dysfunction. However, higher KIR^+ NK cell frequencies were observed in lung recipients with longer cold-ischemic times, suggesting an impact of the ischemia/reperfusion injury. Taken together, this early NK and T cell chimerism may rather be involved in the long-term balance between rejection and tolerance in the lung transplant setting.

MATERIALS AND METHODS

Patients and Sample Collection

Blood samples and clinical data of 51 patients undergoing DLTx were collected and preserved as peripheral blood mononuclear cells (PBMC). PBMC of patients were isolated using Biocoll Separating Solution (Biochrom/Merck, Darmstadt, Germany) as described. The study was approved by the ethics committee at the Hannover Medical School (no. 122-2007, 2500/2014) and all patients provided written informed consent. Venous whole blood samples were collected at the following time points: before (pre), 4-5 hours (T0), 24 hours (T24) and three weeks after DLTx (3wk). Donor and recipient demographics of the entire cohort (n=51) and the respective subgroups for the various analyses are summarized in **Table 1**. No induction therapy is applied to recipients and immunosuppression by calcineurin-inhibitors (CNI), steroids and mycophenolate mofetil (MMF) is administered intraoperatively. PGD scores were ranked according to oxygenation index and the presence of pulmonary radiographic infiltrates (15). Donor lungs were perfused with 2-3 L of Perfadex/Celsior perfusion solution and kept on cold storage in the same solution (perfusate). Perfusates of donor lungs were collected at the end of cold ischemic phase, cold ischemic times (CIT) and cross-clamp times (CCT) were documented. For collection of immune cells migrating or washed out of the allograft, perfusates were centrifuged at 453 x g for 15 min, cells were analyzed by flow cytometry and cell-free perfusate was stored at -20°C.

Detection of Donor-Derived Passenger Leukocytes in Peripheral Recipient Blood by Flow Cytometry

PBMC were thawed and incubated with live/dead yellow fluorescent reactive dye (ThermoFisher Scientific, Waltham, MA, USA), washed and stained with unconjugated donor HLA allele-specific or isotype control antibodies (**Supplementary Table 1**) for 30 min on ice, washed twice, followed by staining with secondary PB-, PE- and FITC-labeled goat-anti mouse IgG or IgM (GaM) mAb (**Supplementary Table 1**). T and NK cell subsets were identified with fluorochrome-conjugated lineage marker mAb (summarized in **Supplementary Table 1**), including mAb specific for KIR2D and 3D receptors. Of note, inhibitory and activating KIR genes cannot be distinguished by these mAb due to the identical extracellular domains of long inhibitory and short activating KIR. All staining steps were

TABLE 1 | Demographic characterization of lung transplanted patients.

Characteristics	Study cohort (n = 51)	HLA panel (n = 39)	NK/T panel (n = 33)	KIR panel (n = 14)	Degranulation assay (n = 10)
Donor					
Age at donation, y±SEM	47.37±2.21	48.72±2.56	45.36±2.7	51.71±4.53	40.6±5.3
sex, n (% male)	27 (53%)	20 (51%)	15 (45%)	4 (29%)	4 (40%)
Recipient					
Age at LTx, y±SEM	48.8±2.01	48.46±2.18	49.48±2.58	52.5±2.87	42.6±5.91
Sex, n (% male)	26 (51%)	19 (49%)	17 (52%)	6 (43%)	4 (40%)
Transplant indication, n (%)					
COPD	15 (29%)	11 (28%)	10 (30%)	5 (36%)	1 (10%)
IPF	17 (33%)	13 (33%)	11 (33%)	5 (36%)	4 (40%)
CF	10 (20%)	8 (21%)	6 (18%)	1 (7%)	3 (30%)
PIPH	3 (6%)	2 (5%)	2 (6%)	0 (0%)	1 (10%)
Others	6 (12%)	5 (13%)	4 (13%)	3 (21%)	1 (10%)
CCT, min±SEM	561.77±17.63	569.65±20.43	561.94±23.79	609.82±38.84	536.8±41.47
CIT, min±SEM	446.86±18.77	435.38±22.12	442.39±22.64	351.64±41.32*	463.2±34.54
PGD (2-3), n (%)	12 (24%)	12 (31%)	6 (18%)	4 (29%)	3 (30%)
T24	10 (20%)	10 (26%)	6 (18%)	4 (29%)	3 (30%)
T48	10 (20%)	10 (26%)	5(15%)	3 (21%)	3 (30%)
T72	3 (6%)	3 (8%)	1 (3%)	1 (7%)	1 (10%)
CLAD at end of follow-up, n (%)	9 (18%)	7 (18%)	5 (15%)	2 (14%)	2 (20%)
BOS	6 (12%)	4 (10%)	5 (15%)	2 (14%)	1 (10%)
RAS	3 (6%)	3 (8%)	0 (0%)	0 (0%)	1 (10%)
Treatment group, n (%)					
SOC	39 (76%)	27 (69%)	27 (82%)	7 (50%)	9 (90%)
EVLP	12 (24%)	12 (31%)	6 (18%)	7 (50%)	1 (10%)

BOS, bronchiolitis obliterans syndrome; CCT, cross clamp time; CF, cystic fibrosis; CIT, cold ischemic time; CLAD, chronic lung allograft dysfunction; COPD, chronic obstructive pulmonary disease; EVLP, ex-vivo lung perfusion; IPF, idiopathic pulmonary fibrosis; PGD, primary graft dysfunction; PIPH, primary idiopathic pulmonary hypertonia; RAS, restrictive allograft syndrome; SOC, standard of care; y, years.

Data are mean values ± standard error of mean (SEM). Asterisks show significant ($p < 0.05$) differences between HLA/BD/KIR/degranulation panels and study cohort (unpaired t-test, Mann-Whitney test).

performed for 30 min at 4°C and PBMC were washed with FACS buffer (PBS plus 0.1% sodium azide, Sigma-Aldrich, Munich, Germany, 1% FBS ThermoFisher Scientific, Waltham, MA, USA). Multi-color FACS analyses were performed using LSR II flow cytometer and the Diva software (8.0.1, BD Biosciences, San Diego, CA, USA).

Functional NK Cell Degranulation Assay

Degranulation assays were performed using recipient PBMC as previously described (16). PBMC were consecutively stained with unconjugated donor HLA allele-specific and secondary PE-labeled GaM mAb at room temperature for 30 minutes respectively, washed and incubated with FITC-labeled CD107a mAb and K562 target cells (16, 17) (1:1) for 4 h at 37°C/5% CO_2 with addition of 50 µM monensin (Sigma-Aldrich, St. Louis, MO; USA) after the 1st h. After two washing steps, cells were stained with CD56, CD16 for NK cells and CD3 as exclusion of T cells for 30 minutes at 4°C, washed twice and resuspended with FACS buffer. Cells were acquired using LSR II flow cytometer and the Diva software.

Statistical Analysis

All statistical analyses were generated using GraphPad Prism (Version 8, San Diego, CA, USA). D'Agostino-Pearson omnibus normality test and Kolmogorov-Smirnov normality test were applied to assess data distribution. Statistical analyses (two-way ANOVA with Sidak´s multiple comparison test and Tukey´s multiple comparison test, one-sample t-test, paired t-test, Wilcoxon test and Pearson correlation) were performed as indicated in the Figure legends. P values <0.05 were considered significant.

RESULTS

The Proportion of NK Cells Increases While the Frequency of T Cells Decreases in the Periphery of DLTx Recipients Directly After Transplantation

In order to define the kinetics of lymphocyte subsets in lung transplant recipients within the first three weeks after DLTx, PBMC of DLTx patients (n=33 patients, **Table 1**) were analyzed for their NK and T cell subsets using multicolor flow cytometry (**Figures 1A, B**). Directly after DLTx (T0), a trend towards an increase in the proportion of NK cells within $CD45^+$ leukocytes ($p<0.12$) could be observed before decreasing significantly ($p<0.001$) at T24 and three weeks to an even lower level compared to baseline pre Tx (pre 12.78% *vs.* 3wk 7.2%; $p<0.05$; **Figure 1A**). This peak at T0 resulted primarily from $CD56^{dim}CD16^{hi}$ NK cells, with a relative increase compared to stable $CD56^{dim}CD16^{lo}$ and $CD56^{bri}$ NK subsets. In parallel, the proportion of T cells displayed a substantial decrease at T0, which, however, did not reach statistical significance (**Figure 1B**) with primarily $CD4^+$ T cells disappearing post DLTx, returning to baseline levels after 3wk post DLTx. $CD8^+$ T cells seemed to disappear less intensely, which resulted in transiently significant changes in the $CD4^+/CD8^+$ ratio. This dynamic change in the NK and T cell subset composition within the first 24h after DLTx indicates a regulated process in recipient blood, with differential effects on these lymphocyte subsets.

Donor-Derived Lymphocytes Influence NK and T Cell Frequencies in the Periphery of DLTx Recipients

The existence of donor-derived 'passenger' lymphocytes migrating from the implanted lung into the periphery of the recipient has been known for a long time, although subset composition and fate of these donor cells remain rather unclear. Therefore, we investigated the kinetics of donor lymphocytes focusing on NK and T cell subsets (**Figures 1C, E**). To test our hypothesis that donor passenger lymphocytes contribute to these postoperative changes, NK and T cells were stained for donor HLA class I alleles at the same time points. Due to the HLA mismatch in DLTx, donor and recipient cells can easily be distinguished using HLA allele-specific Ab, which were available for 39 of the 51 patients (**Tables 1**, **2**). Donor and recipient NK and T cell subsets were then defined by their respective phenotype (**Figure 1C**). Directly post DLTx (T0), the highest frequencies of donor cells could be detected in general, with NK cells as predominant lymphocyte subset (**Figures 1C, D**), followed by $CD8^+$ T cells. Control stainings for donor cells pre DLTx showed 0-0.8% background staining, which defined the detection limit of >1% (**Supplementary Figures A, B**). The majority of donor NK cells displayed a $CD56^{dim}CD16^{hi}$ phenotype at T0 with a downregulation of CD16 over the course of three weeks (**Figures 1C, E**). At three weeks post DLTx, all donor lymphocyte subsets decreased significantly compared to T0 ($p<0.0001$). Nevertheless, donor cells, primarily NK cells, were still detectable three weeks after DLTx. Thus, our results demonstrate a transient chimerism by donor NK and T cells during the first three weeks after lung transplantation in peripheral blood of recipients that contributes to the observed changes in NK and T cell frequencies.

Higher Frequencies of KIR^+ Subsets in Donor NK and T Cells Are Present Within the First Three Weeks After DLTx Compared to Recipient NK and T Cells

To better understand the composition of donor cells after DLTx, NK and T cells in recipient blood were analyzed regarding their KIR repertoire in 14 of the 51 patients (**Table 1**). NK cell activity is among other factors, regulated by the KIR expression at the clonal level. Furthermore, KIR can also be expressed by $CD8^+$ T cells and influence their activity, including TCR signaling. Therefore, KIR2DL/S1, KIR2DL/S2/3 and KIR3DL/S1 expression in NK and T cells was assessed during the first three weeks after lung transplantation (**Figure 2**). Among all donor NK cells, the frequency of KIR2DL/S1 was increased significantly ($p<0.05$) at 3wk post DLTx compared to T0 and T24 (**Figures 2A, B**). Moreover, on donor cells, KIR2DL/S1 was mainly expressed by $CD56^{dim}CD16^{lo}$ NK cells (**Figures 2C, D**). Regarding KIR2DL/S2/3$^+$ donor NK cells, a trend towards a

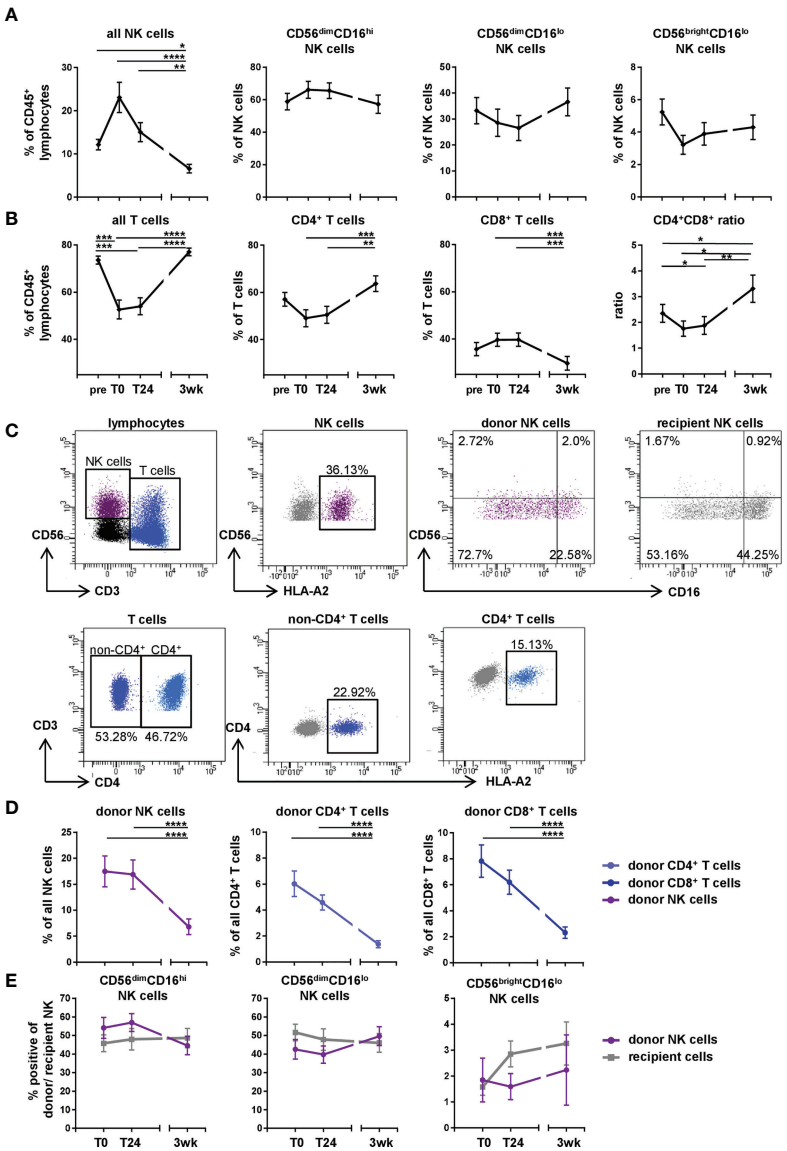

FIGURE 1 | Changes in NK and T cell subsets in first three weeks after DLTx are also mediated by donor cells. **(A, B)** Frequencies of NK and T cell subsets (n = 33 of the 51 patients, **Table 1**) in peripheral blood of DLTx recipients were analyzed pre, directly post (T0), 24 hours (T24) and three weeks after transplantation (3wk). **(A)** NK cells were gated as $CD45^+CD3^-CD56^+$ and NK cells subsets were further discriminated based on CD56 and CD16 expression. **(B)** T cells were identified as $CD45^+CD3^+CD56^{-/+}$ ('all T cells'), $CD3^+CD4^+$ ('$CD4^+$ T cells') and $CD3^+CD8^+$ ('$CD8^+$ T cells'). **(C)** Gating strategy for donor NK and T cells in peripheral blood of one representative patient #14 directly post DLTx is shown. Donor and recipient cells were discriminated *via* HLA class I mismatch using anti-HLA-A2 specific Ab (**Supplementary Table 1**). Here, $CD3^+CD4^+$ T cell and $CD3^+$non-$CD4^+$ T cell subsets were defined. **(D)** Frequencies of donor NK and T cells (n = 39, **Table 1**) in peripheral blood of DLTx patients directly post, 24 hours post and three weeks after transplantation are shown. **(E)** Frequencies of donor and recipient NK cells (n = 14) regarding their CD56 and CD16 expression were analyzed. Cells were gated as described in panel **(C)** Statistical analysis: one-way ANOVA with Dunn´s multiple comparison test for **(A, B, D)** and two-way ANOVA with Sidak´s multiple comparison test and Tukey´s multiple comparison test for **(E)**. Data are shown as mean ± SEM, asterisks indicate p-values with *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

TABLE 2 | HLA mismatches of transplanted recipients and donated lungs.

Number of HLA class I mismatches							Number of HLA class II mismatches				
0	**1**	**2**	**3**	**4**	**5**	**6**	**0**	**1**	**2**	**3**	**4**
0	0	2	2	13	14	8	1	1	15	13	9

HLA, human leukocyte antigen.

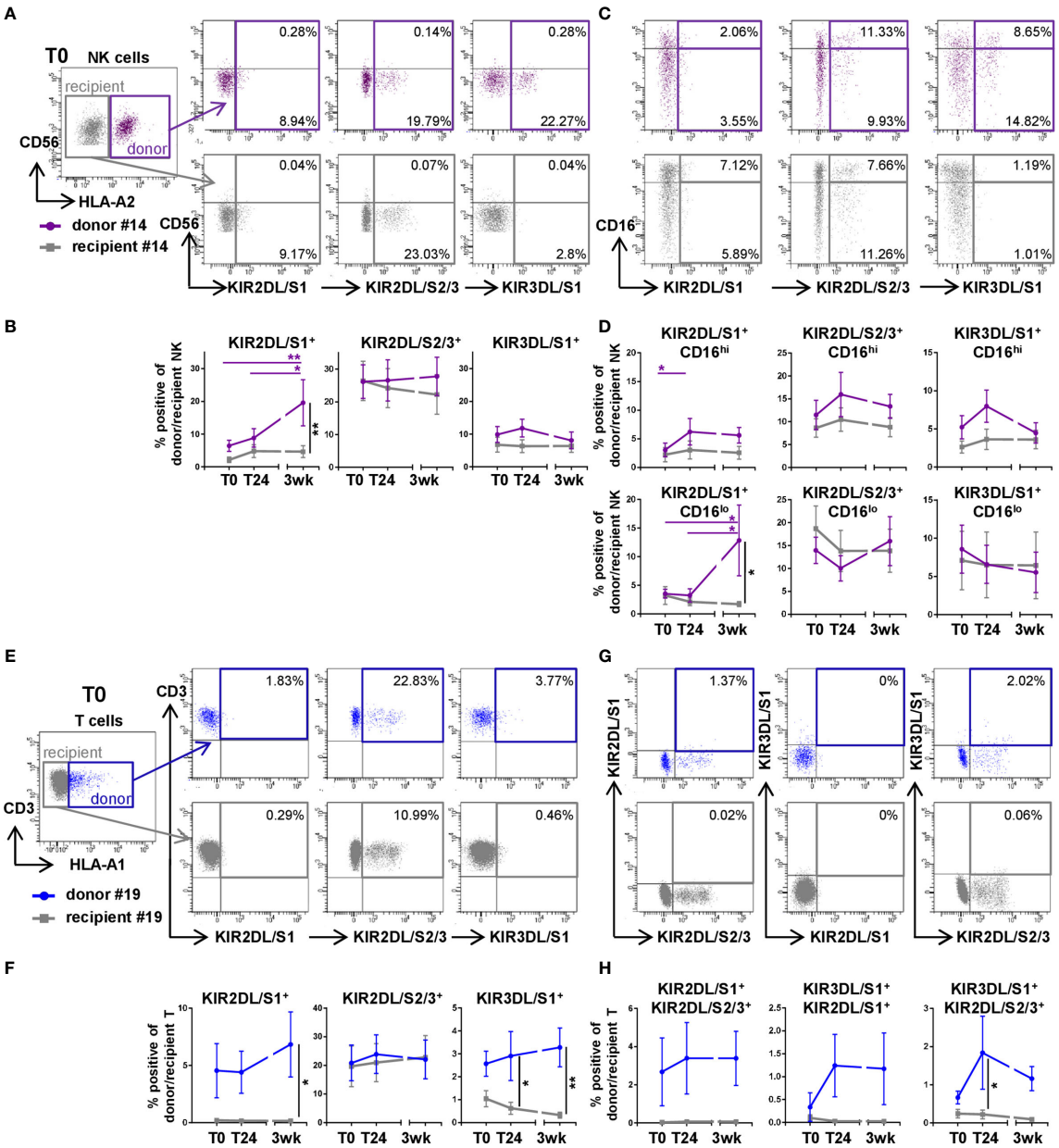

FIGURE 2 | The proportion of killer cell immunoglobulin-like receptors (KIR) on donor NK and T cells is higher compared to recipient cells within the first three weeks after DLTx. **(A–D)** KIR2DL/S1, KIR2DL/S2/3 and KIR3DL/S1 surface expression on NK cells (n=14) was analyzed in peripheral blood of double-lung transplant recipients directly post (T0), 24 hours (T24) and three weeks after transplantation (3wk). Donor and recipient cells were distinguished by HLA mismatch: NK cells of the representative donor #14 were identified by HLA-A2 staining **(A, C)** T cells of the representative donor #19 were identified by HLA-A1 staining **(E, G)**, the gating strategies are shown in **Figures 1C** and **Supplementary Figure 1**. **(A)** Representative FACS plots (T0) and **(B)** frequencies of donor (purple colored squares) and recipient (grey colored squares) KIR on NK cells. **(C)** Representative FACS plots (T0) and **(D)** frequencies showing KIR expression on $CD16^{hi}$ and $CD16^{lo}$ NK cells. **(E–H)** The same KIR repertoire was assessed for T cells (n = 14) in peripheral blood of DLTx patients for the indicated time points. T cells of donor #19 were stained using anti-HLA-A1-specific Ab. **(E)** Representative FACS plots and **(F)** frequencies of KIR on donor (blue colored squares) and recipient (grey colored squares) T cells are displayed. **(G)** Representative FACS plots and **(H)** frequencies describing double positive KIR surface expression on T cells. Statistical analysis: two-way ANOVA with Sidak´s multiple comparison test and Tukey´s multiple comparison test. Data are shown as mean ± SEM, asterisks indicate p-values with *$p < 0.05$, **$p < 0.01$.

switch from $CD16^{hi}$ to $CD16^{lo}$ donor NK cells could be observed within the first 3 weeks after DLTx. The frequency of KIR3DL/$S1^+$ donor NK cells was stably low over time (**Figures 2B, D** and **Supplementary Figure 2A**). This differential dynamic of KIR^+ donor NK subsets was independent from their HLA-C typing (data not shown). During three weeks after DLTx, the frequencies of KIR2DL/$S1^+$ and KIR3DL/$S1^+$ donor T cells were significantly higher compared to recipient T cells (both

p<0.05; **Figures 2E, F** and **Supplementary Figure 2B**), while frequencies of KIR2DL/S2/3$^+$ T cells showed no difference between donor and recipient T cells. Due to the individual variability, no significant changes could be seen for single KIR$^+$ T cells over time. Only very few KIR double-positive donor T cells were detectable after transplantation (**Figures 2G, H**) indicating a rather single KIR$^+$ subset composition. Recipient NK and T cells revealed low and stable KIR$^+$ proportions during the first three weeks after DLTx, except for KIR2DL/S2/3. To uncover a potential age-related increase of KIR expression levels on NK and T cells, donor age was correlated to the KIR$^+$ NK and T cells 24 hours after transplantation (T24) (**Supplementary Figure 3**). Neither a correlation between donor age nor the frequency of donor cells nor the percentage of KIR$^+$ donor NK and T cells could be found, indicating no influence of donor age on the KIR repertoire on donor NK and T cells. Therefore, our results demonstrate a chimerism between donor and recipient NK and T cells in recipient blood during the first three weeks after DLTx with a substantial contribution of donor KIR$^+$ NK cell subsets and a minor fraction of donor KIR$^+$ T cells.

Increased Activity and Functional Capacity of Donor NK and T Cells in the Periphery of the Recipient During the First Three Weeks Post DLTx

To assess activation levels of donor NK and T cells in comparison to recipient cells in peripheral blood, we analyzed the surface expression of the activation marker CD25, IL-2 receptor alpha chain, pre, directly, 24 hours and three weeks post DLTx by flow cytometry (**Figure 3**). Over time, the proportion of CD25$^+$ donor NK and T cells increased up to 20% (p<0.001), whereas recipient CD25$^+$ NK and T cells remained stable at a rather low level of approximately 1% (p<0.0001 for donor *vs.* recipient at 3wk post DLTx) (**Figures 3A, B**). Three weeks post DLTx, activated donor NK cells were characterized as primarily CD56^{dim} (data not shown) CD16^{lo} NK cells (**Figure 3A**). We further analyzed donor and recipient NK cells for their functional capability, i.e. degranulation, by measuring surface expression of CD107a upon exposure to HLA-deficient target cells (K562). Comparing donor and recipient, significantly higher levels of CD107a surface expression could be detected in donor NK cells, already in the absence of K562 target cells (**Figures 3C, D** upper panel). In the presence of K562 target cells, donor NK cells displayed higher degranulation compared to recipient NK cells at all time points (all p < 0.5; **Figures 3C, D** lower panel). However, during the first 3 weeks after DLTx a trend towards increased degranulation capacity could be observed for both donor and recipient cells. Further characterization of NK cell subsets revealed that the main proportion of degranulating donor NK cells displayed a CD56^{dim}CD16^{lo} phenotype. Exposure to K562 showed an additional effect neither on recipient nor on donor NK cell degranulation at any time point indicating a suppressive effect *via* immunosuppression also for donor NK cells. Gating on CD16^{bri} NK cells showed a rather poor degranulation potential, as expected (16). Regarding the degranulation capacity of T cells, again donor CD4$^+$ and CD8$^+$ T cells displayed significantly higher proportions of CD107a$^+$ cells over the course of three weeks without K562 (p<0.01 compared to T0). CD8$^+$ donor T cells also showed overall higher levels of degranulation compared to CD8$^+$ T cells of the recipient (p< 0.0001 at 3wks post DLTx; **Figure 3E**). Since K562 does not stimulate T cells, these data are not shown. The presented results indicate that donor NK and T cells, predominantly CD56^{dim}CD16^{lo} NK and CD8$^+$ T cells, reached *per se* an activated and functional state in the periphery of the recipient, expressing higher levels of activation markers compared to the recipients own NK and T cell repertoire.

Predominantly KIR2DL/S1- and KIR2DL/S2/3-Positive T Cells and CD56^{dim}CD16^{lo} NK Cells From the Lung Allograft Are Found in the Recipient Periphery Early Post DLTx

We next compared donor NK and T cell subsets for their phenotypes including KIR expression in recipient blood *vs.* perfusion solution, i.e. the storage solution of the lung during the *ex situ* phase (**Figure 4**). In perfusion solution, the major NK cell phenotype was CD56^{dim}CD16^{hi}, while donor NK cells in recipient blood at T0 demonstrated a CD56^{dim}CD16^{lo} phenotype indicating previous activation. The frequency of CD56^{bri}CD16^{lo} NK cells was significantly higher (p<0.05) in perfusion solution compared to recipient periphery (**Figure 4A**). Comparing the KIR repertoire on donor NK cells in perfusion solution with recipient blood, high frequencies were observed without significant differences except for KIR2DL/S1with higher proportions on donor cells in recipient blood (**Figure 4B**). In donor T cells, higher KIR2DL/S1 and KIR2DL/S2/3 (both p=0.05) surface expression in recipient blood compared to perfusion solution was detectable (**Figure 4C**). To reveal a potential age-related influence on the KIR repertoire of NK and T cells in the perfusion solution, we correlated donor age to KIR$^+$ donor NK and T cells in perfusion solution. Our results could not reveal an impact of donor age on the KIR repertoire of donor NK and T cells in perfusion solution (**Supplementary Figure 3**). Our data suggest that predominantly CD56^{dim}CD16^{hi} NK cells leave the donated lung during preservation into the perfusion solution, whereas CD56^{dim}CD16^{lo} NK cells may migrate primarily into recipient blood directly after DLTx. KIR expression on donor T cells was higher in the recipient blood compared to perfusion solution while this did not affect KIR$^+$ subsets substantially.

The KIR Repertoire on Donor NK and T Cells in DLTx Recipient Blood Does Not Correlate With Primary Graft Dysfunction

Next, we wanted to analyze the clinical impact of donor NK and T cells in the recipient periphery and their KIR repertoire on primary graft dysfunction (PGD), a major cause of early graft failure and poor transplant outcome. Therefore, lung transplant recipients were divided into two groups according to their PGD scores. PGD was assessed 24 hours post DLTx and graded in PGD 0-1 (no and low degree of severity) and PGD 2-3 (high

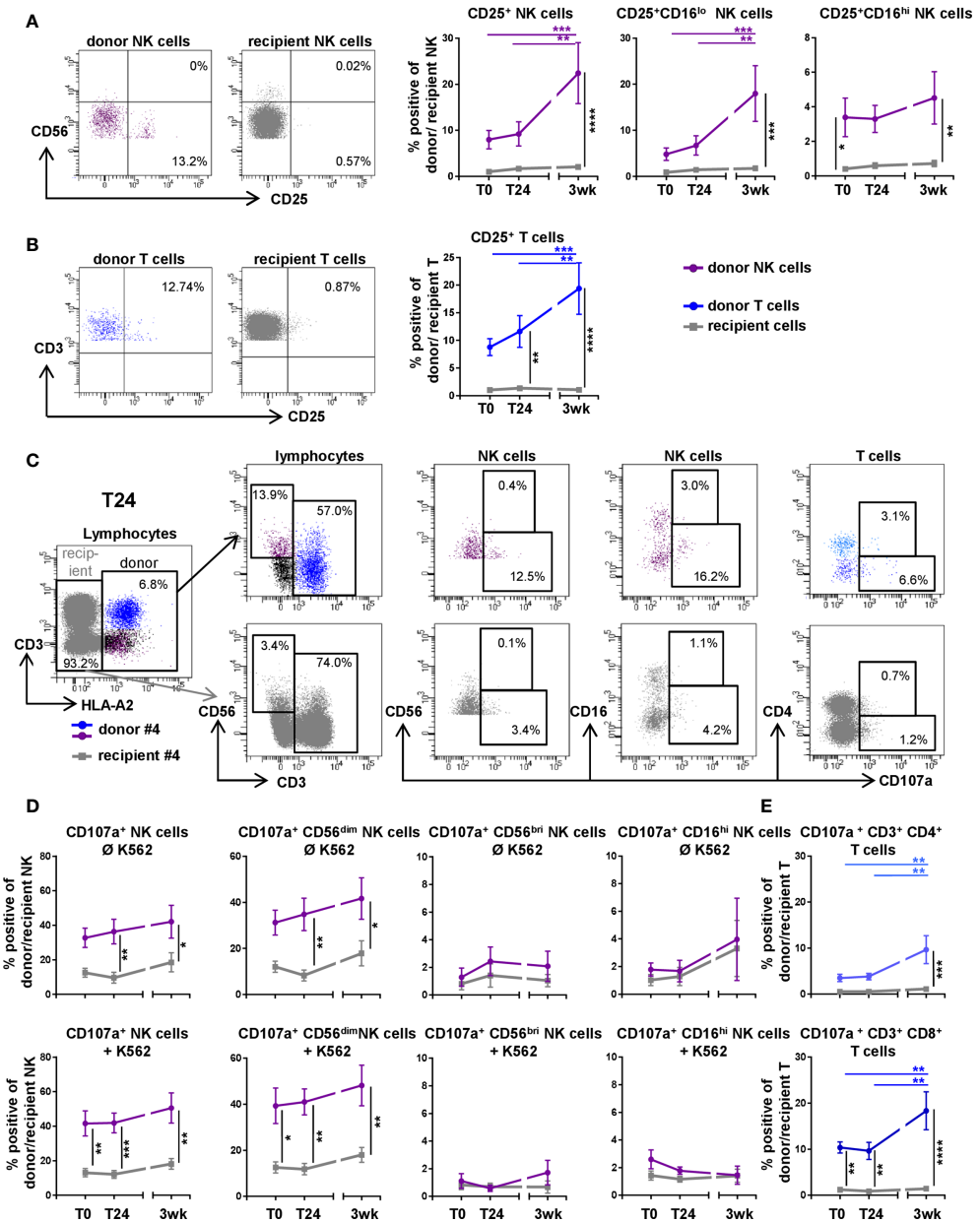

FIGURE 3 | Donor NK and T cells show a higher activation level and degranulation capability three weeks after double-lung transplantation (DLTx) in comparison to recipient cells. **(A, B)** CD25 surface expression was analyzed on NK (n = 14) and T cells (n = 14) in peripheral blood of lung transplant recipients post (T0), 24 hours post (T24) and three weeks after transplantation (3wk). Underlying gating strategy is displayed in **Figure 2**. Representative FACS plots (3wk) and frequencies of **(A)** NK cells and **(B)** T cells are shown. **(C–E)** CD107a surface expression was investigated on donor and recipient NK (n = 10) and T cells (n = 10) at indicated time points with and without exposure to K562, respectively. **(C)** Gating strategy for analysis of CD107a$^+$ NK and T cells in peripheral blood at T24 without K562 is shown. Events were gated on singlets. Lymphocytes were defined through FSC/SSC. Donor and recipient cells were discriminated *via* mismatch for HLA-A2. Cells were gated on CD3$^-$CD56$^{dim/bri}$ for NK cells and CD3$^+$CD56$^-$ for T cells. **(D)** Frequencies of CD107-expressing donor and recipient NK as well as NK cell subsets with and without exposure to K562 and **(E)** CD107-expressing donor and recipient T cells without contact to K562. Statistical analysis: two-way ANOVA with Sidak´s multiple comparison test and Tukey´s multiple comparison test. Data are shown as mean ± SEM, asterisks indicate p-values with *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

degree of severity). Despite a very small sample size, both groups were compared with respect to their donor NK, T cell frequencies, and the KIR repertoire on donor and recipient cells. In general, no differences could be detected for the frequencies of donor NK and CD4$^+$ or CD8$^+$ donor T cells between both PGD groups (**Figure 5A**). Furthermore, the data revealed no significant distinction between PGD0-1 and PGD2-3 focusing on KIR repertoire on donor and recipient NK and T

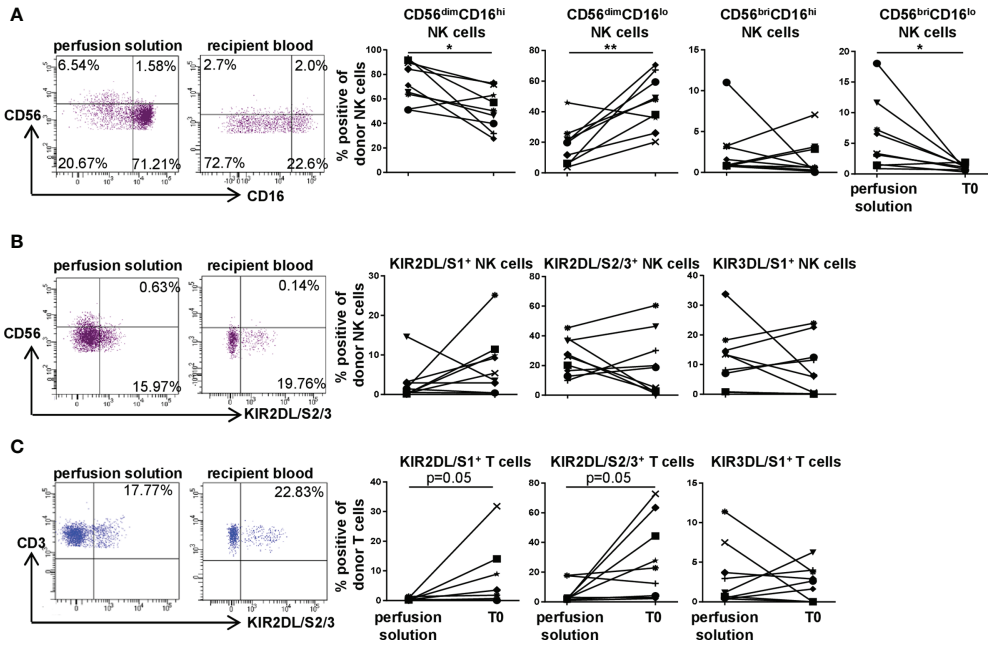

FIGURE 4 | KIR repertoire on donor NK and T cells in perfusion solution distinguishes from the one in recipient blood directly post DLTx (T0). KIR2DL/S1, KIR2DL/S2/3 and KIR3DL/S1 surface expression by donor NK and T in perfusion solution and recipient blood of 9 DLTx patients was determined. Cells were gated for $CD56^{dim}$ and $CD56^{bri}$ NK cells as described in **Figure 1C** and for KIR as illustrated in **Figures 2A, E**. **(A)** Representative FACS plots and frequencies of donor $CD56^{dim}$ or $CD56^{bri}$ and $CD16^{hi}$ or $CD16^{lo}$ NK cells in perfusion solution and recipient blood are shown. **(B)** Representative FACS plots of KIR2DL/S2/3 and frequencies of KIR2DL/S1, KIR2DL/S2/3 and KIR3DL/S1 surface expression on donor NK cells are demonstrated. **(C)** Representative FACS plots of KIR2DL/S1 and frequencies of KIR2DL/S1, KIR2DL/S2/3 and KIR3DL/S1 expression on donor T cells. Statistical analysis: paired t-test was calculated for normally distributed data, otherwise Wilcoxon test. Asterisks indicate p-values with *p < 0.05, **p < 0.01.

cells, although the frequency of KIR^+ NK and T cells was slightly lower in DLTx patients suffering PGD2-3 (**Figures 5B, C**). The association of PGD and the activation marker CD25 was also analyzed. No differences in the CD25 surface expression on donor NK cells comparing PGD 0-1 and PGD2-3 could be shown (**Figure 5D**). In contrast, an increase in the frequency of $CD25^+$ donor T cells ($p<0.05$) in patients with PGD2-3 over time could be demonstrated (**Figure 5E**). The proportion of $CD25^+$ donor T cells in PGD-01 patients also slightly increased without reaching statistical significance. The difference in $CD25^+$ donor T cells between PGD2-3 and PGD0-1 patients at three weeks was also not significant. Taken together, our results indicate that neither frequency nor KIR repertoire of donor NK and T cells did affect the early clinical outcome, i.e. PDG, after lung transplantation.

Frequencies of KIR^+ Donor NK Cells Directly After DLTx Tend to Increase With Longer Cold Ischemic Times (CIT)

Little is known about the impact of the cold static preservation on the explanted lungs in terms of NK and T cell kinetics in recipient blood and clinical outcome. Therefore, we correlated cold ischemic times (CIT) with frequencies of donor NK and T cells at T0 (**Figure 6**). Our data mirrored a positive correlation between the duration of CIT and frequencies of donor NK as well as $CD4^+$ and $CD8^+$ T cell subsets in recipient blood directly post DLTx (**Figure 6A**). Especially the proportion of $CD4^+$ donor T cells increased with longer CIT (r=0.4; p=0.01). Regarding the influence of CIT on the KIR repertoire of donor NK cells at T0 (**Figure 6B**), a trend for a correlation was found between CIT and all analyzed KIR on donor NK cells ($KIR2DL/S1^+$ r=0.48, p=0.09; $KIR2DL/S2/3^+$ r=0.5; p=0.07 and $KIR3DL/S1^+$ r=0.52; p=0.06). Particularly the $KIR2DL/S2/3^+CD56^+$ NK cells showed a positive correlation to CIT. In contrast, we could not show any correlation between CIT and the KIR repertoire on donor T cells (**Figure 6C**, **Supplementary Figure 4**). In summary, these results imply that both donor NK and T cells generally are influenced by CIT. Furthermore CIT has an impact on KIR^+ NK cell subsets whereas the duration of the *ex situ* time of the lung has no influence on the KIR repertoire on donor T cells. These results may have a so far underestimated impact on the donor/recipient distribution in lung transplantation.

DISCUSSION

Recently, the impact of NK cells and the genetics associated with the KIR ligand mismatch has been shown for kidney transplantation (5), which raises also the question for other organs, especially those with tissue-resident cells like the lung. Passenger lymphocytes can be found in the peripheral blood of lung transplant recipients (13, 18), but the knowledge on their

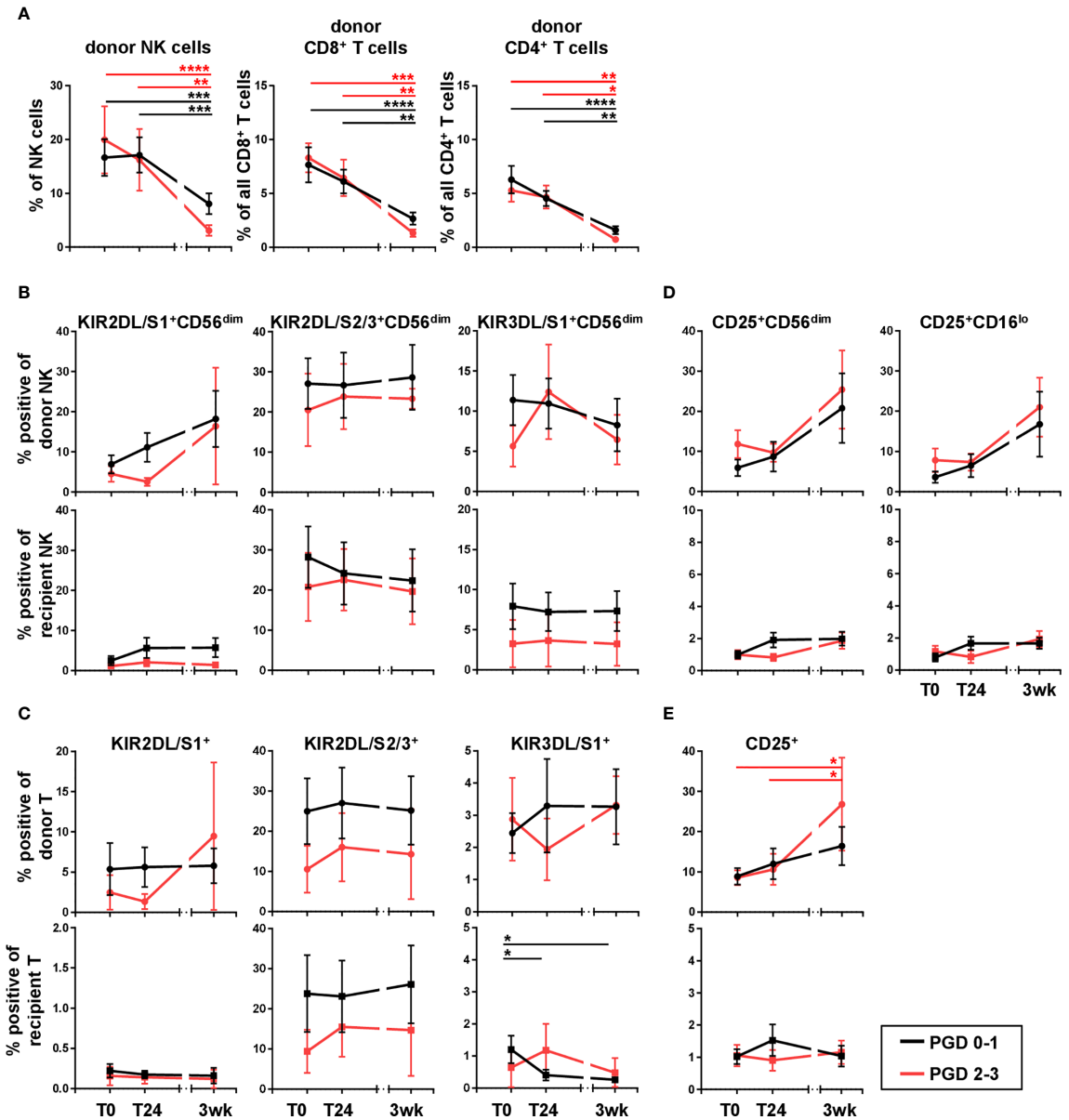

FIGURE 5 | KIR repertoire of donor and recipient NK and T cells does not influence primary graft dysfunction (PGD). **(B, D)** KIR2DL/S1, KIR2DL/S2/3, KIR3DL/S1 and **(C, E)** CD25 expression on NK and T cells was analyzed in peripheral blood of lung transplant recipients post (T0), 24 hours (T24) and three weeks after transplantation (3wk). Donor and recipient cells were distinguished by HLA mismatch using anti-HLA-A2/A1 specific Ab. Cells were gated as described in **Figure 1C**. DLTx recipients were divided into two groups, PGD0-1 [n = 29 for **(A)**; n = 10 for **(B–E)**] and PGD2-3 [n = 10 for **(A)**; n = 4 for **(B–E)**], concerning their severity of PGD at 24 hours post DLTx. **(A)** Frequencies of donor NK and donor T cell subsets are shown. **(B, D)** Illustration of frequencies of KIR positive NK and T cells and **(C, E)** their CD25 expression. Statistical analysis: two-way ANOVA with Sidak´s multiple comparison test and Tukey´s multiple comparison test. Data are shown as mean ± SEM, asterisks indicate p-values with *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

kinetics, phenotype and relevance in the lung transplant setting is still rather scarce. Here, we aimed to elucidate donor NK and T cell characteristics in recipient blood during the first three weeks after lung transplantation with particular focus on their KIR repertoire and their impact on clinical outcome. To the best of our knowledge, these dynamics within the first three weeks after DLTx have not been studied in detail before. In the current study, we unexpectedly observed substantial changes in the composition of lymphocyte subsets in the recipient periphery immediately after DLTx. In general, our analyses revealed higher frequencies of NK cells, dominated by $CD56^{dim}CD16^{hi}$ NK cells at T0, and a lower proportion of T cells, primarily $CD4^+$ T cells, directly post DLTx compared to baseline pre DLTx. Furthermore, a significant decrease in the $CD4^+/CD8^+$ T cell ratio directly post DLTx was detected. In order to identify a potential link between donor passenger cells and the dynamics in

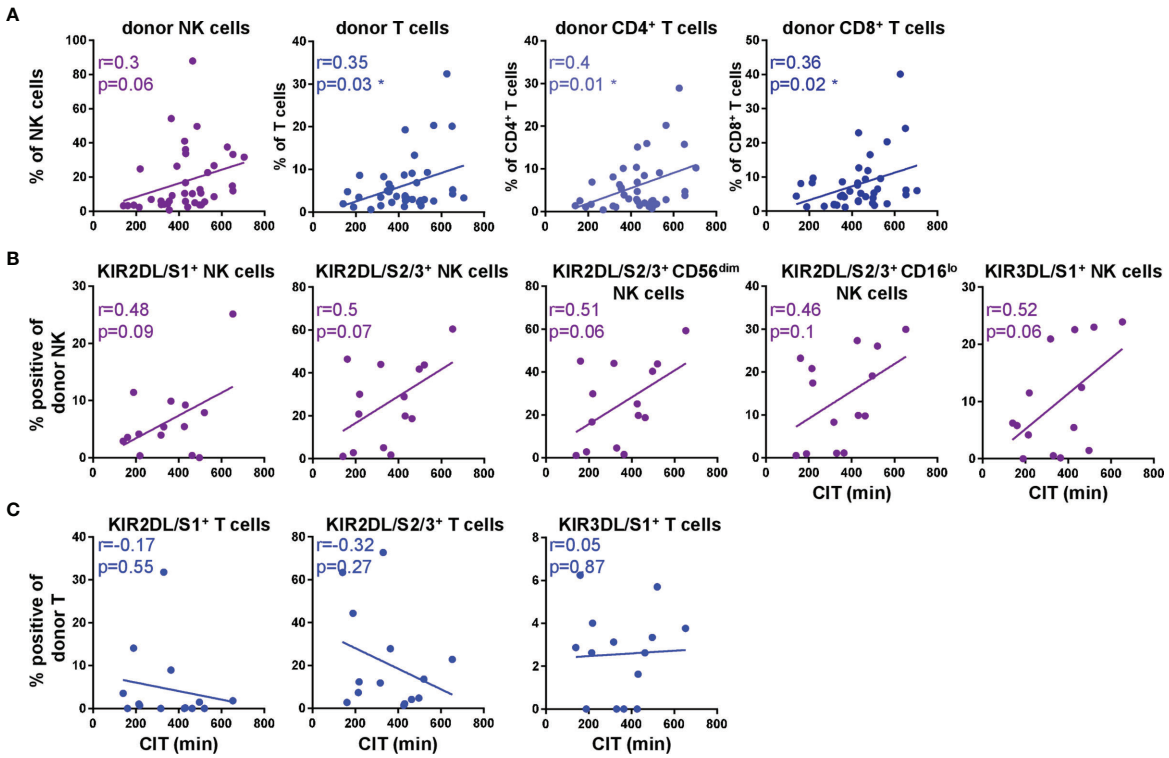

FIGURE 6 | KIR$^+$ donor NK cell subsets increase with longer cold ischemic time (CIT). **(A)** Scatter plots show linear regression of CIT to donor NK and T cell subsets (n = 39) and **(B)** KIR surface expression on donor NK cells (n = 14) and **(C)** T cells (n = 14). Each dot represents one patient. Gating strategy for donor NK and T cells is shown in **Figure 1C** and for KIR in, **(Figures 2A, E)** Statistical analysis: linear regression and correlation analysis by Pearson correlation. Asterisks indicate p-values with *p < 0.05.

lymphocyte subsets, we focused our analyses on the first three weeks, especially the first 24 hours. This time frame was also supported by previous studies detecting donor passenger leukocytes in recipient peripheral blood during the first four weeks following lung transplantation (14).

With the HLA-based discrimination between donor and recipient leukocytes, we could proof that a transient lymphocyte chimerism, primarily by NK and T cells but not B and myeloid cells (data not shown) is involved in the dynamic changes in lymphocyte subsets after DLTx. Within the NK and T cell subsets, donor NK cells represented the highest proportion at all three time points, followed by CD8$^+$ and CD4$^+$ T cells. The frequency of donor CD8$^+$ T cells among all CD8$^+$ T cells was elevated compared to CD4$^+$ T cells and, therefore, can partially explain the generally decreased CD4$^+$/CD8$^+$ T cell ratio directly post DLTx, which was also mediated by substantially decreased recipient CD4$^+$ T cells. The reduced frequency of all T cells and the simultaneously increased proportion of NK cells directly post DLTx cannot be explained only by the augmented frequency of donor NK cells leaving the transplanted lung into the recipient periphery. Hence, it is conceivable that T cells leave the periphery, and may potentially migrate into the transplanted lung, as an immune response to the transplanted organ. This scenario has been proposed for NK cells (19). Alternatively, T cells may migrate to the lymphatic system, i.e. lymph nodes and spleen, which can also be observed during infection. Bidirectional movements of donor and recipient T and NK cells between peripheral blood and the lung allograft are capable to evoke profound changes in leukocyte subsets directly post DLTx.

Next, we aimed for a more detailed characterization of the phenotype of donor NK cells with respect to the major peripheral subsets, i.e. CD56^{dim}CD16^{hi} and CD56^{bri}CD16^{neg} NK cells. We focused on KIR expression, since activating and inhibitory KIR are major surface receptors regulating NK and T cell activity and consequently, might play a crucial role in the context of lung transplantation (11). To get further insights into the KIR expressing donor NK and T cell subsets, we analyzed the surface expression of the most relevant KIR on donor as well as recipient NK and T cells after DLTx. We found higher proportions of KIR2DL/S1, KIR2DL/S2/3 and KIR3DL/S1 expressing subsets in donor NK and T cells compared to recipient cells. Our results furthermore demonstrated a significant increase in frequency of KIR2DL/S1$^+$ donor NK cells during the first three weeks post DLTx, whereas the frequency of KIR2DL/S1$^+$ recipient NK cells remained stable at low levels over time. In addition, we studied the functionality of these donor NK and T cells in recipient blood and found donor NK cells to exhibit a higher degranulation capability compared to recipient NK cells. The poor capability of both donor and

recipient NK cells to respond to HLA class I-deficient K562 cells in terms of vitro degranulation is likely caused by the onset of immunosuppression in the patient intraoperatively (20, 21). In addition, KIR surface expression increases with progressing maturation of NK and T cells (22, 23). Both aspects, enhanced degranulation capability and increased KIR expression on donor cells, suggests donor NK and T cells to have a more functional phenotype compared to recipient cells after DLTx.

In order to investigate the potential origin of donor NK cells in more detail, we also used perfusion solutions to identify the donor NK cell composition and to compare it to the passenger cells in the recipient periphery directly after DLTx (data not shown). NK cells in perfusion solutions were predominantly composed of $CD56^{dim}CD16^{hi}$ NK cells. Since donor NK cells in recipient blood were mainly represented by $CD56^{dim}CD16^{lo}$ NK cells, this change in CD16 surface expression argues for at least some degree of activation in this $CD56^{dim}$ NK cells subset. This $CD56^{dim}$ NK subset has also been identified as lung-resident subset in human lung parenchyma by Marquardt et al. (24). Since we primarily detected the $CD56^{dim}CD16^{hi}$ NK cell subset in perfusion solution, we propose that this subset leaves the donor lung early during preservation, whereas directly after DLTx, the $CD56^{dim}CD16^{lo}$ NK cell subset is migrating out of transplanted lung into the recipient periphery. In addition, the low CD16 expression can be an indicator for a partial activation, which may also explain their spontaneously high CD107a expression level even in the absence of K562 target cells.

Our group has previously demonstrated that downregulation of CD16 surface expression on NK cells in patients after kidney transplantation was associated with activation and induction of interferon-γ (20). Here, we detected a downregulation of CD16 surface expression on NK cells up to three weeks after DLTx, thereby showing a similar CD16 modulation also in lung transplantation. In the DLTx cohort, CD16 downregulation was even more pronounced in donor compared to recipient NK cells. In parallel, the activation marker CD25 was significantly higher expressed on both donor NK and T cells, which argues for a higher activation level in donor *vs.* recipient cells. This higher activation status of donor NK and T cells was observed continuously within the first three weeks after DLTx and may be explained by a permanent recognition of the recipient, which may be driven by the HLA mismatch, especially in KIR ligand mismatch constellations between donor and recipient, according to the 'missing self' hypothesis. This is rather likely to be the case since in our cohort, approximately 90% of recipients show more than three HLA class I and three HLA class II mismatches to the respective donor HLA alleles. In conclusion, we characterized donor NK and T cells to exhibit a more activated and functional phenotype compared to recipient cells in peripheral blood of lung recipients during the first three weeks following DLTx. Thus, future studies are required to further elucidate whether activated NK cells may be associated with rejection also in lung transplantation as it was shown for $CD69^{+}CD56^{dim}$ NK cells in AMR biopsies of kidney transplant recipients (25).

The clinical outcome after DLTx is still limited, predominantly due to early, i.e. PGD, and late chronic allograft dysfunction (CLAD). Since the impact of a transient chimerism has not been addressed clinically in DLTx, we investigated the relevance of donor NK and T cells in terms of PGD and CIT. In BAL of lung recipients, it has been recently shown that certain proportions of donor-derived T cells with a tissue-resident memory phenotype are associated with a lower incidence of primary graft dysfunction (14). In contrast to BAL, donor T cells were not detectable in recipient blood in these analyses, which started 4 weeks after transplantation and, hence, did not cover the very early phase directly after DLTx. Therefore, we can assume that the chimerism detected in our setting is likely to be transient and may wean after several weeks, which is also the case by own observations (data not shown).

To elucidate the function and relevance of the KIR family in the lung transplant setting, we compared the KIR repertoires on donor *vs.* recipient NK and T cells and correlated these to PGD severity, representing a major complication early after DLTx, as well as CIT. In this small cohort, we could not show a correlation between the proportions of KIR^{+} donor NK or T cells and PGD, since patients suffering from PGD2-3 did not display higher KIR frequencies. Hence, the KIR NK and T cell repertoire does not seem to have a direct effect on clinical outcome, i.e. PGD during the first 72 hours after DLTx. However, the preservation period may impinge on the frequency of donor NK cells since we observed a positive correlation between CIT and KIR^{+} NK subsets. Interestingly, the frequencies of KIR-expressing donor T cells did not increase with longer CIT, further underlining the important role of NK cells. This unexpected finding points towards a physiological impact of ischemia reperfusion injury on the mobilization of NK and T cells in lung transplantation, which is likely influenced by the inflammatory milieu of the lung during the *ex situ* phase. In addition to the known ischemic mechanisms (26), extended preservation times may also be critical for the transient chimerism and, rather indirectly, maybe also for early clinical outcome. Our findings are also supporting a previous study demonstrating a crucial role of NK cells on clinical outcome within the early phase after kidney transplantation (27). However, the mechanisms may be unrelated since in kidney transplantation, no passenger cells have been identified (28).

Acute cellular rejection (ACR), driven mainly by alloreactive cytotoxic T cells, can lead to acute graft failure, and with regards to the long-term outcome, can also result in CLAD development. Furthermore, besides NK cells, inhibitory KIRs are expressed by $CD8^{+}$ T cells and, therefore, regulate their functions and survival in i.e. viral infections (22, 29, 30). In our DLTx cohort, we detected a significantly higher KIR3DL/S1 expression on donor compared to recipient T cells. It will be interesting to validate these findings in a larger patient cohort and to relate them to the long-term-survival of the allograft. Still, iKIR are not able to downregulate all aspects of T cell activation, as demonstrated by elegant experiments in a transgenic mouse system (31). Therefore, inhibitory KIR may be important for the fine-tuning and adjustment of T cell functions. In addition, it has been shown that NK and T cells share many functional and phenotypical properties (32). Thus, it might be possible to transfer – at least in part – conclusions for donor NK cells onto donor T cells. Yet, future studies will be needed to further elucidate the functional consequences of iKIR expression by

(donor) $CD8^+$ T cells in the context of lung transplantation with a focus on ACR development.

Based on the known impact of age on NK and T cell subset distributions, we correlated the KIR^+ subsets to donor age with the aim to show whether, increasing KIR proportions may be elevated with progressing age indicating enhanced maturation, as many groups have demonstrated (22, 23). Surprisingly, no correlations between KIR expression and donor age were found indicating that the donor age only has a weak effect on the KIR repertoire on donor NK and T cells. Furthermore, due to limitations of donor lung availability these results imply that even older people could serve as potential donors in the lung transplant setting.

In conclusion, our findings reveal donor NK and T cells in the periphery of lung transplant recipients as highly activated and functional subsets and therefore might be crucial in the lung transplant setting. Further studies investigating regulatory receptors on donor NK and T cells are necessary to better understand their contribution to graft tolerance thereby improving patient survival after lung transplantation.

Limitations of the Study

The results presented here are derived from a single transplant center with a limited sample size. Thus, expanding the patient number is desirable to substantiate our findings. Due to the incomplete HLA-C typing, we were unable to define the HLA-C KIR ligand mismatches in detail. Moreover, based on the limited availability of allele-specific α-HLA monoclonal antibodies, only selected patient combinations with donor-specific HLA class I alleles could be analyzed for the discrimination of donor *vs.* recipient cells. Moreover, it would be interesting to identify the KIR-expressing NK and T cells subsets in BAL samples representing airway compartment. The clinical correlations were limited to the early events like PGD and CIT but in the future, we plan to investigate also a possible impact on intermediate outcome like acute rejection as well as long-term outcome, i.e. CLAD. These aspects are part of ongoing studies.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by ethics committee at the Hannover Medical School (no. 122-2007, 2500/2014). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AMH and KAB performed experiments, analyzed data and wrote the manuscript. BW coordinated sample collection and contributed to writing of the manuscript. RBS and FW helped with statistics and writing of the manuscript. EC revised statistics, figures, tables and text of the manuscript. JK, KB, LMH and CN performed and supported key experiments. FI, WS, MA, CK, IT, JS, TS, AH and GW performed DLTx and were instrumental for sample and clinical data collection. CSF and JFK supervised the work, designed experiments, analyzed data and wrote 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.2021.778885/full#supplementary-material

Supplementary Figure 1 | Flow cytometry gating strategy for the discrimination of donor *vs.* recipient cells, exemplary shown for an HLA-A2-positive donor (lung) transferred into an HLA-A2-negative recipient. **(A)** Cell doublets were excluded based on the area/height ratio. In the next step, dead cells were excluded based on the life/dead staining. $CD45^+$ cells were then gated and finally lymphocytes were gated based on their CD45 expression and granularity (SSC). Subsequently, $CD56^+CD3^-$ NK cells as well as $CD3^+CD56^{+/-}$ T cells were defined. NK cells were further discriminated into either donor or recipient origin based on HLA-A2 expression. In this example, the donor is $HLA\text{-}A2^+$ and donor NK cells ($CD56^+HLA\text{-}A2^+$) are displayed in pink, whereas the recipient is $HLA\text{-}A2^-$ and recipient NK cells ($CD56^+HLA\text{-}A2^-$) are shown in purple. T cells were further divided into $CD3^+CD4^+$ T cells and $CD3^+$non-$CD4^+$ ("non-$CD4^+$") T cells. Finally, donor as well as recipient origin for both $CD4^+$ and non-$CD4^+$ T cells was determined based on HLA-A2 expression (donor T cells $HLA\text{-}A2^+$, recipient T cells $HLA\text{-}A2^-$) and are displayed in different blue shades. **(B)** High proportions of donor NK and T cells can be detected in recipient blood directly post DLTx. Frequencies of donor NK and T cell subsets in peripheral blood of DLTx recipients were analyzed pre, directly post (T0), 24 hours (T24) and three weeks after transplantation (3wk). Donor and recipient cells were discriminated *via* HLA mismatch using anti-HLA-A2 specific Ab. Gating strategy is shown in **Figure 1C**. Statistical analysis: one-way ANOVA with Dunn´s multiple comparison test, (n = 39). Data are shown as mean ± SEM, asterisks indicate p-values with ****$p < 0.0001$.

Supplementary Figure 2 | Exemplary flow cytometric analyses for KIR2DL/S1, KIR2DL/S2/3 and KIR3DL/S1 surface expression on donor NK and T cells in peripheral blood of double-lung transplant recipients directly post (T0), 24 hours (T24) and three weeks after transplantation (3wk). Donor and recipient cells were distinguished by HLA mismatch: NK cells of the representative donor #14 were identified by HLA-A2 staining **(A)**; T cells of the representative donor #19 were identified by HLA-A1 staining **(B)**, the gating strategies are shown in **Figure 1C** and **S1**. **(A)** Representative FACS plots of one patient of donor#14 ($HLA\text{-}A2^+$; purple) and recipient#14 ($HLA\text{-}A2^-$; grey) KIR on NK cells directly after DLTx (T0), 24 hours (T24) and 3 weeks following DLTx. **(B)** Representative FACS plots of one patient of donor#19 ($HLA\text{-}A1^+$; blue) and recipient#19 ($HLA\text{-}A1^-$; grey) KIR on T cells.

Supplementary Figure 3 | Donor age might have no impact on the KIR repertoire on donor NK and T cells directly post DLTx (T0). Linear regression of donor age to **(A)** donor NK and T cell subsets (n = 39) directly post DLTx (T0) and to KIR on **(B)** donor NK cells (n = 14) and **(C)** donor T cells (n = 14) directly post DLTx (T0) is shown. Each dot represents one patient. Gating strategy for donor NK and T cells is

shown in **Figure 1C** and for KIR in **Figures 2A, E**. Statistical analysis: linear regression and correlation analysis by Pearson correlation.

Supplementary Figure 4 | Donor age may has no impact on the KIR repertoire on donor NK and T cells in perfusion solution. Linear regression of donor age to KIR on **(A)** donor NK cells (n = 9) and **(B)** donor T cells (n = 9) in perfusion solution is illustrated. Gating strategy for KIR in **Figures 2A, E**. Each dot represents one patient. Statistical analysis: linear regression and correlation analysis by Pearson correlation.

Supplementary Table 1 | Antibodies for flow cytometry. APC, allophycocyanine; BV, brilliant violet; ECD, energy coupled dye (phycoerythrin-texas red conjugate); FITC, fluorescein isothiocyanate; PB, pacific blue; PE, phycoerythrin; PerCP, peridinin-chlorophyll-protein complex.

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Chimerism-Based Tolerance to Kidney Allografts in Humans: Novel Insights and Future Perspectives

Manuel Alfredo Podestà[1] *and Megan Sykes*[2*]

[1] Renal Division, ASST Santi Paolo e Carlo, Department of Health Sciences, University of Milan, Milano, Italy, [2] Columbia Center for Translational Immunology, Department of Medicine, Department of Surgery, Department of Microbiology and Immunology, Columbia University, New York, NY, United States

Chronic rejection and immunosuppression-related toxicity severely affect long-term outcomes of kidney transplantation. The induction of transplantation tolerance – the lack of destructive immune responses to a transplanted organ in the absence of immunosuppression – could potentially overcome these limitations. Immune tolerance to kidney allografts from living donors has been successfully achieved in humans through clinical protocols based on chimerism induction with hematopoietic cell transplantation after non-myeloablative conditioning. Notably, two of these protocols have led to immune tolerance in a significant fraction of HLA-mismatched donor-recipient combinations, which represent the large majority of cases in clinical practice. Studies in mice and large animals have been critical in dissecting tolerance mechanisms and in selecting the most promising approaches for human translation. However, there are several key differences in tolerance induction between these models and humans, including the rate of success and stability of donor chimerism, as well as the relative contribution of different mechanisms in inducing donor-specific unresponsiveness. Kidney allograft tolerance achieved through durable full-donor chimerism may be due to central deletion of graft-reactive donor T cells, even though mechanistic data from patient series are lacking. On the other hand, immune tolerance attained with transient mixed chimerism-based protocols initially relies on Treg-mediated suppression, followed by peripheral deletion of donor-reactive recipient T-cell clones under antigenic pressure from the graft. These conclusions were supported by data deriving from novel high-throughput T-cell receptor sequencing approaches that allowed tracking of alloreactive repertoires over time. In this review, we summarize the most important mechanistic studies on tolerance induction with combined kidney-bone marrow transplantation in humans, discussing open issues that still need to be addressed and focusing on techniques developed in recent years to efficiently monitor the alloresponse in tolerance trials. These cutting-edge methods will be instrumental for the development of immune tolerance protocols with improved efficacy and to identify patients amenable to safe immunosuppression withdrawal.

Keywords: chimerism and tolerance, kidney, transplantation, mixed chimerism, clinical protocol

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***Correspondence:**
Megan Sykes
megan.sykes@columbia.edu

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INTRODUCTION

Renal transplantation is the established treatment of choice for kidney failure, as it confers both the highest survival and the best quality of life compared to other renal replacement therapies (1). Despite continuous advances in the field of solid organ transplantation, long-term outcomes of kidney allografts have only modestly improved in the last decades. Immunosuppressive therapies consistently control acute rejection, but have little effect on chronic rejection, which leads to graft loss in 50% of cases at 10 years (2). In addition, approximately half of the kidney transplants lost are due to death with a functioning graft: the impact of chronic immunosuppression has potentially devastating consequences in terms of cardiovascular disease, infection and malignancy (3–5), and may severely impair recipients' quality of life.

The induction of tolerance, i.e. the lack of destructive immune responses to a transplanted organ in the absence of immunosuppression, could potentially overcome both of these limitations. Tolerance in kidney transplantation can be functionally defined by stable renal function and absence of histologic, immune and molecular signs of rejection on a kidney biopsy obtained after complete withdrawal of immunosuppression for at least one year. Spontaneous tolerance is unfortunately a rare and unpredictable event that has been described in a small minority among the patients who choose to discontinue their immunosuppression, who retained graft function despite complete withdrawal of immunosuppression (6).

Among the different methods used to induce tolerance in animal models of kidney transplantation, few have been successfully translated to clinical application. Those protocols that have succeeded in patients entail combined kidney and bone marrow transplantation (CKBMT) as a strategy to induce chimerism, a state wherein donor hematopoietic cells engraft into the recipient bone marrow at a level sufficient to be detected by conventional (as opposed to sensitive PCR-based) methods.

Three centers have developed clinical CKBMT protocols, one of which has so far succeeded in achieving tolerance only in the HLA-identical transplant setting (7). Investigators from Stanford University used total lymphoid irradiation combined with anti-thymocyte globulin to facilitate the engraftment of donor hematopoietic stem cells (HSC), which were infused along with a fixed number of donor T cells after kidney transplantation. Mixed chimerism persisting for at least 6 months was achieved in 83% of the 29 HLA-matched patients treated with this protocol. Mixed chimerism was consistently associated with a tolerant state that allowed safe withdrawal of immunosuppression. Unfortunately, when a similar protocol was applied to haplotype-matched donor-recipient pairs, immunosuppressive drug weaning below therapeutic levels led to loss of chimerism and rejection episodes (8, 9).

Only two strategies have succeeded in effectively inducing operational tolerance across HLA barriers so far. As HLA mismatches are commonly present in solid organ transplantation, in this review we will discuss the features of these regimens and the novel mechanistic insights offered by recent studies in the field.

CHIMERISM-BASED PROTOCOLS FOR TOLERANCE INDUCTION ACROSS MHC BARRIERS

Full Donor Chimerism

Animal Studies. More than 60 years ago, Main and Prehn used bone marrow infusion following administration of high-dose, lethal total body irradiation (TBI) to achieve skin allograft tolerance in recipient mice. In this experimental setting, semiallogeneic but not isogenic bone marrow infusion consistently permitted donor-specific skin graft acceptance (10). Subsequent studies from Cobbold and colleagues showed that mice treated with T-cell depleting antibodies along with TBI did not reject MHC-mismatched bone marrow grafts and developed donor-specific tolerance (11). These mice exhibited full donor chimerism, i.e. the entire recipient hematopoietic system was replaced by donor cells (donor cells > 98%), so "self" tolerance of donor T cells was achieved. Later studies suggested that incomplete deletional tolerance of these recipient-reactive donor T cells was achieved, reflecting the absence of a self-renewing source of recipient APCs to ensure complete deletion of host-reactive donor T cells in the thymus. Nevertheless, functional tolerance to the recipient was achieved by a combination of mechanisms that involve thymic stromal cells, which are of recipient origin (12, 13) (**Figures 1A, B**).

Several strategies have been studied to reduce the risk of bone marrow engraftment failure and to curtail the impact of myeloablative conditioning regimens that were initially necessary to allow the engraftment of allogeneic bone marrow stem cells. Ildstad et al. reported the engraftment-promoting effects of a cell product termed "facilitating cells" (FC) in mice treated with high TBI doses. Murine FC include a population of $CD8\alpha^+$ TCR^-, but paradoxically $CD3^+$, plasmacytoid-precursor dendritic cells and also seem to include populations of B cells, NK cells, granulocytes and monocytes. Murine FCs have been reported to provide survival and homing signals to HSC, induce antigen-specific regulatory T cells (Tregs) and expand IL-10-producing Tr1 cells (14–17). These cells were also reported to be present in human bone marrow (18) and have served as the basis for the proprietary product used in the Northwestern University clinical protocol described below.

Clinical Protocols. Investigators from Northwestern University utilized a non-myeloablative conditioning regimen that achieved durable full donor chimerism in humans, attempting to exploit the engraftment-promoting and immunosuppressive effect of FC (19, 20). This regimen builds on the Hopkins protocol that uses post-transplant cyclophosphamide to inhibit GVHD across HLA barriers (21) and includes pre-transplant fludarabine, cyclophosphamide and TBI, which "make space" for HSC engraftment and control anti-donor responses that would otherwise lead to graft rejection (**Table 1**). Kidney transplantation is followed by infusion of a G-CSF +/- plerixafor-mobilized apheresis product treated to retain HSC and FC, as well as a controlled number of donor T cells. While the proprietary method for apheresis product treatment has not been disclosed, the full chimerism achieved in most of these patients, despite non-myeloablative conditioning, suggests a major role for

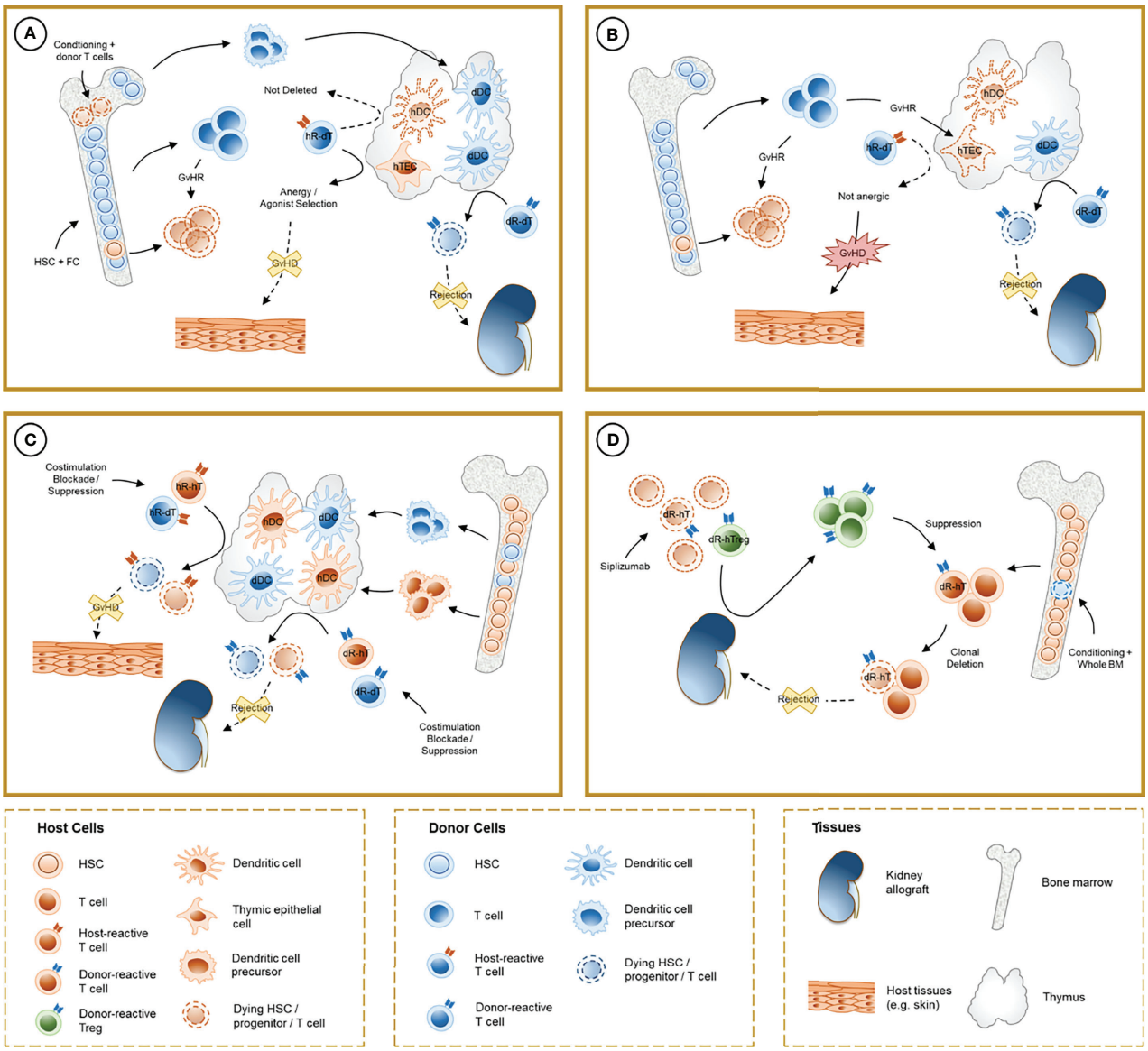

FIGURE 1 | Schematic representation of the mechanisms involved in chimerism-based tolerance to kidney allografts. **(A)** The induction of full-donor chimerism through hematopoietic stem cell (HSC) infusion along with facilitating cells (FC) after non-myeloablative conditioning results in destruction of host HSC, presumably by graft-versus-host reaction (GvHR) from infused donor T cells, and durable engraftment of donor hematopoietic precursors. After thymic repopulation by donor-derived dendritic cells (dDC), donor-reactive T cells from the donor (dR-dT) undergo clonal deletion in the thymus (central tolerance). Host-reactive donor T cells (hR-dT) are incompletely deleted, reflecting the absence of a self-renewing source of recipient APCs, but functional tolerance to the recipient may be achieved by a combination of mechanisms (anergy and selection of host-specific Tregs) that involve recipient thymic epithelial cells (hTEC). **(B)** Destruction of hTEC and thymic structure by GvHR may cause failure of negative selection and precipitate graft-versus-host disease (GVHD). **(C)** In durable mixed chimerism, donor-derived precursors populate the host thymus and differentiate into DC (dDC) without depletion of their host-derived counterparts (hDC). Donor- and host-reactive T cells from both the donor and the host undergo negative selection, allowing allograft tolerance without GVHD. Treg-mediated suppression may also play a role in experimental regimens where clonal deletion is incomplete. **(D)** In CKBMT patients receiving a siplizumab-based conditioning regimen and unprocessed bone marrow, transient mixed chimerism promotes peripheral tolerance. Host Tregs are relatively spared from global T cell depletion, and donor-reactive host Tregs (dR-hTreg) are expanded by antigenic pressure from the graft. Emerging donor-reactive T cells, which are not subjected to central deletion, are suppressed by dR-hTreg and ultimately undergo peripheral deletion over time.

GVH-reactive donor T cells in destroying recipient hematopoietic cells in the bone marrow.

Out of the 37 patients transplanted, 26 exhibited durable donor chimerism (23 developed full-donor chimerism) and were successfully weaned off immunosuppression after one year from transplant. These subjects showed significantly better kidney function compared to matched controls receiving conventional immunosuppression. Two graft losses due to opportunistic

TABLE 1 | Tolerance-inducing protocols for kidney transplantation across MHC barriers.

	Northwestern University	Massachusetts General Hospital	Samsung Medical Center
Type of Chimerism	Durable Full-Donor	Transient Mixed	Transient Mixed
Donor Cells	G-CSF-mobilized HSC (up to ~17×10^6/Kg) + T cells (~4×10^6/Kg) + FC (0.5-12×10^6/Kg). Infused at +1.	Whole BM (2–3×10^8/Kg)	Whole BM (0.6-2.2×10^8/Kg, with HSC 0.8-3.2×10^6/Kg)
Conditioning (KTx day 0)	FLU (30mg/m^2, -5/-4/-3), CYC (50mg/Kg, -3/+3), TBI (200 cGy, -1)	(*NKD03*) CYC (60mg/Kg, -5,-4), siplizumab (0.6mg/Kg, -2/-1/0/+1), TI (700 cGy, -1). (*mNKD03*) NKD03 + ritux (375mg/m2, -7/-2) + pred from 0 to +10 (*ITN036*): NKD03, + ritux (375mg/m2, -7/-2/+5/+12) + pred from 0 to +20 (*TBI-Pilot*): ITN036 + TBI (1.5 Gy, -5/-4) instead of CYC.	(*Protocol-1*) mNKD03 + rATG (1.5 mg/Kg, -1/0/+1) instead of siplizumab + pred up to 3-6 months (*Protocol-2*) Protocol-1 + FLU (15 mg/m2, -6/-5/-4/-3) + rATG (1.5 mg/Kg, +2) (*Protocol-3*) Protocol-1 + FLU (10 mg/m2, -6/-5/-4/-3) + SIR (from month 1) instead of TAC.
Maintenance IS	TAC tapered after 1-year protocol biopsy.	CYA (NKD03 and mNKD03) or TAC (ITN036 and TBI-Pilot) tapered after 6-month protocol biopsy.	TAC (or SIR) tapered after protocol biopsy at 1 year. pred tapered and discontinued at 3-6 months.
Tolerant patients (> 1 year off IS)/transplanted patients	26/37	7/10	5/8
Fatal SAEs	3	0	0
GVHD cases	2	0	0
Graft losses	2	6 (3 after > 10 years)	2

BM, bone marrow; CYC, cyclophosphamide; FC, facilitating cells; FLU, fludarabine; HSC, hematopoietic stem cells; G-CSF, granulocyte colony stimulating factor; pred: prednisone; rATG, rabbit anti-thymocyte globulins; ritux, rituximab; SIR, sirolimus; TAC, tacrolimus; TBI, total-body irradiation; TI, thymic irradiation.

infections were recorded in the first year after transplantation, and one tolerant recipient died due to sepsis. Studies in mice have highlighted that full donor chimeras are somewhat immunoincompetent (22, 23) due to the absence of recipient APCs in the periphery, which are needed to optimally present antigens to T cells that are positively selected by recipient thymic epithelium. Indeed, cytotoxic T cells generated in chimeric mice lacking shared MHC alleles between the donor and recipient are unable to clear virally infected donor cells (24), which thereby serve as a viral reservoir that can result in chronic illness (23). While viral reactivation and other opportunistic infections occurred quite frequently in patients on this study, patients with full donor chimerism nevertheless could be successfully vaccinated after immune cell reconstitution, likely reflecting, at least in part, persistence of immune memory and immunity carried by donor T cells in the hematopoietic cell transplant (25). Additional complications included acute rejection in two patients with transient chimerism that were non-compliant with medications, and one death due to lung cancer. A potentially alarming toxic effect was recorded after a longer observation period: despite the use of post-transplantation cyclophosphamide, two subjects ultimately developed graft-versus-host disease (GVHD). One patient was diagnosed with grade 3 intestinal GVHD and CMV infection that led to a fatal outcome. Although relatively limited in frequency (5% of treated patients), the risk of GVHD in our view outweighs the benefits obtained with approaches based on full donor chimerism for tolerance induction.

Mixed Chimerism

Animal Studies. Mixed chimerism defines a state wherein, unlike full donor chimerism, the host hematopoietic system is not completely destroyed and replaced by the donor's, and hematopoietic cells of both the recipient and the donor coexist in the bone marrow.

Sharabi and Sachs demonstrated that durable mixed chimerism and tolerance could be induced in mice conditioned with T cell-depleting antibodies, low-dose TBI and thymic irradiation (TI) (26). This method overcomes peripheral and intra-thymic rejection (27) of donor HSCs and facilitates bone marrow engraftment, which in turn provides a durable supply of progenitors that migrate to the thymus, differentiating into lymphocytes and dendritic cells. Tolerance to donor and recipient in these models is achieved *via* intra-thymic negative selection of alloreactive T cell clones, mediated by both donor- and recipient-derived antigen-presenting cells (28, 29) and regulatory mechanisms are notably absent in the long-term tolerance maintenance phase (30). Durable mixed chimerism can also be achieved through co-stimulation blockade combined with bone marrow transplantation, which resulted in anergy and peripheral deletion of donor-reactive clones (31–33). Peripheral tolerance of donor-reactive CD4 and CD8 T cells relied on distinct mechanisms, with a role for NFAT, LAG3, TGFβ, PD1 and recipient CD4 T cells, B cells and MHC class II for the CD8 T-cell anergy followed by deletion (34–38) and a pathway involving CD4 T cell-intrinsic CTLA4 and recipient CD80 and CD86 without regulatory mechanisms, leading to peripheral CD4 cell deletion (32, 39). The caspase 9-dependent intrinsic and cell-extrinsic Fas-FasL apoptosis pathways have both been implicated in clonal deletion in these models (40, 41). Notably, alternative mixed chimerism-based regimens that do not achieve complete deletion of donor-reactive T cells also rely on alloreactive Treg-mediated suppression to induce donor-specific tolerance (42, 43) (**Figure 1C**).

Before human application, non-myeloablative conditioning regimens for the induction of allograft tolerance were tested in

non-human primates [extensively reviewed in (44)], a key step to assess the safety and efficacy of these protocols. These experiments underscored that the rate of success and the stability of chimerism induction in primates is considerably lower compared to rodents, partly due to the higher abundance of memory T cells in the former, which are more resistant to conventional T cell-depleting agents (45). The addition of splenectomy (or co-stimulation blockade) and a short course of cyclosporine could partially overcome this barrier in a significant fraction of animals, but mixed chimerism was only transient in all of them (46, 47). Contrary to initial assumptions, tolerance to renal allografts developed in more than 60% of recipients, providing the first proof of principle that durable chimerism is not essential for tolerance induction in primates, thus paving the road to human translation.

Clinical Protocols. Mixed chimerism-based approaches to induce tolerance to kidney allografts have been tested at the Massachusetts General Hospital (MGH) in patients with and without hematologic malignancies. Differences between these regimens have been reviewed in detail elsewhere (48), and we will focus our current discussion on patients without malignancy, as these protocols have the highest potential for translation to routine clinical practice in the future.

Initial studies used a non-myeloablative conditioning regimen that included cyclophosphamide, the anti-CD2 T cell-depleting monoclonal antibody siplizumab and thymic irradiation (TI) (**Table 1**) (49, 50). Unprocessed donor bone marrow was infused on the day of kidney transplantation, and subjects also received calcineurin inhibitors and a short course of corticosteroids postoperatively. Pre- and peri-transplant rituximab doses were introduced after evidence of antibody-mediated rejection in one patient and *de-novo* DSA development in 2 additional patients. After this modification, all patients remained immunosuppression-free for the duration of the study. Transient mixed chimerism for up to 3 weeks was induced in all recipients, without evidence of GVHD. Maintenance immunosuppressive drugs were slowly tapered after 6 months in patients with normal protocol biopsy, and the primary endpoint of 24-month immunosuppression-free kidney allograft survival was achieved in 7 of the 10 patients enrolled. Three of these subjects later (at 4 to 7 years post-transplant) experienced chronic rejection or glomerulonephritis recurrence, which led to reintroduction of immunosuppressive drugs and ultimately resulted in graft loss more than 10 years after transplantation. Of note, these patients were successfully retransplanted with conventional immunosuppression, and there were no significant opportunistic infections in any of them.

In parallel with early host T cell recovery, 9 patients unexpectedly developed severe acute kidney injury. Renal histology was consistent with engraftment syndrome, entailing capillary endothelial injury with vascular leak and lympho-monocytic infiltrating cells in peritubular and glomerular capillaries. Renal function normalized in all but 2 recipients, one of whom experienced graft loss due to acute humoral rejection as a consequence of preformed DSA that were undetectable on a pre-transplant ELISA, but were subsequently confirmed by Luminex. In the other patient, acute kidney injury was initially misdiagnosed as rejection and was treated with higher doses of tacrolimus, which triggered thrombotic microangiopathy. Finally, one patient developed severe cellular rejection after a pyelonephritis episode following immunosuppression withdrawal. Protocol biopsies (at 2-8 years) in tolerant subjects showed either completely normal histology or minimal alterations, including focal glomerular basement membrane duplication and mild podocyte foot process effacement (50).

An additional protocol was tested at MGH based on further observations from studies conducted in non-human primates (46, 51). Compared to previous regimens, cyclophosphamide was substituted with TBI to prevent engraftment syndrome. Renal function remained stable in the two patients enrolled, but one did not develop sufficient chimerism to allow immunosuppression weaning. Immunosuppressive drugs were successfully discontinued in the other patient, but were resumed after more than 4 years due to evidence of humoral rejection on a protocol biopsy (52).

Investigators at the Samsung Medical Center initially used a nearly identical protocol to those outlined above, but the anti-CD2 monoclonal antibody siplizumab was substituted with ATG due to local unavailability (52, 53). To curtail the risk of engraftment syndrome, fludarabine and an additional dose of ATG were added in a second protocol iteration, which allowed reduction of the dose of cyclophosphamide. Due to development of BK nephritis, ATG and fludarabine dose was subsequently decreased, and tacrolimus was substituted with sirolimus one month after transplantation. Overall, mixed chimerism was achieved transiently (at least 3 weeks) in all 8 enrolled subjects. Immunosuppression was successfully discontinued for more than one year in 5 patients, even though one of them experienced acute cellular rejection after a respiratory tract infection, which led to reintroduction of tacrolimus.

MECHANISTIC STUDIES IN HUMANS AND METHODS TO TRACK TOLERANCE

Full Donor Chimerism

The mechanism that underlies tolerance to kidney allografts associated with full-donor chimerism hypothetically involves central tolerance of donor T cells to donor antigens, with donor progenitor cells migrating to the recipient thymus, differentiating into antigen-presenting cells and finally mediating negative selection of "self"-reactive donor T cell clones. Bulk functional assays, including mixed-lymphocyte reactions (MLR) and cell-mediated lympholysis (CML), demonstrated donor-specific hyporesponsiveness in tolerant patients. However, the same effect was observed in recipients who exhibited only transient chimerism and developed rejection after immunosuppression withdrawal (54), suggesting that these assays cannot be relied upon to infer a tolerant state. On the other hand, development of full donor chimerism was the single most accurate predictor of tolerance in these patients (54). An intra-graft signature of tolerance was also described for these

patients, which was characterized by upregulation of genes involved in B cell regulation and pro-tolerogenic plasmacytoid DC enrichment, as well as the induction of regulatory pathways involved in the control of inflammation and maintenance of tissue homeostasis (55). Overall, however, studies elucidating the mechanism of tolerance in these subjects are currently lacking.

Given the full donor chimerism achieved in these patients, the achievement and mechanism of GVH tolerance is also worthy of investigation. Studies in mouse models discussed above would suggest that *de novo* GVH tolerance might be characterized by a combination of clonal deletion, anergy and regulatory T cell-mediated mechanisms. However, GVH tolerance has not been demonstrated in these patients and the inclusion in the infused product of mature donor T cells that eliminate host hematopoiesis suggests that an ongoing GVH reaction may occur, which has culminated in GVHD in several patients. Whether or not GVH reactions in patients without overt GVHD results in thymic injury and failure to negatively select host-reactive T cells, as reported in murine models (56–59), has not been investigated.

Mixed Chimerism

The mechanisms of tolerance in protocols based on transient mixed chimerism have been the topic of extensive studies in recent years. Central tolerance is unlikely to be the main mechanism operating in these CKBMT patients, since transient chimerism is likely insufficient to allow long-term thymic repopulation with donor antigen-presenting cells.

Preliminary studies with bulk functional assays were partly inconclusive, since a lack of post-transplant donor-specific responses was observed both in tolerant patients and in the patient who developed acute rejection after immunosuppression withdrawal in the MGH trial (60). Several mechanisms, including T cell anergy and peripheral deletion, could underlie the observed donor-specific hyporesponsiveness, but these assays could not discriminate between them. Nonetheless, these results were extremely informative when compared with those from recipients of bone marrow transplantation conditioned with a similar regimen but without kidney transplantation. In these subjects, donor-specific reactivity reappeared after chimerism was lost, indicating that the kidney allograft is likely to play a pivotal role in tolerance development in CKBMT recipients (61).

The advent of platforms to perform high-throughput sequencing of the TCRβ CDR3 hypervariable region led to the development of novel tools to analyze the T cell alloresponse. We hypothesized that a significant fraction of the donor-reactive repertoire could be identified in a pre-transplant MLR, by sequencing sorted recipient T cells that divided in response to donor stimulation. These sequences were compared with those of sorted unstimulated recipient $CD4^+$ and $CD8^+$ T cells to define a fingerprint of the anti-donor T cell repertoire. Thresholds for detection were based on a uniform clonal frequency (to normalize for sample size variability over time) and on a minimal fold-expansion (to avoid capturing highly abundant but not specifically donor-reactive clones), while computational methods were used to account for sorting errors (62). This fingerprint was then longitudinally compared with samples obtained at different post-transplant time points to track circulating donor-reactive clones over time. Both tolerant and non-tolerant patients, as well as kidney transplant recipients under conventional immunosuppression, had considerable repertoire turnover, reflecting the use of T cell depleting agents in the conditioning regimens. However, all tolerant patients analyzed displayed a progressive and specific reduction in both donor-reactive $CD4^+$ and $CD8^+$ T cell clones, whereas no significant change was identified in the non-tolerant patient (60), and conventional kidney transplant recipients showed expansion of $CD4^+$ T clones. These results suggest that clonal deletion is involved in the development of tolerance and may serve as a marker to identify patients amenable to safe immunosuppression weaning. Conversely, T cells in the non-tolerant patient were probably anergic, but were re-activated after immunosuppression withdrawal by the infective episode, thus precipitating acute rejection.

The existence of a suppressive mechanism in these patients was initially suggested by re-emergence of anti-donor responses in bulk functional assays performed with Treg-depleted samples from the first post-transplant year. However, samples obtained at later time points failed to show a similar response, suggesting that suppression could be relevant only as an early mechanism (63). Consistent with this hypothesis, limiting dilution assays conducted after the first post-transplant year failed to show an increase in response at higher dilution, which usually indicates the presence of suppressive cells at a lower frequency than responder cells (60).

Phenotypic analysis of circulating mononuclear cells in tolerant patients identified an early expansion of Tregs (80% of $CD4^+$ T cells during the first week) with evidence of peripheral proliferation, possibly recent thymic emigration and, in one patient, conversion from conventional T cells (64). Expression of CD45RA declined after two weeks from transplant (64), suggesting that previously resting Tregs acquired an activated phenotype (65). The presence of a highly demethylated FoxP3 Treg specific region, an epigenetic hallmark of stable Tregs, confirmed the results from phenotypic data.

Subsequent studies demonstrated that the anti-CD2 monoclonal antibody siplizumab could induce costimulation blockade and T cell depletion, but selectively spared Tregs and promoted the expansion of alloreactive Tregs *in vitro* (66). *In vivo*, this process may be further amplified by the lymphopenia-driven expansion state that follows global T cell depletion. Interestingly, siplizumab predominantly reduced the frequency of effector memory T cells, which express the highest CD2 levels among T cell subsets (66, 67). This additional effect may be relevant for tolerance induction, since cross-reactive memory T cells are abundant in humans, and constitute a barrier to the establishment of chimerism and tolerance. Indeed, these cells are more resistant to depletion with ATG, depend less on costimulatory signals and are less susceptible to Treg-mediated suppression (68).

By using the same sequencing approach detailed above, we interrogated donor-reactive sequences that mapped to the unstimulated sorted Treg pool, but these sequences were

detected at a very low frequency due to the low numbers of Tregs in the circulation. The method was therefore optimized by expanding the donor-reactive Treg pool with activated donor B cells instead of performing a conventional MLR. Expansion of donor-specific Tregs with activated donor B cells greatly increased the number of unique donor-specific Treg sequences identified and the specificity and potency of these cells in suppressing anti-donor responses was markedly increased, demonstrating that truly donor-specific Tregs were enriched in this repertoire. Using this method of pre-transplant donor-specific Treg repertoire identification, tolerant patients were found to display significant expansion of donor-specific Tregs at 6 months from transplantation, while the single non-tolerant subject did not (69). This study also showed that the majority of expanded Tregs in tolerant subjects mapped to the pre-transplant unstimulated Treg pool rather than conventional T cells, suggesting that expansion of pre-existing Tregs rather than induction of donor-specific Tregs was the major mechanism for increased donor-specific Tregs in these patients.

Overall, these data indicate a central role for early Treg-mediated suppression in the development of tolerance in combined kidney bone marrow transplantation. It could be speculated that prolonged stimulation of donor-reactive T cell clones by graft antigens under constant restraint by Tregs might mediate anergy and subsequent peripheral deletion of these cells. This suppressive effect loses potency over time as gradual clonal deletion of donor-reactive T cells eliminates the alloresponse needed to maintain expanded donor-specific Treg populations (**Figure 1D**).

FUTURE PERSPECTIVES

Even though tolerance induction has been achieved in humans through chimerism development, these regimens still need to be refined before they can be translated to routine clinical practice. We believe that the ultimate aim will be to develop a protocol capable of reproducibly inducing tolerance through durable mixed chimerism.

Albeit progressively refined over the course of the last decades, conditioning regimens still bear potentially significant systemic toxicity, which results in both short- and long-term clinically relevant complications. The development of costimulation blockers and other novel drugs targeting specific cell populations and molecular moieties could help to refine conditioning regimens further, thus limiting side effects. Avoidance of engraftment syndrome observed in current regimens represents a realistic short-term goal, which may be achieved with revised protocols in the near future. Studies in animal models and humans have outlined that several mechanisms for tolerance coexist, and future strategies may exploit this knowledge to induce a more robust tolerant state. A future, intriguing possibility to promote durable chimerism without increasing the risk of GVHD is represented by peri-transplant infusion of *ex-vivo* expanded recipient Tregs. Administration of polyclonal Tregs was able to induce mixed chimerism in mice in the absence of cytoreductive therapy (70), and promoted more durable mixed chimerism and tolerance, that permitted delayed kidney transplantation without immunosuppression, in primates treated with non-myeloablative conditioning (71).

Reproducibility in humans remains a key issue of translational research in transplantation, especially in the context of tolerance trials, where a universally accepted and validated biomarker of the tolerant state has been lacking so far. The newly developed methods based on TCR sequencing to track donor-reactive T cell/Treg clones deserve further exploration as a tool that may be useful for the identification of patients amenable to safe immunosuppression withdrawal in a personalized manner. Furthermore, this approach has considerable potential to further identify the role of and elucidate mechanisms of host-vs-graft and graft-vs-host reactivity and tolerance, respectively, in recipients of hematopoietic cell transplantation for the purpose of allograft tolerance induction.

Tolerance studies will be also pivotal to pave the way to clinical xenotransplantation, considered to be the next frontier in solid organ transplantation due to its potential to overcome the severe shortage of human organs. Murine models have shown that mixed chimerism induction can promote tolerance to xenografts through several concomitant mechanisms, including deletion of xenoreactive B cells (72–75) with disappearance of natural antibodies to xenoantigens, as well as tolerization of xenoreactive T (76) and NK cells (77). These results have been replicated by induction of porcine mixed chimerism in immunodeficient mice with human immune systems (78–81). However, immune barriers to xenogeneic mixed chimerism induction are considerably greater than those to allogeneic chimerism, particularly due to the rapid destruction of porcine cells by human macrophages (82, 83), which can be at least partially overcome by the introduction of a human CD47 transgene into the pig (84–86). Current protocols will need to be optimized before clinical translation can be safely attempted.

AUTHOR CONTRIBUTIONS

MP and MS jointly wrote the first draft of the manuscript and revised its content critically. All authors contributed to the article and approved the submitted version.

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Successful Induction of Specific Immunological Tolerance by Combined Kidney and Hematopoietic Stem Cell Transplantation in HLA-Identical Siblings

Thomas Fehr[1,2†], Kerstin Hübel[1,3†], Olivier de Rougemont[3], Irene Abela[4], Ariana Gaspert[5], Tayfun Güngör[6], Mathias Hauri[6], Birgit Helmchen[5], Claudia Linsenmeier[7], Thomas Müller[1], Jakob Nilsson[8], Oliver Riesterer[7], John D. Scandling[9], Urs Schanz[10‡] and Pietro E. Cippà[1,11‡]*

[1] Division of Nephrology, University Hospital Zurich, Zurich, Switzerland, [2] Department of Internal Medicine, Cantonal Hospital Graubuenden, Chur, Switzerland, [3] Department of Surgery and Transplantation, University Hospital Zurich, Zurich, Switzerland, [4] Institute of Medical Virology, University of Zurich, Zurich, Switzerland, [5] Department of Pathology, University Hospital Zurich, Zurich, Switzerland, [6] Division of Stem Cell Transplantation, University Children's Hospital Zurich – Eleonore Foundation & Children`s Research Center (CRC), Zurich, Switzerland, [7] Department of Radiation Oncology, University Hospital Zurich, Zurich, Switzerland, [8] Laboratory for Transplantation Immunology, University Hospital Zurich, Zurich, Switzerland, [9] Division of Nephrology, Stanford University School of Medicine, Stanford, CA, United States, [10] Department of Medical Oncology and Hematology, University Hospital Zurich, Zurich, Switzerland, [11] Division of Nephrology, Ente Ospedaliero Cantonale, Lugano, Switzerland

Induction of immunological tolerance has been the holy grail of transplantation immunology for decades. The only successful approach to achieve it in patients has been a combined kidney and hematopoietic stem cell transplantation from an HLA-matched or -mismatched living donor. Here, we report the first three patients in Europe included in a clinical trial aiming at the induction of tolerance by mixed lymphohematopoietic chimerism after kidney transplantation. Two female and one male patient were transplanted with a kidney and peripherally mobilized hematopoietic stem cells from their HLA-identical sibling donor. The protocol followed previous studies at Stanford University: kidney transplantation was performed on day 0 including induction with anti-thymocyte globulin followed by conditioning with 10x 1.2 Gy total lymphoid irradiation and the transfusion of CD34+ cells together with a body weight-adjusted dose of donor T cells on day 11. Immunosuppression consisted of cyclosporine A and steroids for 10 days, cyclosporine A and mycophenolate mofetil for 1 month, and then cyclosporine A monotherapy with tapering over 9–20 months. The 3 patients have been off immunosuppression for 4 years, 19 months and 8 months, respectively. No rejection or graft-versus-host disease occurred. Hematological donor chimerism was stable in the first, but slowly declining in the other two patients. A molecular microscope analysis in patient 2 revealed the genetic profile of a normal kidney. No relevant infections were observed, and the quality of life in all three patients is excellent. During the SARS-CoV-2 pandemic, all three patients were vaccinated with the mRNA vaccine BNT162b2

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Nina Pilat,
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Tecnológico de Monterrey, Mexico

***Correspondence:**
Thomas Fehr
thomas.fehr@uzh.ch

[†]These authors have contributed equally and share first authorship

[‡]These authors have contributed equally and share last authorship

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(Comirnaty®), and they showed excellent humoral and in 2 out 3 patients also cellular SARS-CoV-2-specific immunity. Thus, combined kidney and hematopoietic stem cell transplantation is a feasible and successful approach to induce specific immunological tolerance in the setting of HLA-matched sibling living kidney donation while maintaining immune responsiveness to an mRNA vaccine (ClinicalTrials.gov: NCT00365846).

Keywords: chimerism, hematopoietic stem cell transplantation (HSCT), tolerance, kidney transplantation, immunocompetence, COVID - 19

1 INTRODUCTION

Kidney transplantation is the primary option for treatment of end stage renal failure in patients without contraindication for life-long immunosuppression. Since kidney allograft survival early after transplantation has substantially improved, the focus of research and clinical care has turned to improving long-term patient and allograft survival (1). Under long-term immunosuppression, patient survival is shortened due to neoplastic, infectious, and cardiovascular complications, whereas allograft survival is limited due to chronic rejection, drug toxicity, infections (such as BK virus nephropathy) or unspecific allograft injury and fibrosis. All these complications could either be controlled, reduced or completely avoided if successful immunologic tolerance was induced (2).

In pre-clinical models, various approaches have been successfully tested to induce tolerance to fully mismatched allografts, including co-stimulation blockade, donor-specific transfusion, or transfer of different types of regulatory cells (such as regulatory T cells, macrophages or tolerogenic dendritic cells). However, the only approach that was successfully translated into non-human primate models and clinical studies relies on hematopoietic stem cell transplantation (HSCT) leading to mixed lymphohematopoietic chimerism and transplantation of a kidney from the same donor (3).

Three groups in the United States have independently developed protocols to achieve this goal, using various conditioning regimens, stem cell preparations and timings (pre- versus post-kidney transplant conditioning) (4–6). An overview of these approaches is shown in **Supplementary Table 1**. Only the group in Stanford established a protocol that uses post-kidney transplant conditioning and HSCT, which theoretically allows to translate this approach also to deceased donor transplantation. Therefore, we decided to implement a similar protocol for the first trial of combined kidney transplantation and HSCT in Europe. Here we report the results of the first three patients enrolled in this trial (swisstolerance.CH).

2 METHODS

2.1 Trial Design

This is an open-label feasibility study of combined HLA-matched (10/10; Loci A/B/C/DR/DQ) sibling kidney and hematopoietic stem cell transplantation to induce donor-specific immunological tolerance to the kidney allograft.

The *primary endpoint* of the study was renal allograft acceptance and ability to discontinue immunosuppressive therapy at 1 year.

Secondary endpoints were engraftment of donor hematopoietic stem cells (chimerism) measured at 6 months, absence of graft-versus-host disease (GVHD) after 6 and 12 months, absence of renal allograft rejection at 6 and 12 months, T cell recovery and immune reconstitution, absence of opportunistic infections (immune competence) and quality of life.

Chimerism is defined as evidence of donor-derived hematopoietic cells in peripheral blood measured by Variable number tandem repeats (VNTR).

2.2 Patient Population

2.2.1 Inclusion Criteria

All patients aged 18-70 with end-stage renal failure under evaluation for kidney transplantation at the University Hospital Zurich were considered for this clinical trial. Subjects had to have an HLA-matched sibling donor 18-70 years of age and be able to understand and provide informed consent.

2.2.2 Exclusion Criteria

The following exclusion criteria were applied:

- Evidence of uncontrolled active infection (including replicating HIV, HCV and HBV), serologic positivity to HIV
- Contraindication to therapy with any one of the proposed agents
- Women of childbearing age in whom adequate contraception could not be maintained, pregnant women or nursing mothers.
- Malignancy within the past two years, for which waiting time for transplantation is required by Israel Penn Registry consult, thereby excluding non-melanoma skin cancer and carcinoma *in situ* of the cervix.
- Relevant liver, cardiac or pulmonary disease
- ABO blood group incompatibility in the host-vs-graft direction (major incompatibility)
- Panel reactivity antibody >20%.
- Very high risk of primary kidney disease recurrence (mainly focal segmental glomerulosclerosis or atypical hemolytic uremic syndrome)

2.2.3 Patient Recruitment

The kidney transplant center in Zurich performs approximately 90 kidney transplants per year, among those around 25 living donations. Every kidney transplant candidate is systematically evaluated for a potential living donation. If a candidate had a

potential sibling donor, they were introduced to the concept and the protocol of the swisstolerance.CH trial. Between 2016 until 2020, eight HLA-identical sibling pairs were evaluated, and 3 were included into this pilot trial. The other five pairs were excluded either due to ABO incompatibility, or the donor had contraindications to living donation. Among those, three pairs were transplanted regularly outside the protocol.

2.3 Study Protocol

2.3.1 Interventions Before Transplantation

Donor and recipients were screened according to the established internal guidelines for living donor kidney and HSCT of the Transplantation Center of the University Hospital Zurich. For the planning of the total lymphoid irradiation (TLI) a planning CT was performed 2-4 weeks before transplantation and repeated on the day after kidney transplantation to shield the transplanted kidney.

Donor-derived hematopoietic progenitor cells were isolated from peripheral leukocytes after apheresis by positive selection (CD34+ cells) *via* magnetic cell sorting (CliniMACS, Miltenyi®, Germany) according to SOPs of the certified Stem cell laboratory of the University Children's Hospital Zurich. CD34-negative cells were analyzed by flow cytometry to determine the number of CD3-positive cells (for T cell add-back). CD34+ cells and flow through cells were frozen in liquid nitrogen until use.

2.3.2 Kidney Transplantation (Day -11)

Living kidney donation (laparoscopic approach) and transplantation were performed according to standard procedures. Immunosuppression in the first weeks after kidney transplantation included prednisone, mycophenolate mofetil and cyclosporine A (details of dosing: see 3.3.4). In addition, rabbit anti-thymocyte globulin (ATG, Thymoglobulin®) at a dose of 1.5 mg per kg body weight was applied from day -11 to day -7 (**Figure 1**).

2.3.3 Hematopoietic Stem Cell Transplantation (HSCT, Day 0)

The conditioning regimen consisted of total lymphoid irradiation (10 daily doses of 120 cGy = total dose 12 Gy) each to the supradiaphragmatic lymph nodes, thymus, subdiaphragmatic lymph nodes and spleen. The treatment started 1 day after kidney transplantation (d-10).

On day 0, the isolated CD34+ hematopoietic progenitor cells (≥4x10^6 cells/kg of the recipient`s body weight) were thawed an infused together with 1x10^6 CD3+ T cells/kg from the CD34- fraction to promote the engraftment of hematopoietic progenitor cells (T cell add-back)

2.3.4 Immunosuppression and Anti-Microbial Prophylaxis

Immunosuppression post-transplant was guided as follows:

- Methylprednisolone/Prednisone: steroids were rapidly tapered during the first days after transplantation. All patients were off steroids 14 days after kidney transplantation.
- Mycophenolate mofetil: 2 g per day (in 2 doses), started at day 0 (4 to 6 hours after HSCT) and discontinued 1 month after HSCT.
- Cyclosporine A: first 6 months whole blood through level (C0) 250-300 µg/L, after 6 months cyclosporine was tapered and discontinued if the following criteria were fulfilled: Sustained chimerism for at least 180 days, no clinical signs of rejection, protocol biopsy showing no evidence of acute or chronic rejection, no clinical signs of GvHD.
- Anti-microbial prophylaxis was performed as follows: Amoxicillin/clavulanic acid 2.2 g preoperatively; Sulfamethoxazole/Trimethoprim 3x/week for 6 months; Valganciclovir: a) low risk (D-R-) – no prophylaxis; b) intermediate risk (R+) – prophylaxis with valganciclovir 450 mg once daily, starting one month post-kidney transplant; c) high risk (D+R-) – prophylaxis with valganciclovir 450 mg once daily, starting immediately after kidney transplant.

2.3.5 Post-Transplant Monitoring

Standard follow-up procedures for living kidney donors and recipients as established in the transplant center in Zurich were applied. In addition, during immunosuppression tapering and in the first months off immunosuppression renal function was weekly monitored for an early detection of rejection episodes. GVHD was monitored clinically at each regular visit as well as by measurement of liver function tests.

Donor chimerism level in peripheral blood was regularly assessed. Immune reconstitution was analyzed by flow cytometry of peripheral blood leukocytes.

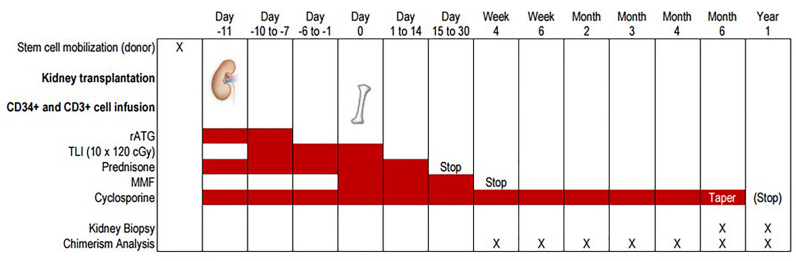

FIGURE 1 | Overview over the trial protocol. Schematic overview over the trial protocol showing the timing of kidney and hematopoietic stem cell transplantation, immunosuppressive medication and monitoring with peripheral blood chimerism analyses and allograft biopsies.

Kidney allograft biopsy was performed at 6 months post-transplant and immediately before full withdrawal of cyclosporine A.

2.3.6 Assessment of SARS-CoV-2-Specific Immunity

During the COVID-19 pandemic, all three trial patients were vaccinated against SARS-CoV-2 with the mRNA vaccine BNT162b2 (Comirnaty®, Pfizer/BioNTech), and the SARS-CoV-2-specific antibody and T cell responses were assessed several months after vaccination.

The SARS-CoV-2 antibody response was assessed by the commercially available ELISA assay (Elecsys®, Roche). To assess the neutralizing capacity of these antibodies, an additional assay developed by the Institute of Medical Virology (IMV, University of Zurich) was used (ABCORA®).

The SARS-CoV-2 T cell response was assessed with an *in vitro* T cell stimulation assay established in the laboratories of the Division of Clinical Immunology (University Hospital of Zurich). As a positive control, the T cell responses against a mitogen (Concanavalin A) and a bacterial superantigen (Staphylococcus aureus SEA/SEB) were evaluated. To assess the specific anti-viral T cell response, the SARS-CoV-2 spike protein and a CMV protein were used as antigens.

2.4 Ethical Approval and Trial Registration

This pilot trial was approved by the Ethical Committee for clinical research of the Canton of Zurich (KEK_ZH, application No 2013-0603). The trial is registered at ClinicalTrials.gov (Identifier NCT02176434).

3 RESULTS

3.1 Individual Patients

3.3.1 Patient 1

Our first patient was a 57-year-old Caucasian woman with end stage renal disease due to a glomerulopathy, that could not be specified at the time of diagnosis (**Table 1**). She received a preemptive kidney transplant from her 53-year-old HLA-identical brother. Immunosuppression followed the standard trial protocol (including ATG, cyclosporine, mycophenolate mofetil and steroids) during the first month, which was then continued with cyclosporine A monotherapy starting at month 2. Two kidney biopsies were performed according to protocol, the first at month 6, the second before discontinuation of cyclosporine at months 11 (**Supplementary Table 1**). Cyclosporine was gradually tapered after the first biopsy which did not show any signs of rejection, and it was discontinued after the second biopsy eleven months after transplantation (**Supplementary Figure 1**), still without signs of rejection. Whole blood donor chimerism at that time was around 50%.

The patient was never re-hospitalized after transplantation. Her first year was characterized by only few medical problems. She developed a calcineurin inhibitor pain syndrome (CIPS) with typical features on bone scintigraphy, 3 months after transplantation. It was well controlled with analgesics and disappeared when cyclosporine A was reduced and then stopped according to the trial protocol. One urinary tract infection, treated with antibiotics, occurred ten months after transplantation. Furthermore, asymptomatic low-level cytomegalovirus (CMV) reactivation was observed, which did not require treatment (maximum titer: 600 IU/mL). The patient returned to work two and a half months after transplantation.

Around one-year post-transplant, a low-level albuminuria was observed, which increased to about 1g/d by the end of the second year (**Figure 2**). The third kidney biopsy was performed 18 months post-transplant and revealed a primary glomerulonephritis (recurrent or *de novo*), which could not be further classified. The patient was treated for 10 weeks with mycophenolate mofetil, which however had no effect on proteinuria and was stopped due to gastrointestinal side effects. The patient was then switched to aliskiren (7).

The patient is now in her fifth year after transplantation with stable kidney function and albuminuria around 2 g/d.

3.3.2 Patient 2

The second patient was a 61-year-old Caucasian woman with end stage renal disease due to a not further specified

TABLE 1 | Patient and transplant characteristics.

Patient No		Patient 1	Patient 2	Patient 3
Recipient	Age, sex	57, F	61, F	49, M
	Renal disease	GN, unknown	GN, unknown	ADPKD
	Other diseases	M. Meniere	Breast cancer (9y before transplantation) Osteoporosis Neuroborreliosis	Nephrolithiasis
	Dialysis	None, preemptive transplant	Peritoneal dialysis (33 months)	Peritoneal dialysis (4 months)
	Transplant No	First	First	First
Donor	Age, sex	53, M	54, F	46, F
HLA typing	HLA class I	A1, A2; B8; B64(16); Cw7, Cw8	A2, -; B7, B62(15); Cw9; Cw10	A1, A3; B7, B8; Cw7, -
	HLA class II	DR17(3), DR7; DR52, DR53; DQ2, -; DP1, DP4	DR15(2); DR 13(6); DR51, DR52; DQ6(1), -; DP2, -	DR17(3); DR12(5); DR52, -; DQ2, DQ7; DP4, -
HSCT	No of CD34+ cells	8.78x10^6/kg BW CD34+	5.61x10^6/kg BW CD34+	12.3x10^6/kg BW CD34+
	No of CD3+ cells (T cell add-back)	1x 10^6/kg BW CD3+	1x 10^6/kg BW CD 3+	1x10^6/kg BW CD3+

ADPKD, adult polycystic kidney disease; BW, body weight; F, female; GN, glomerulonephritis; HSCT, hematopoietic stem cell transplantation; M, male; No, number.

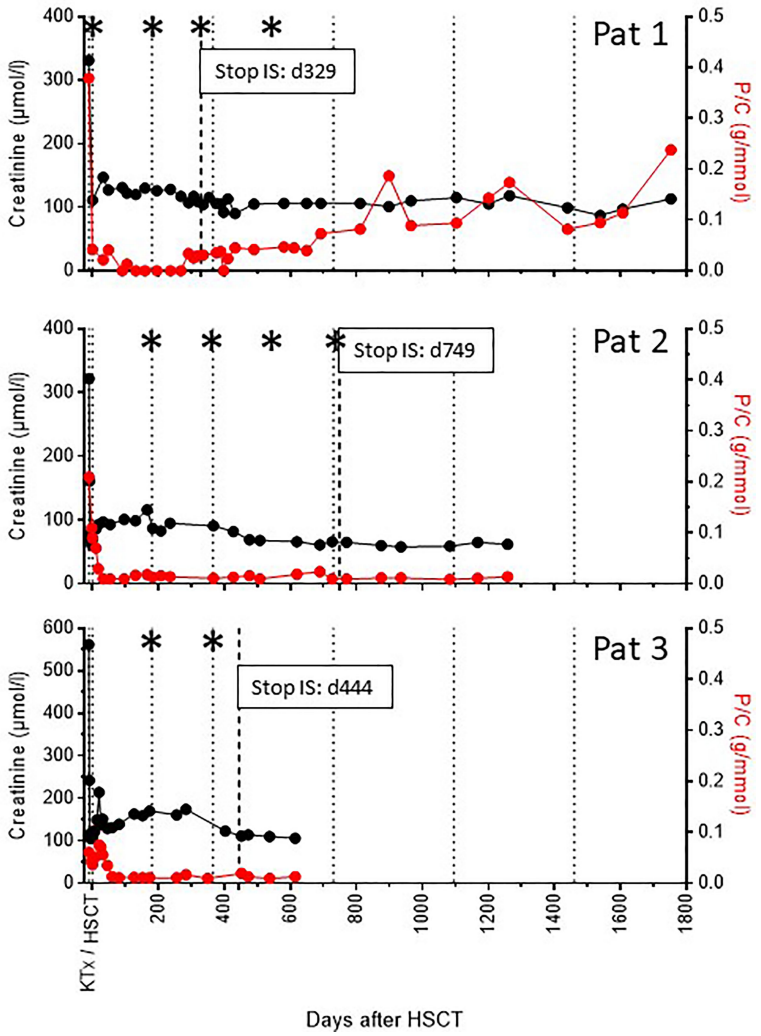

FIGURE 2 | Synopsis of renal function and proteinuria over time. The course of allograft function (serum creatinine, black dots) and proteinuria (protein/creatinine ratio, red dots) over time is shown for all three recipients. Dotted lines indicate time intervals of 6 and 12 months post-transplant and yearly thereafter. The hatched line indicates the time point of stop of all immunosuppressive treatment (IS). Asterisks indicate time points of allograft biopsies (see also **Supplementary Table 1**).

glomerulopathy. She was on peritoneal dialysis for 2 years and nine months before she received the kidney of her 54-year-old HLA-identical sister. Immunosuppression followed the standard trial protocol and was continued with cyclosporine A monotherapy starting at month 2. The donor chimerism level achieved was low. Therefore, we maintained the cyclosporine A whole blood through levels between 200-250 µg/L for 12 months (**Supplementary Figure 1**).

The first biopsy after 6 months showed very few lymphocytes in the peritubular capillaries (not diagnostic for peritubular capillaritis). We started cyclosporine A tapering after a normal second allograft biopsy one year after transplantation. Due to a very low chimerism level, tapering was performed very slowly. Cyclosporine A was finally stopped two years after transplantation (**Supplementary Figure 1**) and with a normal biopsy at that time point. The patient is now 3 years post-transplant and nineteen months without immunosuppressive therapy with stable kidney function and no proteinuria.

Since the transplantation, the patient has never been hospitalized. The only major complication was a Helicobacter-negative gastric ulcer, which was successfully treated by a proton pump inhibitor.

3.3.3 Patient 3

The third patient was a 49-year-old Caucasian male with end stage renal disease due to adult polycystic kidney disease. He was on peritoneal dialysis for three and a half months before receiving a living donor kidney from his 46-year-old HLA-identical sister. Immunosuppression followed the standard trial protocol and was continued with cyclosporine monotherapy

starting at month two. Early post-transplant, the patient had to be hospitalized for fenestration of a lymphocele.

The first allograft biopsy 6 months post-transplant did not show any signs of rejection (**Supplementary Table 2**). However, because of a rapid decline of donor chimerism, we maintained cyclosporine A whole blood though levels at 200-250 µg/L until months 9 and started tapering only thereafter. The second biopsy after 12 months during cyclosporine A tapering showed a BK-polyomavirus nephropathy. At this time point BK viremia was detected at very low level of about 975 IU/mL. Cyclosporine tapering was therefore continued, and since month 14 after transplantation the patient is without any immunosuppression (**Supplementary Figure 1**).

The patient is now 2 years post-transplant and 10 months without immunosuppressive therapy with stable kidney function, no signs of proteinuria (**Figure 2**), and no BK viremia. He returned to work 10 weeks post-transplant.

3.4 Synopsis of Renal and Hematologic Outcome

3.4.1 Renal Outcome

All three patients achieved an immediate and excellent allograft function until the last follow-up 5 years, 3.5 years, and 1.7 years post-transplant (**Figure 2**). Proteinuria is normal in two of the patients, whereas patient 1 developed albuminuria up to 2g/d due to a glomerulonephritis (*de novo* or recurrent).

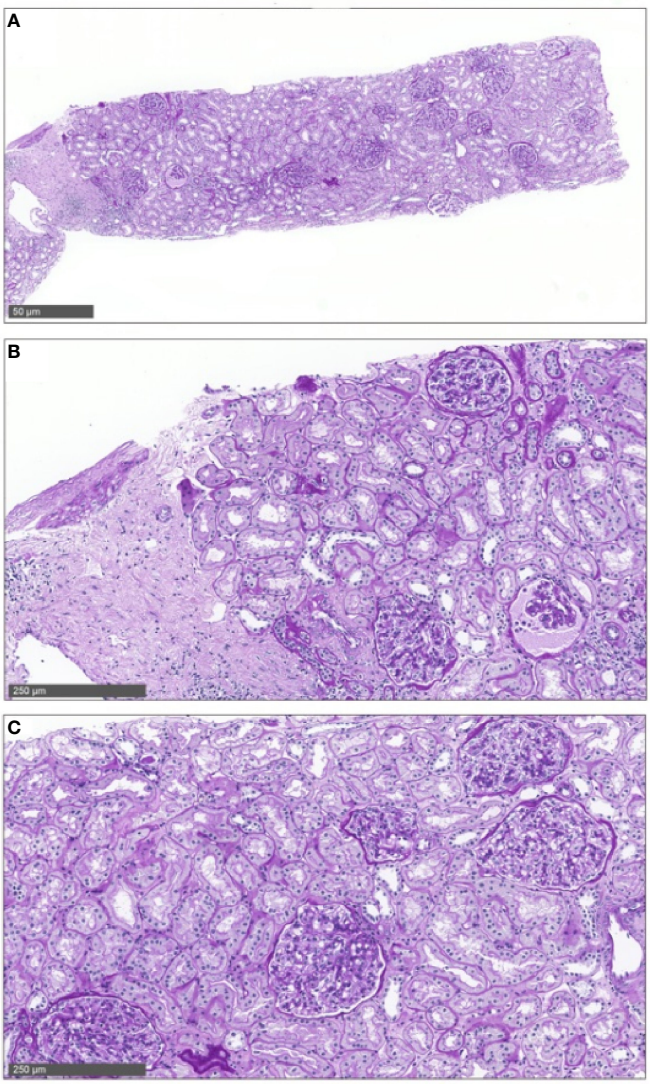

FIGURE 3 | Allograft biopsy no 3 in patient 2. Allograft biopsy of patient 2 18 months post-transplant showing minimal glomerular alterations without signs of acute rejection. This biopsy was taken under minimal immunosuppressive therapy (cyclosporine A level at 13 ug/L), at the same time as the molecular microscope analysis shown in **Figure 4**.

In a total of 9 allograft biopsies no signs of rejection were seen (**Supplementary Table 2**). In patient 3, the 12 months biopsy surprisingly showed a BK polyomavirus nephropathy. A low-level BK viremia was also found, which however immediately disappeared with stop of immunosuppression 14 months post-transplant. Kidney function remained stable over time (**Figure 2**).

In patient 2, the biopsy 18 months post-transplant showing minimal glomerular alterations (**Figure 3**) was analyzed in addition with the molecular microscope technology (MMDx), including an mRNA microarray of 60 genes, as previously described (8). This analysis showed a completely normal gene expression as seen in normal kidneys from living donors (**Figure 4**).

3.4.2 Hematological Outcome

Allogeneic HLA-identical transplantation of peripherally mobilized hematopoietic stem cells was successfully performed in all three recipients without any transplant-related complications. Donor 2 did not mobilize sufficient stem cells in a first harvest; therefore, a second stimulation and harvest had to be performed. Eventually, we were able to transplant between 5.6 and 12.3x 10^6 selected CD34+ stem cells/kg body weight in these three recipients, which were infused together with a T cell add-back of 1x 10^6 CD3+ T cells/kg body weight (**Table 1**). No signs of graft-versus-host disease (neither acute nor chronic) were seen during the whole follow-up in all three recipients.

All patients developed as expected a profound lymphopenia around the time of HSCT (**Figure 5**). Hemoglobin and platelet levels remained stable. Only patient 3 developed transient neutropenia (nadir 600 neutrophils/µl) three weeks post-kidney transplant, which however resolved again two weeks later. The other two recipients never experienced neutropenia. We did not observe any severe infectious complications in any of the patients neither early nor late post-transplant (**Table 2**)

The evolution of donor chimerism followed different patterns (**Figure 6**) (9):

- Patient 1 achieved a maximum whole donor blood chimerism of 62%, which then very slowly declined over time, but remained stable between 20-30% until 4 years post-transplant. She was the only recipient who also developed a long-lasting donor T cell chimerism.
- Patient 2 received the lowest number of donor CD34+ cells and experienced the lowest level of whole blood donor chimerism of maximally 25%, which was never stable and slowly declined over time. Therefore, cyclosporine A tapering was started later and delayed in this patient. She finally lost whole blood chimerism by day 500. Immunosuppression was anyway withdrawn by d749, and tolerance is maintained more than 18 months after stop of cyclosporine A.
- Patient 3 achieved the highest levels of whole blood donor chimerism early on (71%), which then rapidly declined and seemed to stabilize on a much lower level. Cyclosporine tapering was therefore started only 10 months post-transplant, and it was stopped 5 months later. This patient remains tolerant 8 months after stopping immunosuppression. This patient never developed

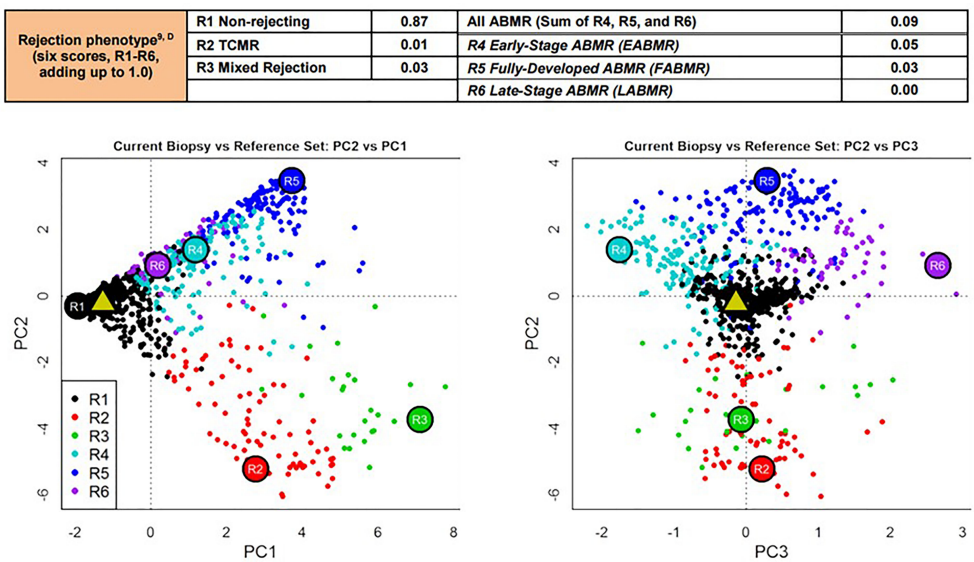

FIGURE 4 | Molecular microscope analysis of allograft biopsy no 3 in patient 2. In this analysis, an mRNA microarray of 60 genes was performed to arrive at a molecular diagnosis of T-cell-mediated, antibody-mediated or mixed rejection. This analysis was performed 18 months post-transplant under very low levels of cyclosporine monotherapy (trough level of 13 µg/L, **Supplementary Figure 1**) and showed a completely normal gene expression pattern as seen in normal kidneys from living donors (black dots are normal kidney, the green triangle represents our patient).

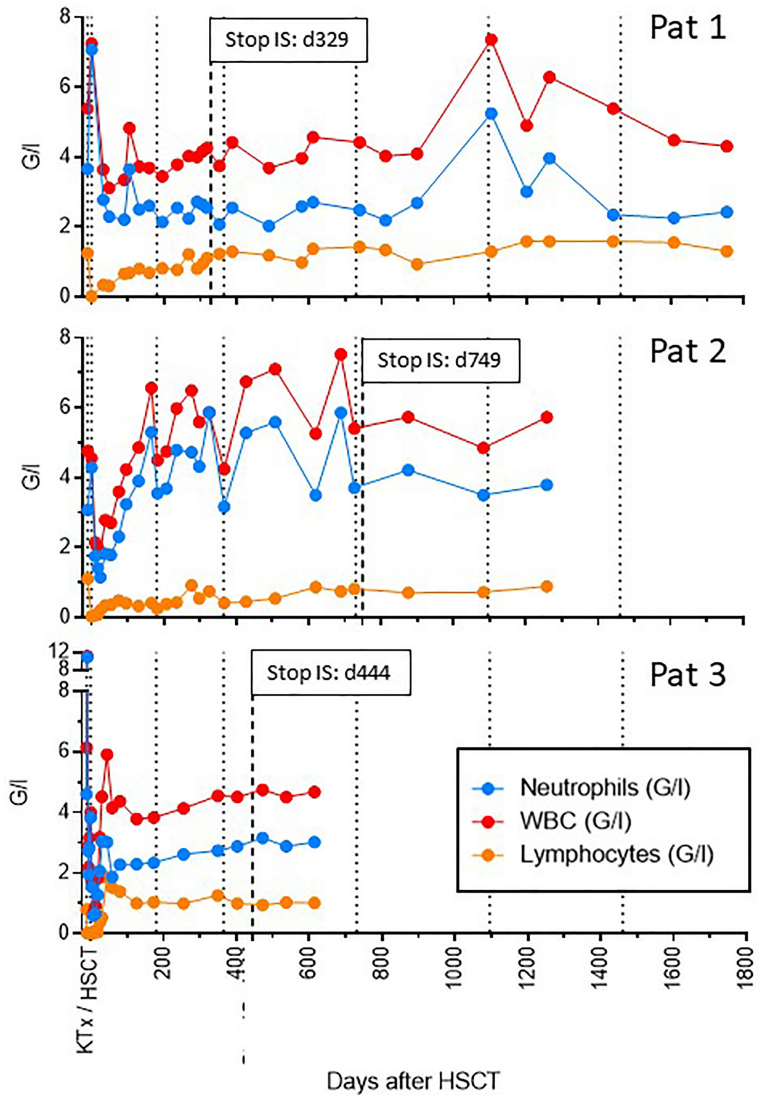

FIGURE 5 | Synopsis of hematological parameters over time. The number of total leukocytes, neutrophils and lymphocytes is shown over time for all three recipients. Dotted lines indicate time intervals of 6 and 12 months post-transplant and yearly thereafter. The hatched line indicates the time point of stop of all immunosuppressive treatment (IS). Profound lymphopenia around the time of transplantation was seen in all three recipients. However, only patient 3 also experienced transient neutropenia, which resolved by 4 weeks post-transplant.

substantial T cell chimerism, and lost whole blood chimerism after stop of cyclosporine A.

3.4.3 Specificity of Tolerance and Impact During the COVID-19 Pandemic

Renal transplant recipients have been shown to develop SARS-CoV-2-specific antibodies in only about 50% of cases after application of two vaccine doses. In case of SARS-CoV-2 infection (COVID-19) morbidity and mortality in this patient population is high.

During the COVID-19 pandemic, all three of our trial patients were vaccinated against SARS-CoV-2 with the mRNA vaccine BNT162b2 (Comirnaty®, Pfizer/BioNTech). None of the patients suffered from COVID-19. Several months post-vaccination, specific antibody as well as T-cell responses were assessed in all three patients. All three patients developed antibody responses with titers considered to be protective. A SARS-CoV-2-specific T cell response could be detected in 2 out of the three patients. In addition, all of them showed evidence of CMV-specific cellular immunity (**Table 3**).

These vaccine-induced immune responses during the COVID-19 pandemic demonstrated that the immunological tolerance achieved in our trial patients is indeed specific to the donor, while maintaining the ability to mount an effective immune response against viral spike proteins by this novel mRNA vaccine.

TABLE 2 | Trial outcome overview.

Patient No		Patient 1	Patient 2	Patient 3
Kidney transplant	Primary graft function	Yes	Yes	Yes
	Acute rejection	No	No	No
	Other diagnoses	Yes, biopsy-proven GN	No	Yes, polyomavirus nephropathy
HSCT	Chimerism 1 mts	Yes	Yes	Yes
	Chimerism 12 mts	Yes	Yes	Yes
	Chimerism 24 mts	Yes	No	NA
	Acute GvHD	No	No	No
	Chronic GvHD	No	No	No
Immuno-suppression	Standard trial immunosuppression until months 6	Yes	Yes	Yes
	Cyclosporine A weaning initiation	Month 7	Month 13	Month 10
	Cyclosporine A Stop	Month 11	Month 25	Month 15
Complications	Viral infections	Asymptomatic CMV reactivation	None	BK viremia with nephropathy
	Bacterial infections	Uncomplicated urinary tract infection	None	None
	Non-infectious complications	Calcineurin inhibitor pain syndrome (CIPS)	Gastric ulcer	Lymphocele (→ fenestration)

CMV, cytomegalovirus; GN, glomerulonephritis; GvHD, graft-versus-host disease; HSCT, hematopoietic stem cell transplantation; mts, months; NA, not applicable; No, number.

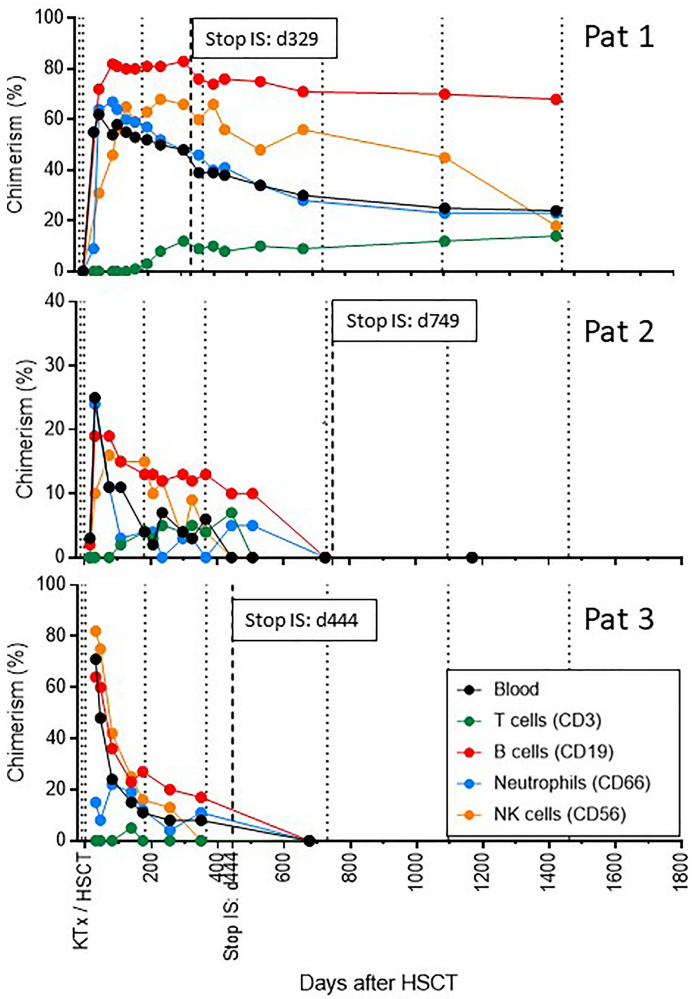

FIGURE 6 | Synopsis of donor chimerism over time. The level of whole blood as well as lineage-specific donor chimerism is shown over time for all three recipients. Dotted lines indicate time intervals of 6 and 12 months post-transplant and yearly thereafter. The hatched line indicates the time point of stop of all immunosuppressive treatment (IS).

TABLE 3 | SARS-CoV-2-specific vaccination and immunity.

Patient No		Patient 1	Patient 2	Patient 3
Vaccination	Vaccine	Comirnaty ®	Comirnaty ®	Comirnaty ®
	1st vaccination	29.1.2021	29.4.2021	14.1.2021
	2nd vaccination	5.3.2021	18.5.2021	11.2.2021
SARS-CoV-2-specific antibody response	Elecsys ® (Roche), anti-NP IgG (<1.0)	Not reactive, 0.074	Not reactive, 0.076	Not reactive, 0.075
	Elecsys ® (Roche), anti-Spike IgG (<0.8)	Positive, 1488 U/ml	Positive, 1951 U/ml	Positive, 919 U/ml
	ABCORA ®, (IMV) neutralization score (protective score > 17)	Protective, 28.2	Protective, 83.9	Protective, 40.3
SARS-CoV-2-specific T-cell response (net stimulation, % CD3+)	Concanavalin A	42.7%	55.2%	63%
	St. aureus superantigen	66.9%	67.7%	71%
	CMV antigen	(17%)*	(10%)*	76%
	SARS-CoV-2 SP subunit 1	0	27.1%	5%
	SARS-CoV-2 SP subunit 1	1.1%	14.5%	6%

CMV, cytomegalovirus; NP, nucleoprotein; IMV, Institute of Medical Virology (University of Zurich); SARS-CoV-2, new Coronavirus 2; SP, spike protein; St. aureus, Staphylococcus aureus
**These two analyses were performed in a separate assay. Thus, the absolute levels cannot be directly compared to the Concanavalin A/Superantigen response, but the responses are clearly positive.*

4 DISCUSSION

The SARS-CoV-2 pandemic confirmed the necessity to continue searching for the holy grail of transplantation medicine: finding solutions to perform solid organ transplants without the need for life-long immunosuppression. In an era of a global pandemic solid organ recipients are particularly vulnerable due to life-long immunosuppression to prevent allograft rejection. Immunosuppressed solid organ recipients (i) suffer more often from common and opportunistic infections; (ii) infectious diseases tend to have a more severe course and worse outcome compared to non-immunosuppressed patients (10); and (iii) immunosuppressed organ recipients respond less well to vaccines (11–13). More than 50 years of research since the seminal experiments on chimerism and tolerance performed by Billingham, Brent, and Medawar in the 1950s (14) were required until the first clinical trials were performed for combined kidney and hematopoietic stem cell transplantation from the same living donor (15). Meanwhile, 3 US centers have established successful programs for such procedures (Stanford, Boston, and Chicago; **Supplementary Table 1**), and up to 100 patients may have benefited so far from these programs to receive an allogeneic kidney without long-term immunosuppression (16–19).

According to our knowledge, swisstolerance.CH is the first European trial applying an established tolerance induction protocol by an independent group. Replicating the clinical protocol required a dedicated interdisciplinary team, but the outcome data are consistent with the results reported by the group of Strober et al. at Stanford University. We demonstrate that this elaborated protocol – despite its complexity – can be replicated by an independent group in another part of the world. The primary endpoint in our study was achieved by the first three patients presented here: acceptance of an HLA-identical allogeneic kidney without long-term immunosuppression, without acute allograft rejection and without graft-versus-host disease. The tolerance to the graft was further demonstrated by a molecular microscope analysis in one of the patients, where the gene expression pattern was indistinguishable from a normal living donor kidney (8).

The benefits of immunosuppression-free allograft acceptance are expected to become particularly important in the long-term, but several observations suggest that our patients have already taken advantage of this. The number of infection-related complications was low, and none of the patients developed post-transplant metabolic disorders. Patient 1 experienced CIPS, which rapidly resolved after stopping cyclosporine A. BK-polyomavirus nephropathy had a very favorable clinical evolution after tapering of immunosuppression in patient 3.

Tolerance induction, in contrast to general immunosuppression, becomes even more attractive in the unique context of the COVID-19 pandemic. All three patients were transplanted before the pandemic reached Europe. None of them suffered from COVID-19. The patients could be vaccinated with the mRNA vaccine BNT162b2, when they were off immunosuppression, and mounted strong and protective neutralizing SARS-CoV-2-specific antibody responses, and 2/3 also specific T cell responses. The number of patients is not sufficient for a conclusive analysis, but the data suggest a better immunological response to the vaccine in comparison to kidney transplant recipients under immunosuppressive therapy (11–13). This peculiar epidemiological setting, with the opportunity to study in patients the immune response to a novel virus, was instrumental to demonstrate the specificity of the immunological tolerance achieved with this protocol, which allowed acceptance of an allogeneic kidney while maintaining fully protective anti-vaccine responses.

Freedom of immunosuppression was only achieved in the second year after transplantation in 2 out of 3 patients. The reason was a slower cyclosporine A tapering than initially planned due to low (patient 2) or rapidly declining (patient 3) whole blood chimerism. This fact reveals one of the limitations of this current protocol: the donor chimerism levels achieved in an individual recipient-donor pair are unpredictable – in terms of absolute levels, stability, and duration (9). When referring to murine experiments, intermediate stable and multilineage chimerism (including T cells) confers the most robust tolerance status (20, 21), and different approaches have been developed to facilitate the induction of stable mixed chimerism (22–24). However, in non-human primate experiments it was demonstrated that also transient chimerism (if present high and long enough) can lead to tolerance towards an allograft, which is maintained beyond the loss of blood chimerism (25). It is

considered that non-deletional tolerance mechanisms (such as regulatory cells of any type) and local adaptations within the graft maintain tolerance in these patients (26, 27). Whether this tolerance status is as robust as in stable mixed chimeras is currently unknown.

Our pilot trial also indicates that one major problem of late kidney allograft loss, namely the recurrence of the primary kidney disease, cannot be solved by the induction of mixed chimerism, at least in the HLA-identical setting (patient 1). The non-myeloablative, minimal intensity protocol for HSCT does not allow for a complete reset of the immune system, and autoantigen presentation will also not be impaired, if the donor displays an identical set of HLA molecules. However, individual case reports indicate that HLA-mismatched HSCT may help improving some primary glomerulopathies, such as IgA nephropathy (28).

In conclusion, by replicating the Stanford protocol we confirm that donor-specific immune tolerance can be achieved in selected patients by mixed hematopoietic chimerism. The first three patients enrolled in our tolerance program were successfully withdrawn from all immunosuppression while maintaining stable allograft function and without signs of rejection or GvHD. Immunocompetence was demonstrated by protective immune responses against the SARS-CoV-2 vaccine in all three patients. The main challenge for the future will be the further development of this protocol across HLA barriers.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethikkommission der Kantons Zürich, Schweiz. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TF, JS, US, and PC designed the trial. TF and PC wrote the ethical board applications and applied for funding. TF, PC, KH, and US wrote the manuscript. KH, OdR, and TM cared for the patients. TG, MH, and US were responsible for preparation of cellular transplant products and for stem cell transplantation. CL and OR were responsible for total lymphoid irradiation procedures. AG and BH analyzed allograft biopsy specimens. JN and IA performed immunological analyses. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.796456/full#supplementary-material

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REVIEW
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Establishment of Chimerism and Organ Transplant Tolerance in Laboratory Animals: Safety and Efficacy of Adaptation to Humans

Robert Lowsky[1] *and Samuel Strober*[2*]

[1] Division of Blood and Marrow Transplantation and Cancer Cellular Therapy, Stanford University School of Medicine, Stanford, CA, United States, [2] Division of Immunology and Rheumatology, Stanford University School of Medicine, Stanford, CA, United States

The definition of immune tolerance to allogeneic tissue and organ transplants in laboratory animals and humans continues to be the acceptance of the donor graft, rejection of third-party grafts, and specific unresponsiveness of recipient immune cells to the donor alloantigens in the absence of immunosuppressive treatments. Actively acquired tolerance was achieved in mice more than 60 years ago by the establishment of mixed chimerism in neonatal mice. Once established, mixed chimerism was self-perpetuating and allowed for acceptance of tissue transplants in adults. Successful establishment of tolerance in humans has now been reported in several clinical trials based on the development of chimerism after combined transplantation of hematopoietic cells and an organ from the same donor. This review examines the mechanisms of organ graft acceptance after establishment of mixed chimerism (allo-tolerance) or complete chimerism (self-tolerance), and compares the development of graft versus host disease (GVHD) and graft versus tumor (GVT) activity in complete and mixed chimerism. GVHD, GVT activity, and complete chimerism are also discussed in the context of bone marrow transplantation to treat hematologic malignancies. The roles of transient versus persistent mixed chimerism in the induction and maintenance of tolerance and organ graft acceptance in animal models and clinical studies are compared. Key differences in the stability of mixed chimeras and tolerance induction in MHC matched and mismatched rodents, large laboratory animals, and humans are examined to provide insights into the safety and efficacy of translation of results of animal models to clinical trials.

Keywords: chimerism, transplant tolerance, immune suppression, animal models, human trials

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***Correspondence:**
Samuel Strober
sstrober@stanford.edu

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INTRODUCTION

Currently, about 500,000 patients with end stage renal disease are undergoing dialysis in the US of which over 100,000 are on a wait list for a deceased or living donor transplants (1). In addition, there are patients who have yet to start dialysis and require a kidney transplant for end stage renal disease. The treatment of choice for these patients is kidney transplantation, since transplantation allows the

best chance for a return to a near normal life style, and an improved life expectancy (2–4). Recipients of living and deceased donor kidneys require strict and lifelong adherence to combinations of immune suppression (IS) medications to prevent immune mediated rejection of the transplanted kidney. Most commonly, recipients require a two or three-drug IS regimen consisting of a purine metabolism inhibitor, a calcineurin inhibitor, and steroids (5, 6).

However, even with the use of these potent drug combinations there is continued and progressive loss of the kidney graft due to immune mediated graft rejection: median graft survival of living donor HLA mismatched kidneys is ~15 years, and recipients of deceased donor kidney transplants have graft survival averaging 8-12 years (7–9).

Long-term patient survival after graft loss is poor. In addition to graft rejection, the IS drug regimens themselves cause significant medical comorbidities that include chronic allograft vasculopathy, new onset diabetes after transplant (NODAT), infections, cancer, heart disease, hypertension, renal dysfunction, and osteoporosis and osteopenia (10–13). It has been shown that the number of IS medications per day and the occurrence of IS-related adverse effects have tremendous impact on a patient's quality of life and adherence to treatment (10, 11). Moreover, dosage adjustments to reduce the IS medications have been associated with acute rejection (10, 11). Patient noncompliance with IS drug regimens is the third leading cause of graft loss in renal transplantation, after chronic allograft nephropathy and death with a functioning graft (14). Both chronic allograft nephropathy and death with a functioning graft are related, directly and indirectly, to chronic IS drug regimens. Thus, the main limitations to successful and safe organ transplants are immune mediated graft rejection, and the medical comorbidities induced by the combinations of IS medications that help deter or delay but not prevent rejection. Strategies that result in IS drug minimization and/or complete withdrawal while maintaining normal graft function would represent a significant health benefit to patients undergoing transplantation. The unmet medical need is to eliminate the lifelong requirement of IS drug combinations with their attendant side effects, and to prevent immune mediated rejection of donor organ transplants. From pioneering studies of more than 70 years ago, it is long known that the establishment of hematopoietic cell chimerism in organ transplant recipients would lead to normal graft function without IS medications and without evidence of histologic rejection. This review will concentrate on preclinical models of chimerism-based transplantation tolerance, and highlight the bench-to-bedside adaptation of the models by combining living donor kidney and hematopoietic cell transplantation to purposefully withdraw IS medications from patients while maintaining normal graft function.

Definitions of Mixed Chimerism, Complete Chimerism, and Immune Tolerance to Organ Transplants

Seminal observations of the survival of skin grafts in adult dizygotic cattle that shared a placenta *in utero* with the skin graft donor, and in adult mice that had been given injections of genetically disparate bone marrow cells from the skin graft donor strain during the fetal/neonatal period led to the concept of "tolerance" as opposed to the expected "rejection" of the grafts (15–17). The specificity of the tolerant state was demonstrated by the ability of the recipients to reject third party but not donor skin grafts (16, 17).

Tolerance was linked to "genetical chimerism" in these skin graft recipients, and the term was used to mean that two or more cell lineages with different genotypes (allogeneic) were present in the recipients (15–17). Subsequent studies of erythrocyte chimeric cattle twins showed that skin grafts exchanged between them had prolonged survival, but were eventually rejected (18). In this model system, immune rejection was attenuated, and not completely prevented. Thus, the skin graft recipients did not meet the criteria of tolerance that includes lack of evidence of rejection of the donor graft.

At present the term "mixed chimerism" is used to identify recipients with a mixture of two or more hematopoietic and immune cell lineages in the blood forming and lymphoid tissues (19–21). Numerous preclinical studies have shown that the recipient and donor immune cells of mixed chimeras show a mutual state of immune tolerance (specific unresponsiveness) to donor and recipient alloantigens, but not to third party alloantigens (18–21). The term "mixed chimerism" is currently used to distinguish human or non-human recipients from those with "complete chimerism".

Studies with complete chimerism in humans involve recipients who were treated with blood and marrow transplants as curative therapy for a hematologic malignancy. In these patients the donor hematopoietic and immune cells completely replaced the recipient hematopoietic and immune cells (22–25). The development of complete chimerism is desirable to prevent tumor relapse because complete chimerism associated with beneficial alloreactive graft-versus-host reactions necessary for immune mediated eradication of residual host-derived cancer cells. However, complete chimeras have a higher risk of developing harmful graft versus host disease (GVHD) as compared to mixed chimeras (25, 26). The risk of GVHD in human complete chimeras has been mitigated even in HLA mismatched transplant patient pairs by the development of several strategies including the administration of posttransplant cyclophosphamide (PTCy) (27, 28).

Reports of cancer patients with complete chimerism after bone marrow transplantation and who thereafter developed end stage renal disease (ESRD) accepted a kidney transplant from their bone marrow donor, and without the need for immunosuppressive (IS) drugs (29, 30). The acceptance of the organs in the latter recipients did not reflect a proof of concept of "immune tolerance", rather these cases represent acceptance of "self" histocompatibility antigens shared between the cells of the donor organ and the cells of the donor bone marrow, since there were only chimeric donor immune cells and no residual detectable recipient immune cells in the blood or marrow (29, 30). Complete chimeras can develop donor immune cells that are specifically unresponsive to recipient alloantigens

(31, 32). This represents graft versus host (GvH) acceptance. The use of pretransplant host conditioning using total lymphoid irradiation (TLI) combined with anti-thymocyte globulin (ATG) or the administration of cyclophosphamide shortly after bone marrow transplantation in laboratory animals and in humans has been shown to establish GvH acceptance that prevents GVHD (27, 32, 33).

STUDIES OF TOLERANCE IN LABORATORY ANIMALS

Establishment of Mixed Chimerism and Tolerance to Skin and Organ Grafts in Neonatal Rodents: Stability of Chimerism in the Absence of Immunosuppressive Drugs

The seminal observations of the development of stable mixed chimerism in cattle that shared a placenta *in utero*, and the inability of the adult chimeras to rapidly reject skin grafts from each other, but not from third parties, formed the conceptual framework for the linkage of chimerism and tolerance (15–17). The key advance was made after the intentional establishment of chimerism and tolerance by injecting neonatal mice of one strain with bone marrow cells from another (34, 35). As in the case of the cattle, the adult mixed chimeras accepted skin grafts from the marrow donor strain but not from third party strains (34, 35). Follow up studies showed that recipient immune cells obtained from the chimeras were specifically unresponsive to the alloantigens of the donor strain and not to third party strains (35–37). Thus, the recipients met the current definition of tolerance by the acceptance of donor grafts, rejection of third-party grafts, and evidence of specific immune unresponsiveness of recipient immune cells to donor antigens (19–21). This is referred to as "host versus graft (HvG) tolerance" or "organ transplant tolerance". As in the case of the chimeric cattle, mixed chimerism was self-perpetuating in the mice, and levels of chimerism remained stable during adulthood in absence of IS drugs (38, 39). Occasionally the injected neonates showed a phenomenon called "runting" that upon further investigation was found to be a consequence of GVHD after the development of complete instead of mixed chimerism (16, 40). The development of GVHD is an indication that GvH tolerance failed. Mixed chimeras develop bidirectional HvG and GvH tolerance.

Establishment of Chimerism and Acceptance of Skin Grafts after Myeloablative Lethal Total Body Irradiation of Adult Rodents

The application of the principles of induction of tolerance after the establishment of chimerism in neonates was initially applied to adult mice conditioned with a lethal dose of total body irradiation (TBI). Irradiated adults from one parental strain were given F1 hybrid MHC matched bone marrow transplants followed by skin grafts from the allogeneic parental strain (34). The use of F1 hybrid marrow avoided the development of GVHD, since the immune cells in the marrow were naturally unresponsive to the tissues of the recipient due to sharing of genetically determined histocompatibility antigens with the recipients. The key observation was that the allogeneic skin grafts were accepted after transplantation of hybrid but not syngeneic marrow (34). Although the studies of irradiated adults shared similar observations of skin graft acceptance with that of studies of graft acceptance in neonates, the radiation chimeras given myeloablative lethal TBI were likely to be complete chimeras rather than mixed chimeras due to depletion of recipient immune and hematopoietic cells, and the complete replacement by donor immune and blood forming cells. Thus, the acceptance of the skin grafts in the adults was not due to residual recipient cells developing immune tolerance to donor alloantigens, but rather due to a failure of the donor hybrid immune cells to reject an organ expressing the alloantigens of the shared parental strain (34).

Subsequent studies of acceptance of skin transplants in complete chimeras given lethal TBI, and bone marrow transplants from fully allogeneic strains (donor parental strain cells instead of F1 hybrid cells) also showed acceptance of allogeneic skin grafts in the chimeras (35). However, a proportion of the recipients developed severe GVHD depending on the level of MHC matching (35). The studies of radiation chimeras and acceptance of skin grafts by complete chimeras were of considerable interest. However, acceptance of organ grafts was based on the failure of the chimeric donor cells to reject donor organ grafts. This failure can be explained by the concepts of the lack of donor immune cell responses to donor self-molecules initially described by Burnet, and not on the concepts of immune tolerance to alloantigens initially described by Medawar and his co-workers [reviewed in (41)]. As pointed out in the latter review, experiments of the Medawar group were interpreted as showing that "fully tolerant recipient mice could be said to show 'central failure' of their own response to the tolerated transplantation antigens". These experiments did not prove that purified chimeric recipient immune cells were specifically unresponsive to donor alloantigens, since the technology to perform such experiments did not exist at that time. Subsequent experiments using adult murine mixed chimeras that were tolerant of organ grafts proved that purified chimeric recipient immune cells were unresponsive to donor alloantigens in association with clonal deletion (see below).

Establishment of Mixed Chimerism and Immune Tolerance to Skin Grafts After Non-Myeloablative TLI of Adult Mice

The risks of myeloablative radiation and of GVHD after allogeneic bone marrow transplantation were justified for adaptation to humans treated for hematologic malignancies, since these diseases were uniformly lethal (22, 42). These risks were mitigated by the use of fully MHC matched transplant pairs that were studied extensively in dogs prior to the application to humans (43). However, the use of lethal TBI described in the

studies of myeloablative radiation mouse chimeras discussed above to achieve acceptance of organ transplants could not be applied to humans undergoing organ transplant surgery due to the inherent dangers of lethal irradiation in the recipients, and the development of GVHD.

An alternative was to condition recipients of combined organ and bone marrow transplants with non-myeloablative radiation regimens that had been proven safe in humans. Accordingly, the laboratory of Strober and his co-workers at Stanford University studied the non-myeloablative radiation regimen of TLI for application to pre- clinical models of bone marrow transplantation with or without organ transplantation (38, 39, 44–52). The TLI radiation regimen was developed by Kaplan, Rosenberg, and their Stanford co-workers for the treatment of patients with early stage Hodgkin's disease (HD) [reviewed, (53)]. TLI radiation was given as multiple small doses over several weeks targeted to the spleen, lymph nodes above and below the diaphragm, and thymus of HD patients (53). All other non-lymphoid tissue areas including the lungs, central nervous system, intestines etc. were shielded with lead. About 50% of the marrow volume was shielded and prevented the development of severe neutropenia and thrombocytopenia observed with myeloablative TBI. TLI was successful in the induction of durable complete remissions in early stage HD, and long-term studies of safety and efficacy were made on thousands of patients starting in the 1960's (53).

A TLI regimen was developed for use in mice and rats by manufacture of a lead jig that allowed for the irradiation of the spleen, thymus, and lymph nodes and shielded the lungs, skull, liver, portions of the intestines, and marrow in the limbs (38, 39, 44–46). Unexpectedly, when adult TLI treated recipient mice were given bone marrow transplants from fully MHC mismatched donor mice stable self-perpetuating mixed chimerism was established without evidence of GVHD (38, 39, 44–46). In contrast, mice conditioned with a single dose of myeloablative TBI and given mismatched bone marrow transplants uniformly died of GVHD (38, 39, 47, 48). In view of the success in achieving persistent mixed chimerism without GVHD in adult mice, TLI treated mixed chimeras were given skin grafts from the marrow donor strain and third-party strains (38, 39, 44). Whereas the donor-type skin grafts were accepted for observation periods of up to 6 months, the third-party grafts were rejected within a few weeks. Purified recipient immune cells collected from the mixed chimeras were shown to be specifically unresponsive to donor alloantigens in the mixed leukocyte reaction, and clonally deleted to the alloantigens (49, 54). Thus, the recipient mice fulfilled the criteria of immune tolerance discussed above, using a safe non-myeloablative regimen without GVHD that could be applied to adult humans (32). Subsequent to the studies of transplant tolerance with mixed chimerism in rodents and dogs by the group at Stanford, Sachs and his co-workers at Harvard, developed a regimen to achieve stable mixed chimerism and immune tolerance without GVHD in MHC mismatched adult mice by injecting a combination of recipient and donor bone marrow cells into recipients conditioned with TBI (55, 56). Thus, establishment of mixed chimerism was clearly linked to the induction of tolerance in adults in this model also. The contribution of clonal deletion of donor-reactive recipient T cells to the development of tolerance in these mixed chimeras was demonstrated in subsequent experiments (54).

Establishment of Mixed Chimerism and Immune Tolerance to Heart Transplants in Adult Rats Using a Completely Posttransplant TLI Conditioning Regimen

The TLI regimen developed in mice was adapted for use in rats given combined MHC mismatched bone marrow given intravenously, and heterotopic heart transplants directly anastomosed to the abdominal aorta and vena cava (44). Lead jigs were manufactured to expose the spleen, thymus and lymph nodes as in the mouse model, and 10 doses of TLI with 5 doses of ATG were used for conditioning (44). Initially the conditioning regimen was administered pretransplant as in the mouse model, and mixed chimerism and tolerance were induced in all recipients (44). However, in order to adapt the conditioning regimen for future use in deceased donor organ transplantation in humans, the timing of the TLI/ATG regimen was changed in subsequent experiments, and administered starting one day after rather than 14 days before the organ transplant (**Figure 1**) (57–60). The change was made because of the uncertainty of the timing of the availability of human deceased donor organ, since the start of a pretransplant conditioning regimen cannot be timed according to the organ availability. The infusion of the donor bone marrow cells was performed immediately after the last dose of TLI administered on day 12 posttransplant (57–60). In humans, this would require cryopreservation of donor cells, and thawing at the completion of TLI.

When the completely posttransplant regimen was used, mixed chimerism was established in all rat recipients, and the heart transplants were not rejected during an observation period of up to 6 months (57–60). In further studies, a course of the calcineurin inhibitor, cyclosporine was administered to the rats given the combined bone marrow and heart transplants after the posttransplant TLI/ATG conditioning regimen was completed in order to determine whether posttransplant immunosuppressive drugs would enhance or interfere with chimerism and the induction of tolerance (61). The results showed that the administration of cyclosporine enhanced the establishment of stable mixed chimerism, and tolerance in this model system (61).

Additional experiments were performed to determine whether an infusion of purified donor peripheral blood mononuclear cells (PBMC) or purified blood monocytes could substitute for the infusion of donor bone marrow cells in recipients given heart transplants and TLI/ATG conditioning (58, 59). Although the survival of heart grafts was markedly prolonged after the PBMC or monocyte infusions as compared to that of controls given no donor cell infusion, biopsies of the surviving heart transplants obtained more than 100 days posttransplant showed evidence of moderate to severe chronic rejection on microscopic analysis (58–60). In contrast, control recipients given infusions of donor bone marrow cells showed no

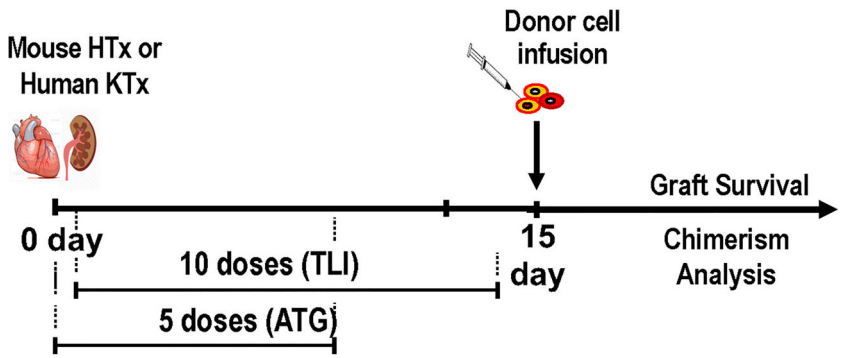

FIGURE 1 | Experimental Scheme for Establishment of Mixed Chimerism and Tolerance in Laboratory Animals and Patients After Organ Transplantation; Donor organ transplantation (heterotopic heart in mice, kidney in humans) is performed on day 0, and the first of 5 daily doses of ATG is given on day 0. On day 1 the first of 10 daily doses of TLI is given. Animals receive 5 doses during the first week and 5 doses during the second week posttransplant. Patients receive 4 doses during the first week and 6 during the second week. Donor hematopoietic cells (cryopreserved and thawed in humans, and fresh in mice) are infused immediately after the last dose of TLI. Serial tests of chimerism and organ graft function are performed thereafter.

evidence of acute or chronic rejection associated with the development of mixed chimerism (60). The results indicated that the latter chimeric recipients were protected from chronic rejection as compared to the non- chimeric recipients given PBMC or monocytes (60).

Studies of Regulatory Immune Cells Required for the Establishment of Mixed Chimerism and Tolerance in MHC Mismatched Mice Given Combined Bone Marrow and Heart Transplants Using the TLI/ATG Posttransplant Conditioning Regimen

Our further studies used MHC mismatched C57BL/6 (H-2b) and BALB/c (H-2d) mice as either donors or as recipients conditioned with posttransplant TLI/ATG and given combined bone marrow and heart transplants. Four recipient immune cell types were identified that were required for the induction of chimerism and tolerance (47, 48, 61–63). These included CD8+ tolerogenic dendritic cells (DCs), NKT cells, Tregs, and myeloid derived suppressor cells (MDSCs). Deletion of any one of these recipient cells by genetic engineering (ie., Batf-3-/- to selectively delete CD8+DCs and Jalpha18-/- to selectively delete NKT cells) or by administration of depleting mAbs to delete Treg cells and MDSCs abrogated chimerism and tolerance, and add back of these cells restored chimerism and tolerance (47, 48, 61–63).

The first cells in the chain of interactions in this complex network are Batf-3-/- dependent CD8+DCs that take up apoptotic bodies (efferocytosis) that are produced in great quantities by the TLI procedure (**Figure 2**) (63). The uptake induces changes in the receptors, function, and molecules produced by the CD8+DCs such that they become tolerogenic and express negative signaling surface molecules such as PDL-1 and produce the immunosuppressive cytokines such as IDO (63) The induction of apoptosis is a consequence of the TLI radiation triggering the p53/Bcl2 apoptotic pathway (64, 65). Due to the rapid upregulation of expression of the anti- apoptotic Bcl2 gene in NKT and Treg cells, the balance of these radioresistant T cell subsets is changed to favor these regulatory T cells over radiosensitive naïve Tcon cells (64, 65). The tolerogenic host DCs expressed CD1d and Rae-1 surface receptors that interact with the invariant TCR and NKG2D on host NKT cells such that the latter cells become tolerogenic and secrete abundant IL-4 (63). The NKT cells activate Treg cells in an IL-4 dependent manner as shown in the far left of **Figure 2**, and increase Treg immune suppressive function that is dependent on the secretion of IL-10. Host NKT cells also activated host DCs and MDSCs to become immunosuppressive *via* production of molecules such as IDO, and arginase-1 as reported previously (63, 64). In preclinical models of prevention of GVHD after bone marrow transplantation, Stanford investigators have shown that infusion of donor NKT cells activate donor Treg cells and host Gr-1+ MDSCs, and thereby prevent GVHD (65–67). Analogous changes in the development of immunosuppressive human host MDSCs after TLI based conditioning have been identified (68).

Tolerance Induction Regimens in Large Laboratory Animals Given Combined Organ and Bone Marrow Transplants

Subsequent to the tolerance studies described above in rodents, several laboratories investigated organ transplantation with bone marrow transplantation using tolerance induction regimens in large animals that were bred and MHC typed in laboratories including dogs, non-human primates, and mini-swine (56, 69–73). Studies in fully MHC matched dogs given non-myeloablative TBI followed by bone marrow transplantation showed that stable mixed chimerism was established for observation periods of at least a few years even after the withdrawal of IS drugs at 6 months posttransplant (69). The mixed chimeras were given donor organ transplants several months after the bone marrow transplants, and almost all

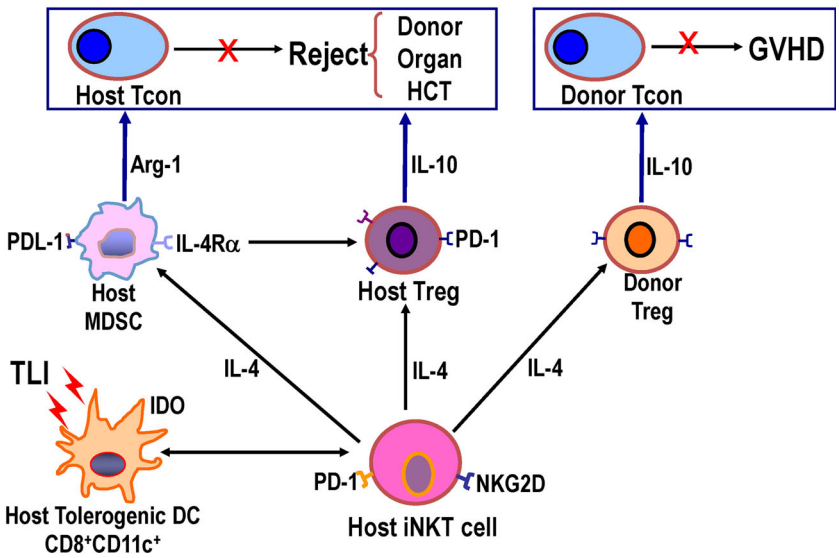

FIGURE 2 | Diagram of network of host and donor regulatory cell interactions after TLI based conditioning regimen and transplantation that are required for establishment of mixed chimerism and tolerance. TLI induced massive apoptosis of lymphocytes, and apoptotic bodies were engulfed by host CD8+DCs that interacted with host NKT cells such that both cell types became immunosuppressive/tolerogenic with NKT cell secretion of IL-4. Host NKT cell interacted with and activated host DCs, host Tregs, host MDSCs, and donor Tregs to upregulate production of immunosuppressive molecules including PDL-1, arginase-1, IDO, IL-10, and PD-1. Knockout or depletion of each of the latter cells or their secreted cytokines abrogated tolerance, and add back of each cell type restored tolerance (62, 63).

recipients accepted the organ without IS drugs without evidence of rejection on biopsies (69). In contrast, third party organ grafts transplanted to the mixed chimeras were rejected rapidly (69). Interestingly, in some experiments the recipient mixed chimeras bearing the donor organ transplants were treated with TBI and recipient leukocyte infusion (RLI) to deplete the donor chimeric cells (73). Despite the loss of chimerism after the RLI, the acceptance of the kidney grafts continued without rejection (73). The results indicated that persistence of mixed chimerism in these fully MHC matched recipients was not required for continued organ graft acceptance (73).

A pretransplant conditioning regimen with non-myeloablative TBI (300 cGy) combined with thymic radiation and ATG allowed for the development of mixed chimerism and acceptance of kidney transplants in MHC mismatched non-human primates given an infusion of donor bone marrow cells at the time of the organ transplant without the need for maintenance IS drugs in the majority of recipients (71, 72). Interestingly the mixed chimerism was transient and lost after a few to several weeks, and the long-term kidney transplants maintained good function without IS drugs despite the loss of chimerism (71, 72). Similar results were achieved in mini-swine given combined MHC mismatched bone marrow and kidney transplants (56). The studies in both the MHC matched dogs and the MHC mismatched non-human primates concluded that transient, but not stable mixed chimerism, was required for organ graft acceptance (70–73). In collaborative studies with investigators at the University of Wisconsin and Stanford University using a TLI/ATG posttransplant conditioning followed by MHC mismatched combined kidney and hematopoietic cell transplantation, long term acceptance of kidney grafts after IS drug withdrawal was also observed after transient mixed chimerism (personal communication, Dixon Kaufmann).

In conclusion, stable mixed chimerism was not required for acceptance of kidney transplants in both dog and non-human primate studies. However, the stability of mixed chimerism in the MHC matched dogs, and the instability in the MHC mismatched non- human primates suggests that there is likely to be a link between stability and MHC matching. This link has not been studied as yet in either of these large animal models, but has been studied in humans (see below).

STUDIES OF NON-CHIMERIC TOLERANCE IN HLA MISMATCHED AND MATCHED PATIENTS

Feasibility of Non-Chimeric Tolerance Induction in Patients Given HLA Mismatched Kidney Transplants From Deceased Donors Using Pretransplant TLI and Posttransplant ATG Conditioning

Although pretransplant TLI conditioning in fully MHC mismatched mice and rats markedly prolonged skin, heart, and kidney transplant survival, tolerance was not achieved without the addition of a hematopoietic cell infusion and the establishment of mixed chimerism (38, 39, 44, 74–76). In contrast, a study in outbred dogs showed that pretransplant TLI and posttransplant ATG

without the infusion of donor cells allowed for acceptance of MHC unmatched heart transplants for more than 1 year in the absence of immunosuppressive drugs (77). The recipients rejected third party transplants and showed specific unresponsiveness to donor alloantigens (77). These recipients showed the feasibility of inducing non-chimeric tolerance to outbred canine kidney transplants using TLI/ATG conditioning.

In view of the results in the canine study, and the safety of the use of TLI to treat patients with Hodgkin's disease, pretransplant TLI and posttransplant ATG was used to treat a series of patients given HLA unmatched deceased donor kidney transplants to determine the feasibility of maintaining graft function with low dose (0.1- 0.2mg/kg/day) prednisone monotherapy as maintenance therapy (78, 79). TLI was targeted to the lymph nodes, spleen, and thymus with lead shields for all other tissues using multiple doses of 100cGy each given 3 times per week until achieving a total dose of 2,000cGy, and then once per week until the donor organ became available (78, 79). The results showed that the 16 patients who completed the regimen were maintained on prednisone alone at the last observation point with good graft function during a follow up period of up to 25 months. The frequencies of patient survival, graft survival and rejection episodes in experimental patients were similar to that of concomitant standard of care patients maintained on prednisone and cyclosporine, and graft function was improved (78, 79).

In a follow up study, the feasibility of completely withdrawing maintenance prednisone therapy from 3 of the patients given deceased donor kidney transplants and pretransplant TLI was determined (80). The 3 patients showed no evidence of rejection while off IS drugs for 10, 24, and 69 months, and specific unresponsiveness to donor alloantigens was demonstrated in the mixed leukocyte reaction and cell mediated lympholysis assays (80). Thus, the patients met the criteria of actively acquired tolerance. One of these patients was studied again 12 years off IS drugs, and had no evidence of rejection (81). Although, this was the first study to show the feasibility of immune tolerance induction in humans, the frequency of rejection episodes and of graft loss among the experimental patients enrolled in the study who did not develop tolerance was similar to that of concomitant standard of care patients (79). In order to increase the success rate of tolerance induction, and to reduce the frequency of rejection episodes and graft loss, recipients of kidney transplants treated with TLI/ATG conditioning in subsequent tolerance studies were given infusions of hematopoietic progenitor cells from fully or partially HLA matched living donors to establish mixed chimerism (see section 11 below).

Induction of Non-Chimeric Tolerance in Patients Given HLA Matched Kidney Transplants From Living Donors Using IS Drugs and Infusions of Donor Hematopoietic Cells Without Conditioning

A study of 20 patients who received HLA matched kidney transplants, and multiple injections of donor hematopoietic progenitor cells in order to withdraw maintenance IS drugs was performed by investigators at Northwestern University (82, 83). Recipients were not conditioned with radiation or chemotherapy, and were given 4 infusions of CD34+ selected donor cells along with standard of care IS drugs. The latter included Alemtuzumab induction, short term tacrolimus and MMF that was switched to sirolimus maintenance therapy before complete IS drug withdrawal at 2 years posttransplant on the basis of transplant biopsies at 12,18, and 24 months without evidence of rejection. Peak transient donor chimerism was above 1% (1.7 to 5.2%) in 3 patients, and 5 of 15 patients withdrawn from IS drugs were off drugs without rejection for 60 to 73 months at the last observation point. Seven out of 15 patients withdrawn from IS drugs developed rejection episodes thereafter, and were returned to maintenance therapy (82–84). The patients who maintained good graft function off drugs were considered "operationally "tolerant", and had increased levels of Treg cells in the blood after transplantation, and a gene signature that was similar to the reported for "operationally" tolerant patients by other investigators (83). Specific unresponsiveness to donor alloantigen as a marker of immune tolerance was not reported.

STUDIES OF TOLERANCE IN HLA MATCHED PATIENTS WITH MIXED CHIMERISM

Establishment of Mixed Chimerism and Immune Tolerance in Patients Given HLA Matched Living Donor Kidney and Hematopoietic Cell Transplants

In order to evaluate the ability of TLI/ATG conditioning to promote the establishment of chimerism in humans Stanford investigators first performed a study of allogeneic hematopoietic cell transplants to treat patients with hematologic malignancies (32). In this and in subsequent studies patients received G-CSF mobilized peripheral blood mononuclear cell (PBMC) transplants without CD34+ purification form HLA matched donors who were related or unrelated to the recipients (26, 32). The goal of the latter study was to establish complete chimerism and eradication of tumor cells without GVHD based on preclinical studies that showed that this outcome can be achieved in rodent models using TLI/ATG conditioning (47, 50, 52). Patients enrolled in the study were not considered candidates for myeloablative conditioning due to medical comorbidities and/or advanced age (32). Since persistent chimerism without severe neutropenia or thrombocytopenia was established in almost all the HLA matched recipients with hematologic malignancies (32), a modified protocol using purified CD34+ cells (>4x10^6 cells/kg) and a defined dose of donor T cells (1x10^6 cells/kg) was applied to HLA matched combined kidney and hematopoietic cell transplant patients (**Figure 3**) (85).

HLA matched donor cells were collected, purified, and cryopreserved about 6 weeks before kidney transplantation

(85–88). Donor cells were injected intravenously immediately after the completion of 10 doses of TLI. Four doses were given in the first week posttransplant along with 5 doses of ATG. Patients were discharged at day 5 or 6 posttransplant, and the remaining 6 doses of TLI were administered in the out-patient clinics during the second week posttransplant. Twenty-nine patients were enrolled in the study, and 24 who developed mixed chimerism for at least 6 months were completely withdrawn from IS drugs within 1 to 8 months thereafter (85–88). Immune suppression (IS) medications were prednisone for 10 days, MMF for 30 days, and a calcineurin inhibitor (cyclosporine or tacrolimus at standard doses) for six months with gradual tapering to discontinuation thereafter. Withdrawal of IS drugs was dependent of establishment of chimerism for at least 6 months, no evidence of GVHD, and no evidence of microscopic rejection on protocol biopsies just before complete IS drug withdrawal (85–88).

Of the 24 patients withdrawn from IS drugs, 22 had no evidence of rejection with up to 15 years follow up (88). Two patients had rejection episodes at about 4 years after IS drug withdrawal, the episodes were reversed with standard of care treatment, and patients were returned standard maintenance therapy with normal graft function thereafter (88).

Ten patients had mixed chimerism that was persistent until the last chimerism test, and 14 patients lost chimerism sometime after the first year posttransplant (88). Despite the loss of chimerism in 14 of the HLA matched recipients withdrawn from IS drugs, the 12 maintained good graft function without evidence of rejection thereafter, and 2 developed rejection episodes as described above. Patients with mixed chimerism showed specific unresponsiveness of PBMC T cells to donor cells in the MLR, and made normal T cell responses to recall antigens *in vitro*. In summary, the 22 of 29 patients developed mixed chimerism and tolerance to their kidney transplants after combined organ and hematopoietic cell transplantation using TLI/ATG conditioning.

There were no graft loses due to rejection, and no GVHD, severe neutropenia or thrombocytopenia in the 29 patients enrolled in the protocol (85–88).

Medeor Therapeutics performed a multi-center randomized trial of tolerance induction in 20 experimental HLA matched kidney transplant patients and 10 standard of care patients based on the results of the clinical study report by investigators at Stanford University (89). Experimental patients were conditioned with posttransplant TLI/ATG, and given an infusion of donor CD34+ enriched cells and a defined dose of T cells to establish persistent mixed chimerism (89). The interim analysis showed the ability to achieve persistent chimerism in all enrolled experimental fully matched patients who were followed for at least six months, and the ability to completely withdraw IS drugs by the end of one year without subsequent evidence of rejection. The primary endpoint of the study was to determine the percentage of patients off IS drugs for 2 years without evidence of rejection (89).

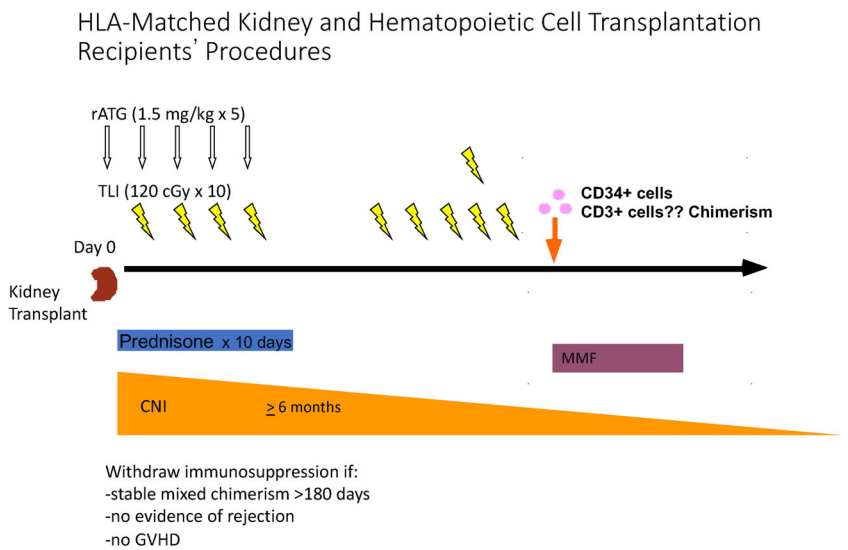

FIGURE 3 | Schema for Establishment of Mixed Chimerism, and Complete Withdrawal of IS Drugs after HLA matched Kidney Transplantation. Kidney transplantation was performed on day 0, and the first of 5 doses of ATG was administered intra-operatively. The first of 10 doses of TLI was administered on day 1, and 3 additional daily doses were administered during the first week. Patients were discharged from the hospital on days 5 or 6 posttransplant, and received and 6 TLI doses in the clinic during the second week posttransplant. Cryopreserved and thawed donor cells were infused immediately after the completion of TLI. Donor cells were collected 2 months before kidney transplantation by apheresis from G-CSF mobilized blood, and CD34 cells were purified. The latter cells and a defined dose of T cells were cryopreserved until infusion after TLI. Prednisone was administered for 10 days, MMF was given for 30 days starting with the day of the donor cell infusion. A calcineurin inhibitor was administered for 6 months posttransplant at standard dosage, and then tapered to discontinuation during the first year posttransplant if chimerism persisted for at least 6 months, there was no evidence of GVHD, and there was no evidence of microscopic rejection on a protocol biopsy performed just before IS discontinuation.

STUDIES OF TOLERANCE IN HLA MISMATCHED PATIENTS WITH MIXED CHIMERISM

Feasibility Study of Tolerance Induction and Mixed Chimerism in Patients Given Combined HLA Mismatched Living Donor Kidney and Hematopoietic Cells Transplants Using TLI/ATG Conditioning Posttransplant

A feasibility study was performed at Stanford University to assess the safety and efficacy of infusing purified donor CD34+ hematopoietic progenitor cells into 4 patients given kidney transplants from HLA mismatched living donors in order to establish mixed chimerism using posttransplant TLI/ATG conditioning (90). Two patient pairs were unrelated and two were related haplotype matched patients, who were given a completely posttransplant regimen of 10 doses of 80cGy each and 5 doses of ATG during the first 10 days after transplantation (90).

Purified CD34+ cells (3-5x10^6 cells/kg) obtained from G-CSF mobilized donor peripheral blood were enriched on Miltenyi columns and infused into recipients shortly after the completion of TLI (90). Donor cells were harvested about 6 weeks before kidney transplantation and cryopreserved until thawing at the time of the cell infusion. T cell contamination of the purified CD34+ cells was below the threshold expected to cause GVHD (<1x10^5 cells/kg). Chimerism was measured using PCR based short tandem repeat analysis of purified subsets of blood cells. Maintenance IS drugs were prednisone and cyclosporine starting at standard of care doses. The goal of the study was to determine whether IS drugs could be withdrawn from patients with mixed chimerism by the end of the first year posttransplant.

Three of 4 patients developed transient mixed chimerism that was lost during the second and third months posttransplant, and none developed GVHD (90).

Immunosuppressive drug withdrawal was attempted in one patient who developed chimerism for about 1 month, and then donor specific unresponsiveness in the MLR after 7 months. IS drug withdrawal was completed at the end of one year without rejection episodes during withdrawal (90). However, the patient developed a rejection episode during the second year and was returned to standard of care maintenance therapy. Complete withdrawal was not attempted in the other two chimeric patients.

Establishment of Transient Mixed Chimerism, Tolerance, and Complete IS Drug Withdrawal in Patients Given HLA Mismatched Living Donor Kidney and Hematopoietic Cell Transplants Using Anti-CD2 mAb, Thymic Radiation, and Cyclophosphamide Conditioning

A study of 10 patients given combined kidney and bone marrow transplants from HLA haplotype matched living donors was performed by investigators at Harvard University based on preclinical data generated in non-human primates and mini-swine (91–93). The preclinical studies showed that tolerance to MHC mismatched kidney transplants could be achieved using a conditioning regimen of TBI, thymic radiation and anti-T cell antibodies with transient chimerism for a few to several weeks (56, 71, 72, 94).

The follow up clinical study used cyclophosphamide instead of TBI for conditioning along with thymic radiation and anti-CD2 monoclonal antibodies. Maintenance IS drugs posttransplant were used for several months before complete discontinuation in 7 patients (91–93). Nine of 10 patients developed an "engraftment syndrome" with injury to the graft vasculature observed on biopsies obtained shortly after the loss of chimerism during the first month posttransplant (95). Nevertheless, 7 patients were subsequently withdrawn completely from IS drugs, and 4 of 7 remained off the drugs for 4 to 10 years without evidence of subsequent rejection (93). Graft loss was observed in 3 of 10 recipients who were not withdrawn from IS drugs during the first 3 years posttransplant, and 3 recipients developed acute and/or chronic rejection a few years after complete withdrawal (93).

The Harvard group examined mechanisms of immune suppression drug withdrawal and maintenance of graft function in their patients receiving HLA-mismatched combined kidney and bone marrow transplantation (CKBMT) that led to transient (< 3 weeks) donor cell chimerism (96, 97). They reported a marked increase in CD3+CD4+CD25highCD127lowFoxp3+ Tregs early after transplant and showed evidence that these cells originated from the thymic emigrants as well as peripheral expansion (96). Few Treg clones were identified as non-Tregs pre-transplantation which suggested that induction of Tregs from non-Tregs present prior to transplant was not a prominent contributor to the increased Treg population. High- throughput TCR sequencing from circulating cells enabled the tracking of clones pre and post-transplant, as well as the clones that infiltrated the kidneys on post transplantation renal biopsies (96). A relatively high proportion of clones in the post-transplant biopsies were also detected in the pre-transplant and post-transplant circulating CD4+ and CD8+ T cell populations. A relatively high percentage of clones in each biopsy of patients successfully weaned off immune suppression were identifiable as Tregs. These patients had a decrease of donor reactive conventional T cell clones identified in the pretransplant MLR such that non-donor reactive T cell clones represented the most abundant T cell clones in tolerant recipients (96, 97). In contrast, there was a relative high percent of donor reactive clones in the post-transplant biopsy of a graft rejector, and in patients who received conventional transplants (96, 97). The results suggested that peripheral immune regulatory mechanisms and clonal deletion reduce donor reactive clones. It is unclear if, and how, thymic irradiation and anti-CD2 monoclonal Ab administration promoted non-chimeric peripheral 'tolerance' in the Harvard protocol. It can be speculated that Tregs may be spared by treatment with siplizumab and thymic irradiation, and that treatment may result in immune modulatory responses that

enhance Treg emigrants from the thymus, as well as their peripheral expansion.

Establishment of Persistent Mixed Chimerism and Partial IS Drug Withdrawal During the First Year Posttransplant in Patients Given HLA Mismatched Living Donor Kidney and Hematopoietic Cell Transplants Using TLI/ATG Conditioning

Based on the results of the HLA matched tolerance induction trial using TLI/ATG conditioning, Stanford investigators performed a follow up trial to determine whether immune tolerance and complete IS drug withdrawal could be achieved in 22 recipients of HLA haplotype matched living donor kidney transplants (87, 88).The study had 3 goals:1) Establish persistent mixed chimerism for at least one year, 2) During the first year reduce IS drugs to tacrolimus monotherapy in persistent chimeras, and 3) Determine whether tacrolimus monotherapy could be discontinues during the second year.

Recipients were given 10 doses of 120cGy each of TLI and 5 doses of ATG as in the fully matched study, and an infusion of donor CD34+ cells and a defined dose of T cells that was escalated from 3x106 cells/Kg to 150x106 cells/Kg in order to facilitate mixed chimerism (87). During the study the CD34+ dose was increased to a minimum of at least 10x106 cells/kg, by adding one dose of the mobilizing agent, plerixafor, to 5 doses of G-CSF administered to donors. Recipients were given maintenance IS drugs during the first year starting with prednisone, MMF, and tacrolimus. The MMF and prednisone were withdrawn, and patients were maintained on tacrolimus monotherapy at the end of one year, if they had persistent mixed chimerism by STR analysis, no evidence of rejection on protocol biopsy, and no evidence of GVHD (87, 88). Ten of the 22 patients developed chimerism for at least one year while reducing three IS drugs to monotherapy. Thus, the first two goals were achieved.

Study of Withdrawal of Tacrolimus Monotherapy in Mixed Chimeras During the Second Year Posttransplant in Patients Given HLA Mismatched Living Donor Kidney and Hematopoietic Cell Transplants Using TLI/ATG Conditioning

In order to achieve the third goal, complete IS drug withdrawal was attempted during the second year in the 10 haplotype matched patients with persistent chimerism at the end of one year on tacrolimus monotherapy (87, 88). Tacrolimus dosing was gradually tapered while monitoring the chimerism levels of whole blood and T cells, as well as serum creatine values monthly. Levels of T cell chimerism in all 10 patients at the end of the first year, and before tacrolimus tapering, were below 20% (mean 10%) (88).

Tapering to subtherapeutic levels or to discontinuation of tacrolimus was associated with loss of chimerism in the first 6 patients (88). Three of the 6 developed mild acute rejection episodes that were resolved with standard of care treatment. The latter patients as well as 3 of 6 who lost chimerism without evidence of rejection were returned to maintenance IS drugs (88). The remaining 4 of 10 patients were maintained on therapeutic levels of tacrolimus monotherapy with persistence of mixed chimerism with up to 5 years of observation. One of the latter patients with proteinuria, and a biopsy that showed glomerulonephritis without rejection was switched to MMF monotherapy. Graft function has remained normal in these patients.

The instability of mixed chimerism and dependence of chimerism on continuation of IS drugs after one year in these patients contrasts with the observations of mixed chimerism in MHC mismatched rodents that were conditioned with TLI/ATG and given combined organ and bone marrow transplants (38, 39, 44, 75). Mixed chimerism observed starting one month after the donor bone marrow infusion was uniformly stable in the absence of IS drugs even with T cell chimerism levels below 20% in the majority of recipients (38, 39, 44, 75). Stability of mixed chimerism was observed in some groups of rodent recipients given a brief course of cyclosporine, and subsequent withdrawal of cyclosporine (61).

The observation of IS drug dependent mixed chimerism in the patients with HLA mismatched kidney transplants, and the IS drug independent mixed chimerism in MHC mismatched rodents using the same TLI/ATG conditioning regimen suggests that the rodent and human immune systems differ in their responses to the combined transplants. The instability of mixed chimerism in studies of patients given hematopoietic cell transplants to treat hematologic malignancies and anemias based on genetic mutations is consistent with the observations observed in the kidney transplant patients.

It is possible that the low levels of T cells chimerism (<20%) observed in the haplotype matched kidney transplant patients at the end of the first year contributed to the instability of chimerism during IS drug tapering during the second year, and that increased levels of T cell chimerism will allow for complete withdrawal of IS drugs without subsequent rejection. Investigators at Stanford are currently intensifying the conditioning regimen to increase the levels of T cell chimerism to determine the impact on the stability of chimerism during taper and discontinuation of IS drugs during the second year posttransplant.

STUDIES OF ORGAN TRANSPLANT ACCEPTANCE IN HLA MISMATCHED PATIENTS WITH COMPLETE CHIMERISM

Establishment of Complete Chimerism and Withdrawal of IS Drugs in Patients Given HLA Mismatched Living Donor Kidney and Hematopoietic Cell Transplants Using Pre and Posttransplant Conditioning With TBI, Fludarabine, and Cyclophosphamide

Investigators at Northwestern University used a conditioning regimen for their studies of kidney and hematopoietic progenitor transplantation that was developed at Johns Hopkins University

for treatment of hematologic malignancies with HLA mismatched bone marrow transplantation (27). The conditioning regimen included administration of pre-and post-transplantation cyclophosphamide (PTCy), a well- established potent inhibitor of HVG and GVH reactions. More than 50 years ago it was demonstrated that cyclophosphamide was effective at delaying the rejection of MHC mismatched mouse skin allografts when given after compared to before the graft (98). Durable MHC mismatched mouse skin allografts however, were attained only when the combination of pretransplant host conditioning was followed by an infusion of ≥50 million allogeneic donor spleen cells and 48-72 hours thereafter by an intraperitoneal injection of high dose cyclophosphamide (33).

The use of PTCy was translated to a clinical protocol in cancer patients receiving HLA haploidentical bone marrow in a two-cohort study intended to determine if cyclophosphamide could improve engraftment (99) by mitigating HVG reactions. In cohort 1, patients were conditioned with fludarabine 30 mg/m 2 on days −6 to −2, TBI 2 Gy on day −1, and PTCy 50 mg/kg on day + 3. Patients received post grafting immune suppression with mycophenolate mofetil and tacrolimus. In cohort 2, patients received the same regimen, plus the addition of pre-transplantation cyclophosphamide. The majority of patients in the second cohort achieved full donor chimerism. In 2008, a two-center study established PTCy as an acceptable platform for GVHD prophylaxis after haplo-matched BMT (100). Patients received intermediate intensity fludarabine/Cy/TBI pretransplant host conditioning with PTCy on days +3 and +4. The majority of patients achieved full donor chimerism, the incidence of all grades of acute GVHD was 34% and that of severe grade III/IV acute GVHD was 6%. The incidence of chronic GVHD was < 25% (100).

The main mechanism by which PTCy mitigates bi-directional alloreactivity to reduce the incidences of graft rejection and GVHD is to eliminate donor reactive intrathymic host T cells and post thymic host T cells that cause graft rejection, and to eliminate the proliferating alloreactive donor T-cells necessary for GVHD (101, 102). Post transplantation cyclophosphamide was reported to spare *foxp3* + regulatory T cells (T regs), possibly due to the high expression of aldehyde dehydrogenase, an enzyme that metabolizes cyclophosphamide (103).

In the Northwestern protocol, donor cells injected into conditioned recipients included CD34+ cells, and α,β T cells and a unique population of facilitator cells (FC). Two major CD8 + FC subpopulations were described, one is CD56dim/− and the other CD56bright. The majority of CD56dim/− FC were also positive for CD3ϵ and HLA-DR and negative for the dendritic cell markers CD11c and CD123. The CD56dim/− FC comprised approximately half of the FC total. The majority of the CD56bright FC subpopulation were CD19+, CD11c+, CD11b+ and CD3ϵ− and comprised just under half of the FC total (104).

The intensity of host conditioning combined with the composition of cells in the donor inoculum resulted in kidney transplant patients with early and complete conversion to donor chimerism in most recipients (104–106). Complete chimerism is not tolerance to donor alloantigens by recipient immune cells, since no recipient immune cells remain after the HCT procedure. As noted above, non-responsiveness to donor antigens in complete chimeras is due to self-tolerance. Rather, the issue in complete chimeras is GVH alloreactivity and its complications of GVHD.

It is difficult to assess the contribution of the unique FC population in promoting conversion to complete chimerism and mitigating GVH alloreactivity. In single and multi- center studies of cancer patients that received haploidentical bone marrow transplants using unmanipulated donor cell infusions with pre- and post-transplantation cyclophosphamide, over 90% of recipients attained complete donor chimerism (107, 108). The cumulative incidence of 100-day grade 3-4 acute GVHD, and immune suppression requiring chronic GVHD was <8% and 20%, respectively (107, 108).

The Northwestern group reported at least one year of follow-up in 37 patients (36 at Northwestern, 1 at Duke University) treated using their protocol (104–106). Recipients were initially maintained on tacrolimus and mycophenolate-based immune suppression. At six months, if stable renal function, and a normal protocol biopsy were noted, then mycophenolate was discontinued in the chimeric patients. Testing for the absence of donor specific antibodies in complete chimeras would not offer information about the potential for allograft rejection as the recipient is all donor type. Tacrolimus was weaned over the six months that followed, and fully withdrawn at one year if patients continued with complete donor chimerism and normal renal function.

Among the 37 patients, 26 achieved full donor chimerism and 23 (62%) were removed from immune suppression medication. Eight patients with transient chimerism required immune suppression. Two patients failed to establish donor cell chimerism at any level. Two patients had kidney allograft loss during the first year after transplant, and two other patients with full donor chimerism developed immune suppression dependent GVHD one of who died from GVHD (104–106).

Summary of Outcomes of the Clinical Tolerance Protocols With Combined Kidney and Hematopoietic Cell Transplantation

The Harvard, Northwestern, and Stanford University protocols vastly differ from one another albeit all use the concept of combining donor hematopoietic cell infusions, host transplant conditioning, and planned immune suppression drug withdrawal from kidney transplant patients. Comparisons of pros and cons are difficult especially given that relatively few patients have been transplanted therefore the accuracy concerning toxicity, complications and outcomes remains somewhat speculative (**Table 1**).

The Northwestern approach was akin to traditional allogeneic HCT for hematologic malignancies with predictable and significant toxicities, some life threatening, and attainment of complete donor chimerism. Immune suppression drug

withdrawal in the setting of 100% donor chimerism does not reflect HvG tolerance, and a concern with this approach is GVH alloreactivity and development of GVHD in some patients. GVHD was not observed in the Harvard and Stanford protocols.

The Stanford protocol was well tolerated and associated with few if any toxicities above and beyond a standard of care transplant. The conceptual framework is predicated on the protection afforded by persistent mixed chimerism that prevents bi- directional HvG and GvH reactions. Immune suppression independent persistent mixed chimerism in HLA mismatched transplants was not reliably been achieved (87, 88). Rather drug minimization with the use of low dose monotherapy was reported to be required for persistence of chimerism (88). The Stanford protocol is adaptable to deceased donors (https://clinicaltrials.gov/ct2/show/NCT04571203) as host conditioning is entirely post kidney transplant.

The Harvard protocol was associated with the side effect of "engraftment syndrome" in 9 of 10 patients in the first month posttransplant (95). The syndrome was a manifestation of vascular injury to the kidney graft shortly after the loss of chimerism (95). The syndrome was not observed in the Northwestern study, nor in the Stanford study of fully HLA matched patients, but was observed in 2 of 22 HLA mismatched patients shortly after the loss of chimerism in the Stanford study (88). Whereas kidney graft loss was observed in the first three years of Northwestern and Harvard studies, it was not observed in the Stanford study (88, 95). The Harvard study focused attention on mechanisms of peripheral tolerance, the role of Tregs, and acquired deletion of donor reactive recipient T cells in patients with transient chimerism who were off drugs (96).

The Stanford study showed evidence of donor specific unresponsiveness in HLA matched and mismatched chimeric recipients, but did not assay for clonal deletion (85–88). The Northwestern study did not assay recipient cell immune responses due the development of complete chimerism.

COMPARISONS OF PROTOCOLS USED TO TREAT PATIENTS WITH HEMATOLOGIC MALIGNANCIES VERSUS PROTOCOLS TO INDUCE TOLERANCE IN KIDNEY TRANSPLANT PATIENTS

Comparison of Intensity of Host Conditioning for HCT Treatment of Hematologic Malignancy Versus Tolerance Induction

That host conditioning pre-hematopoietic cell infusion is essential for the engraftment of donor cells is beyond reproach. There are dozens of published host conditioning regimens that enable donor cell engraftment, and they vary significantly in the chemotherapy and radiation components included, and in their intensity profile.

Defining the intensity of the conditioning regimen is based on the type and dose of chemotherapy and/or radiation administered, the expected duration and severity of pancytopenia (irreversible, prolonged, minimal), and the requirement for stem cell support (essential, required, optional) (109, 110).

It is well established in allogeneic HCT cancer recipients that as the intensity of host chemo-radiation conditioning increases, donor hematopoietic cell engraftment is more easily attained and conversion to complete donor cell chimerism is the result (110, 111). Complete chimerism (>95% donor type) is a desired goal in cancer patients because complete chimerism by definition does not create tolerance, and donor cell tolerance is not advantageous to patients with cancer (112). Rather, post thymic alloreactive donor T cells that accompany the infused donor cell inoculum are required to provide beneficial anti-tumor reactions important in mediating cancer cures. Complete chimerism, however, comes with the risk of acute and chronic GVHD which is mediated by

TABLE 1 | Comparison of Relative Safety of Clinical Tolerance Protocols.

	Northwestern	Boston	Stanford
Intensity of Conditioning*	+2.5, traditional BMT protocol	+1, nonmyeloablative	0, lowest intensity nonmyeloablative
Conditioning Regimen**	Flu-Cy/TBI and PTCy	Cy/TR/Anti-CD-2/Rituxan	TLI-ATG
***Grade 4 neutropenia	100%	100%	< 5%
***Grade 4 thrombocytopenia	100%	100%	< 5%
***Grade 3 Anemia	100%	Not reported	<1%
ICU stay during 1st 90 days	Estimated $\geq$ 5%	Unclear	None
Chimerism goal	Complete	Transient	Persistent mixed
GVHD risk	>10%	No	No
GVHD related death	Yes	No	No
**** Apparent need for medical teams to support post-transplant care beyond SoC renal transplant team	YES: BMT unit, ICU, Pulmonary, Urology, Infectious Diseases, Gastroenterology	YES: BMT unit	No
Ability to directly translate to recipients of deceased donor transplants	No	No	Yes

Transplant conditioning intensity score based on Ref (109).

**Flu, Fludarabine; Cy, cyclophosphamide; TBI, total body irradiation; PTCy, post-transplant Cytoxan; TR, thymic irradiation; TLI, total lymphoid irradiation; ATG, anti-thymocyte globulin.*

***Based on Common Terminology Criteria for Adverse Events (CTCAE; Version 5. https://ctep.cancer.gov/protocoldevelopment/electronic_applications/docs/ctcae_v5_quick_reference_5x7.pdf).*

****SOC, standard of care.*

alloreactive donor immune cells to the target tissues of GVHD (111). The consequences of GVHD are the main limitation to safe allogeneic transplantation. To use common transplant parlance, the 'holy grail' so to speak in allogeneic HCT for cancer patients is to separate the deleterious graft versus host reactions from the beneficial graft versus tumor reactions.

Numerous trials in cancer patients compared clinical outcomes that helped to define conditioning dose intensity. During the initial development of reduced intensity conditioning regimens for cancer patients 2 Gy of TBI was compared to 2Gy TBI combined with 90 mg/m2 fludarabine (Flu) (112). Pretransplant conditioning with 2Gy TBI alone resulted in a predictable 10-14 days of marked cytopenia, yet 20% of the patients recovered endogenous hematopoietic cells that led to donor hematopoietic graft rejection. In order to reduce the high donor cell rejection rate, Flu was added to the 2 Gy TBI, which resulted in a significant decrease in donor hematopoietic rejection to 3%. The median donor T-cell chimerism levels were significantly higher in the TBI-Flu arm compared to the TBI arm at day +28 (90% vs. 61%, $p < 0.001$) owing to the more profound host immune cell depletion with the addition of Flu that facilitated donor cell engraftment. Patients without disease relapse in the TBI-Flu arm converted to complete donor cell chimerism by day +90. For patients on the TBI-Flu arm, the incidence of clinically significant acute and chronic GVHD was 46% and 48%, respectively, and the transplant related mortality at 1 year was 5%. This randomized trial demonstrated the importance of fludarabine in augmenting the ensuring prompt and durable conversion to complete donor cell chimerism (112).

As the intensity of host conditioning increases, the likelihood of developing clinically significant Grades 3, 4 and 5 adverse event (AE) toxicities increase (109, 110). These toxicities include but are not limited to the GI tract (mucositis/colitis/typhlitis), lungs (pneumonitis and diffuse alveolar hemorrhage), heart (chemotherapy induced cardiac dysfunction and radiation induced vascular damage), and liver (sinusoidal obstructive syndrome). With increases in conditioning intensity marked and prolonged cytopenia develops which may lead to severe neutropenic infections, sepsis, as well as the need for red cell and platelet transfusion support. The regimen related tissue toxicities typically resolve within the first eight weeks of the cell infusion yet they are associated with unanticipated hospitalizations, significant patient morbidity, the need for consultation with medical specialty (such as BMT physicians, pulmonary, ICU, gastroenterology, infectious diseases) teams, and increased transplant related mortality.

A transplant conditioning intensity (TCI) scoring system was developed to help investigators standardize nomenclature and allow a comparison of the many differing regimens (110). For example, and with particular relevance to kidney tolerance host conditioning regimens, points would be assigned based on the doses of TBI, Flu, and Cyclophosphamide (110). The higher the TCI score the more intense the regimen. The performance of the TCI score was tested in over 8200 BMT recipients and regimens were grouped as low intensity (score of 1 and 2), intermediate intensity (score of 2.5- 3.5) and regimens with scores of >3.5 were considered high intensity (110).

Intermediate score regimens were highly predictive of increased early (day +100 and +180) regimen related toxicity and mortality compared to low intensity regimens.

The MGH tolerance regimen in their patients without malignancy consisted of 60 mg/kg of cyclophosphamide on days −5 and −4 with respect to transplantation; a humanized anti-CD2 monoclonal antibody (MEDI 507, MedImmune) on days −1, 0, and +1, thymic irradiation (700 cGy) on day −1, +/- rituximab 375 mg/m2 days −7 and −2 (91). In an effort to mitigate the toxicities of the high-dose cyclophosphamide including the severe cytopenia, gastrointestinal side effects and cardiotoxicity, and to help eliminate the "engraftment syndrome" that occurred in 9 of 10 patients the conditioning regimen was changed and total body irradiation (1.5 Gy x 2, total dose of 3 Gy) was substituted for cyclophosphamide (113). The MGH regimens would score as low intensity conditioning with one point assigned for cyclophosphamide, or 3 Gy TBI. Marked cytopenia would be of short duration and the need for a donor stem rescue would be desirable but not essential or required.

The Northwestern kidney tolerance regimen consists of cyclophosphamide 50mg/kg on days -3 and +3, 2 Gy TBI on day -1, and Fludarabine total dose of 90mg/kg divided equally on days −5, −4, −3 (104, 106) is intermediate intensity with a score of 2.5 based on 1 point for TBI dose, 0.5 points for fludarabine, and 1 point for cyclophosphamide.

This degree of intensity is associated with severe neutropenia, the need for G-CSF administration in all recipients, and the requirement for transfusion support. A "rescue" donor cell graft is not essential yet is highly desirable (required) in order to avoid prolonged blood count recovery and the associated significant health risks.

The Stanford conditioning regimen that uses TLI-ATG falls below the TCI scoring system because the combination of TLI and ATG does not induce clinically significant cytopenia or regimen related organ (gastrointestinal, pulmonary or liver) toxicity. There is no need for transfusion support. The unexpected 100-day re-hospitalization rate in over 600 cancer patients transplanted from HLA matched and mismatched related and unrelated donors was 25% compared to over 80% for patients that were transplanted using 2 Gy of TBI or 2 Gy TBI-Flu (114). The 1-year re-hospitalization rate in the 38 recipients of living related HLA matched and mismatched donor kidneys using the Stanford tolerance regimen was 13%, a value that is not different than contemporaneously treated kidney transplant recipients at the same institution using standard of care methods (87, 88).

The assessment of the risk of developing clinically significant AEs and mortality resulting from toxicity of the intensity of host conditioning is based on scores and indices specifically developed to measure the conditioning regimen intensity. Whereas the MGH and Stanford protocols are non-myeloablative, low intensity regimens the Northwestern protocol follows a

traditional BMT reduced intensity conditioning (RIC) regimen of intermediate intensity and that is associated with increased regimen related morbidity and mortality (110).

Comparison of Donor Cell Inoculum in HCT Treatment of Hematologic Malignancy Versus Tolerance Induction

The composition of the donor cell inoculum is also an important determinant in whether engraftment and chimerism is transient, persistent and mixed, or results in conversion to complete door type. The cellular composition contributes to the risks of developing acute and chronic GVHD, and post-transplant infections, and the risk of transplant related mortality (115–119). Despite over 50 years of allogeneic HCT, however, the immune and progenitor cell composition of grafts is typically not well characterized in clinical practice, except for the number of total nucleated cells (TNC), and CD34+ and CD3+ cells. There is wide variation in the cellular composition even when factors like donor age, sex, the method of collection, and source of hematopoietic cells (marrow or mobilized blood) is controlled (120). The time of day of collection can influence the donor cell composition; HSCs and other progenitor populations do not steadily or randomly circulate under homeostasis, but rather follow a physiologically regulated, rhythmic circadian oscillation of release (121). A report of 85 healthy donors confirmed that afternoon apheresis collections, when the level of circulating catecholamines are at their lowest, resulted in significantly higher average CD34+ cell yields compared to products in which donors underwent apheresis in the morning (122).

As a general consideration, donor cell engraftment is facilitated by increasing the number of donor TNC (total nucleated cells), and CD34+ and CD3+ T cells infused. The cellular composition should also be considered in combination with the intensity of host conditioning. For example, as the intensity of host conditioning is reduced higher numbers of TNC, CD34+ and CD3+ T cells were required to support donor cell chimerism (116, 123, 124). Using low intensity TLI-ATG conditioning in cancer patients who received unmanipulated G-mobilized apheresis products that contained roughly 200-400 x106 CD3+ T cells/kg and >5 x106 CD34+ cells/kg only 60% of recipients converted to complete chimerism by +90 days after the cell infusion (26, 114). Even more challenging is the combination of a low intensity conditioning regimen with a low number of CD3+ T cells, and in this scenario mega-doses (>10 x106/kg) of CD34+ cells were important in helping establish engraftment (125–127). In a small series of cancer patients that received low intensity 2 Gy TBI conditioning, followed by the infusion of a graft containing a low number of column enriched CD34+ cells (range of 3-5 × 106/kg) combined with 2 ×106/kg CD3+ cells, 4 of 5 patients had low levels of transient chimerism that was not sustained beyond three months (127). Taken together, these data confirm an important relationship between host conditioning intensity and the cellular composition of the donor inoculum in terms of achieving chimerism.

In assessing the MGH protocol for patients without malignancy the graft source and cell doses were not specified. Based on the body of their work, however, it is reasonable to presume unmanipulated donor bone marrow harvest products were obtained at the time of kidney harvest. Decades of clinical bone marrow transplantation has confirmed that the interquartile (equal to the difference between the 75th and 25th percentiles) range for TNC and CD34+ cells in a bone marrow graft is about 1.5-4.0 x108/kg and 2-4.5 x106/kg, respectively, and CD3+ T cells range between 20-40 x106/kg for (128). When bone marrow harvest cell doses are combined with the low intensity MGH conditioning regimen, and across mismatched HLA barriers, it is not surprising that donor cells were detectable for <21 days post transplantation. A potential limitation to using bone marrow as a graft source is the low number of hematopoietic cells that can be obtained. In contrast, cell products collected by apheresis following a variety of donor mobilization strategies allows far greater numbers of TNC, and CD34+ and CD3+ T cells that can be, or not, manipulated or enriched.

The Northwestern protocol uses a unique donor cell composition that is a challenge for the reader to understand because details are considered proprietary to a commercial entity and was based on preclinical murine models. The publications highlight that using the CliniMACS (Miltenyi Biotec) system mature GVHD-producing and antigen- presenting cells are removed while HSC, FC, and progenitor cells are retained. The dose of CD34+ cells range from about 1-16 x106/kg, the dose of α,β T cells appear set at 3.8 x106/kg and FC range from 2-12 x106/kg (104–106). It is unclear if the α,β T cells represent an enrichment population with few contaminating cells, or simply a volume adjusted fraction of the CD34+ flow through. Nonetheless, the doses of cells combined with intermediate intensity host conditioning is sufficient to result in conversion to complete donor cell chimerism in the majority of recipients.

There is a panoply of cells in the donor inoculum that appear important in promoting engraftment, and that are not associated with inducing GVHD. In this regard, there is abundant preclinical literature to support that Tregs of donor and recipient origin promote donor hematopoietic engraftment or enable persistent mixed chimerism without inducing GVHD (48, 129, 130). The analysis of 32 cancer patients that received allogeneic HCT and achieved persistent mixed instead of complete chimerism showed that the proportion of Treg cells in the circulation was increased in patients with mixed chimerism (131). The Treg cells were comprised of equal numbers of donor and host-derived regulatory cells. The dendritic cells in the patients with mixed chimerism had a tolerogenic programmed death ligand-1 (PD-L1) profile. The T cells from patients with mixed chimerism showed reduced cytotoxicity against host target cells *in vitro* that was restored following depletion of CD4+ Treg cells. The aggregate of data supports the contention that suppression of bi-directional alloimmune responses in mixed chimerism may be enhanced through peripheral cell-based regulation and raises the potential therapeutic options of Treg cells (131). This concept is currently being investigated in a Stanford and Northwestern collaborative phase 1 study (https://clinicaltrials.gov/ct2/show/NCT03943238)

using the Stanford host TLI-ATG conditioning regimen, a donor graft enriched for CD34+ cells combined with a defined CD3+ T cell dose and a recipient Treg cell infusion in HLA mismatched living donor kidney transplant recipients.

Comparison of Post-Transplant Immune Suppressive Antibodies and Drugs for HCT Treatment of Hematologic Malignancies Versus Tolerance Induction

The addition of donor derived hematopoietic cells imparts new immunologic phenomena not previously considered in clinical organ transplantation with a crossing of a double barrier, there is the known HVG reactions yet this is combined with GVH reactions. Traditionally, high intensity myeloablative and immune suppressive host conditioning regimens administered before the donor cell infusion were to eliminate HVG reactions and facilitate donor cell engraftment, while the need for post-infusion immune suppression mitigated the complications of GVH reactions. With the development of lower intensity regimens in which residual host hematopoietic and immune cell compartments persist, the importance of post grafting immune suppression focused attention to the need to mitigate HVG reactions. The Seattle group used DLA- identical littermate dogs given donor bone marrow cell infusions at clinically relevant doses of 1.9 to 4.4 × 108 TNC/kg within 4 hours of completing low intensity 2 Gy TBI (132). Recipient animals received post grafting immune suppression with cyclosporin (CSP) alone, CSP combined with methotrexate, or CSP combined with mycophenolate mofetil (MMF). Dogs that received post grafting immune suppression with the combination of CSP and MMF had sustained mixed chimerism whereas dogs in the other groups had no, or a low level of transient chimerism that did not persist beyond 30 days. Reducing the dose of TBI to 1 Gy combined with post grafting CSP and MMF failed to result in donor cell chimerism. This important contribution confirmed the need of pharmacological immune suppression post grafting to induce mixed chimerism and inhibit HVG reactions.

The Northwestern and Stanford protocols albeit very different from one another, use as a backbone post grafting Tacrolimus combined with MMF, in part because the above article so heavily influenced, in the right direction, the field (85–88, 104–106). Weaning of immune suppression is dependent on clinical outcomes with a planned tapered to cessation within 12-18 months of organ transplantation. The MGH protocol used single agent CSP as a post grafting immune suppression (91–93).

There are additional post grafting immune suppression considerations. The MGH team used pre- and post-grafting anti-CD2 humanized monoclonal antibody (MEDI- 507), siplizumab, in their ten patient published series (91–93). CD2 is a non-essential cell adhesion molecule found on the surface of T cells and natural killer (NK) cells that interacts with lymphocyte function-associated antigen-3 (LFA-3/CD58) on APCs, most commonly macrophages (133). Yet CD2 has additional multifunctionality acting as a co- stimulatory signal on T and NK cells, and is upregulated on memory and activated T cells. Anti-CD2 monoclonal Abs have been shown *in vitro*, and in clinical studies to induce immune modulatory effects that include upregulation of Treg cells (134). The specific mechanism (s) by which anti-CD2 Ab treatment contributed to the ability to wean immune suppression medication remains to be more clearly defined.

The Northwestern group used high-dose, post-transplantation cyclophosphamide (PTxCy). Post-transplantation cyclophosphamide represents the culmination of over 50 years of preclinical research dedicated to inducing immunologic tolerance (27). The use of PTxCy is particularly attractive because the treatment is inexpensive, strikingly effective at depleting alloreactive HVG and GVH T cells, and requires no special manipulation beyond the administration of high dose IV chemotherapy. Post grafting anti-CD2 Ab administration and PTxCy have important roles in contributing to the clinical outcomes of the kidney tolerance protocols.

Approaches to Prevent GVHD After HCT Treatment in Hematologic Malignancies and Tolerance Induction

A main limitation to safe treatment of hematologic malignancy with allogeneic HCT is that donor T cells infused at the time of transplant are the critical mediators of GVHD even when GVHD appears months after the infusion. The intensity of alloreactivity is proportional to the degree of HLA mismatch between donor and recipient (24). Studies that infused a T cell depleted (TCD) donor graft (CD3+ T cells < 105/kg) following high intensity myeloablative host conditioning reported significantly less acute and chronic GVHD (24). Post grafting immune suppression was not needed in these trials because the high intensity conditioning effectively eliminated host immunity and prevented for the most part HVG reactions and graft rejection. The infusion of a product that contained >3-log fold *in vitro* T cell depletion sufficiently removed enough alloreactive T cells that GVHD was markedly reduced (135). Yet despite the marked reduction in GVHD, overall patient survival was not increased in these studies due to a higher rate of disease relapse (there is reduced GVT reactions without sufficient number of donor T cells), and increased patient mortality due to infection. T cell replete syngeneic twin transplants developed little GVHD even in the absence of post-transplant immune suppression medication (136). The infusion of donor lymphocytes (DLI) to convert mixed to complete chimerism, or as treatment for disease relapse induced acute GVHD in a dose dependent manner (137). GVHD was not observed when the dose of CD3+ T cells in an unmanipulated DLI was <10 x 106/kg, whereas 10%, 30% and 50% of recipients experienced clinically significant and severe GVHD when infused with a DLI containing T cells doses of 10 x106 CD3+ T cell/kg, 50 x106/kg and 100 x 106/kg, respectively (137).

The risk of GVHD extends beyond just the dose of infused T cells and is also dependent on the level of chimerism attained. Whereas mixed chimerism protected against GVHD, complete chimerism associated with the risk of developing GVHD. Studies confirmed that there is a precipitous fall in the risk of GVHD once there is more than 10% residual lymphoid (CD3+) cells

(138), and virtually no GVHD risk if the percent donor T cells remained below 75%. The majority of patients treated using the Northwestern protocol rapidly converted to complete donor type and therefore patients on this protocol were at risk for acute and chronic GVHD. In fact, GVHD has been reported by the Northwestern investigators including death from GVHD and its associated complications.

The removal of Tnaive cells from the donor cell inoculum by CD45RA column depletion is a platform to promote donor cell engraftment with a low risk of clinically significant GVHD even after conversion to complete donor chimerism (139, 140).

Enrichment for CD8+ T memory cells following CD45RA depletion also promoted conversion to complete chimerism with perhaps even lower GVHD risk then a CD45RA depleted graft as CD4+ memory T cells may promote GVHD (141, 142). Another graft manipulation strategy that may not significantly affect the likelihood of donor cell engraftment yet that limits GVH reactivity and that is in clinical trials is based on depletion of the lymphocyte population primarily responsible for GVHD, namely, T lymphocytes carrying the αβ chains of the T-cell receptor (TCR), coupled with B-cell depletion accomplished through the use of an anti-CD19 monoclonal Ab (143). These studies confirmed prompt donor cell engraftment with a low risk of GVHD even across major HLA barriers (143). Clinically significant viral infections limit the broad applicability of this strategy. The point being that there are a multitude of cellular components in the donor inoculum that may have significant impact on the outcome. It would be helpful to clinical researchers in the field if the three tolerance programs invested effort to more comprehensively identify the cellular composition of the donor graft used in their trials and this would include providing the details regarding populations of cells with unusual phenotypes.

REALTIONSHIP BETWEEN CHIMERISM AND TOLERANCE IN LABORATORY ANIMALS AND HUMANS

Differences in the Stability and Linkage of Chimerism to Tolerance in Humans Versus Laboratory Animals

The rodent models summarized in this review uniformly demonstrated that mixed chimerism and tolerance were stable after MHC matched and mismatched combined hematopoietic progenitor and organ transplantation (38, 39, 44–52). The observations in the cited rodent models were not reflected in large animal models and in humans. However, the use of additional strains of rodents and additional conditioning regimens may provide improved models for large animals and humans. Mixed chimerism was frequently transient, unstable, and dependent on the presence of IS drugs in humans (87, 88). The strong link between tolerance and persistent chimerism in rodents was also not observed in humans. Acceptance of organ grafts and tolerance of recipient immune cells to donor alloantigens continued in most MHC matched and in some mismatched humans withdrawn from IS drugs even after loss of mixed chimerism (85–88).

In the case of complete chimerism, concordant observations were made in humans and laboratory animals. Complete chimerism was achieved in HLA mismatched patients given combined kidney and hematopoietic progenitor transplants at Northwestern University, and was stable (104–106). Acceptance of donor organ grafts in the complete chimeric patients was based on self-tolerance rather than on tolerance of recipient immune cells to alloantigens.

The tolerance induction clinical trials performed at Stanford and Harvard Universities represented a bench-to-bedside translation of preclinical models of mixed chimerism and organ transplantation tolerance to clinical medicine to establish proof of principle that scientific approaches can be used to treat diseases in humans. Each used a center-specific approach to establish mixed chimerism: In one case there is transient, short duration mixed chimerism, in another persistent and often permanent mixed chimerism. There are pros and cons with each approach and when considered as an aggregate they provide insight into some of the fundamental concepts and mechanisms that underlie immune suppression drug withdrawal chimerism-based tolerance protocols.

Mixed chimerism in these studies was uniformly above 1% of donor type cells in the recipient blood and lymphoid tissues, and in almost all instances above 10%. This level of chimerism is referred to as "macrochimerism". In contrast, some studies of organ with or without hematopoietic progenitor transplantation have reported the development of stable "micro-chimerism". In 1993 Starzl and colleagues reported a small cohort of liver transplant recipients that spontaneously developed very low levels of donor hematopoietic cell chimerism detectable only by high resolution PCR testing that was subsequently termed micro-chimerism (<1% donor type: the liver itself can act as a hematopoietic cell reservoir and bring forth donor hematopoietic progenitor cells after transplantation). Subsequent withdrawal of immune suppression medication with maintenance of normal graft function was seldom accomplished (144, 145). This report led to the development of protocols that evaluated combined deceased donor organ and vertebral body (VB) bone marrow cell infusions to induce chimerism and promote drug minimization and graft survival. By 2003, it was reported that 400 liver, 125 kidney, 28 heart, and 25 kidney and pancreas transplants received VB bone marrow cell infusions from their deceased organ donor (144–149). Yet unlike the mixed chimerism and transplant tolerance approaches used above for HLA mismatched recipients, host conditioning was not given in these early trials and consequently anything beyond transient micro-chimerism was not established.

To provide context to chimerism-based kidney tolerance protocols the lessons learned from more than five decades of clinical allogeneic HCT in more than 1 million cancer patients that received donor grafts should be considered. Patients receiving allogeneic hematopoietic cells are prepared with pre-hematopoietic cell infusion 'host conditioning' that consists of chemotherapy alone or in combination with radiation therapy

and/or antibodies directed to immune cells. The pretransplant host conditioning is essential to establish donor hematopoietic cell chimerism and serves two critical purposes: to deplete and suppress the recipient immune cells that would otherwise reject the infused donor cells, and to deplete host bone marrow progenitor cells from their marrow niches to create 'marrow space' and enable donor stem cell replacement and engraftment.

Hypothesized Relationships Between Chimerism and Tolerance in Humans and Laboratory Animals

In context of protocols that use donor hematopoietic cells, tolerance is perhaps easiest to conceptualize in the setting of persistent mixed chimerism. With persistent mixed chimerism, donor APCs in the thymus present donor Ag to developing host T cells and induce deletion of alloreactive HVG T cells. Similarly, with mixed chimerism host APCs in the thymus present host self Ag to donor T cells and induce deletion of GVH reactive T cells. This bi-directional clonal deletion establishes central tolerance (**Figure 4**). Yet central tolerance is not enough to allow immune suppression drug withdrawal without the risk of HVG (organ graft rejection) and GVH reactions. This is because mixed chimerism following low intensity host conditioning implies there are residual long living host post thymic T cells in the recipient that can mediate organ transplant rejection. Likewise, there are post thymic donor T cells that accompany the hematopoietic graft and that may persist perhaps even for decades and provide GVH reactions. Therefore, it is likely that peripheral mechanisms that help control bi- directional alloreactivity may also be needed. The Stanford protocols induced persistent mixed chimerism in the majority of recipients of HLA matched and mismatched recipients and consequently it is fair to assume based on current paradigms, that central tolerance was achieved (85–88). In the HLA matched setting, and after 6-months of persistent mixed chimerism, immune suppression drug withdrawal resulted in immune suppression independent continued mixed chimerism in about half of patients and a loss of chimerism in half (85–88). Whether mixed chimerism persisted or not after the complete withdrawal of immune suppression medication, kidney allograft rejection episodes were not observed with follow up extending to beyond 12 years (85–88).

These findings suggest that in the HLA matched setting, 6-months of persistent mixed chimerism may be a sufficient condition to induce long-lasting central tolerance that can control HVG alloreactivity and prevent graft rejection. The contribution and need for peripheral regulation in this setting is less defined. In a few instances however, patients who had had persistent mixed chimerism and subsequently lost the donor cells after immune suppression drug withdrawal, years later had an acute rejection episode. This suggests that central tolerance may not be a permanent state in a few patients or that peripheral regulation was lost.

In the HLA mismatched Stanford tolerance protocol low dose single drug immune suppression was required for persistent mixed chimerism as complete drug withdrawal resulted in loss of chimerism that was associated with acute rejection episodes (86, 87). This implied that in the HLA mismatched setting the development of 'central tolerance' may be imperfect. Alternatively, there may also be more need for peripheral

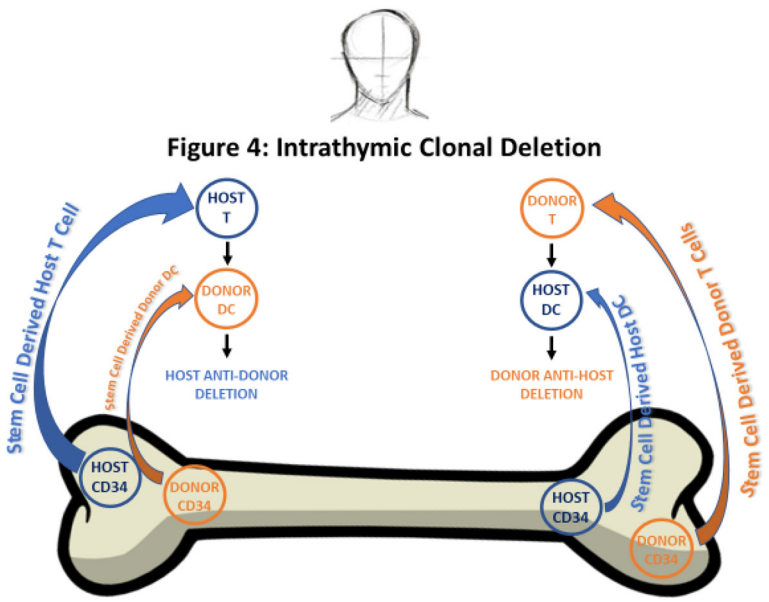

FIGURE 4 | Intrathymic Clonal Deletion: Newly generated donor and host T cells do not cause GVHD or graft rejection in mixed chimeras. Newly generated naïve donor T cells are clonally deleted against host alloantigens when T cell precursors interact with donor intrathymic DCs derived from residual host CD34 cells. Newly generated naïve recipient T cells are clonally deleted when T cell precursors interact with intrathymic DCs derived from injected donor CD34 cells.

immune regulation to prevent HVG alloreactivity mediated by long living post thymic host T cells not eradicated by TLI-ATG. In the newest iteration of the Stanford HLA mismatched protocol a single and very low dose of TBI (0.4-0.8 Gy) will be substituted for the last dose of TLI to provide additional host T cell depletion (decrease HVG reactions) and improve the levels of mixed chimerism; higher levels of chimerism within the first 6 months are expected to improve its stability even after immune suppression drug withdrawal. In a recently completed clinical trial in cancer patients TLI-ATG with a single very low dose of TBI host conditioning resulted in improved early chimerism without the toxicity associated with 2 Gy of TBI (https://clinicaltrials.gov/ct2/show/NCT03734601).

SUMMARY AND CONCLUSIONS

The key observations of the link between chimerism and transplant tolerance were initially developed in rodents, and then studied in large laboratory animals and humans. Whereas stable mixed chimerism and organ graft acceptance without IS drugs was achieved in most MHC mismatched models of tolerance induction in rodents, it has been achieved only in MHC matched large animals and humans at present. Stable mixed chimeras showed specific immune unresponsiveness in both the HvG and GvH directions. Stable complete chimerism was achieved in MHC mismatched laboratory animals and humans given HCT as treatment for hematologic malignancies or for withdrawal of IS drugs with organ transplant acceptance. In the latter complete chimeric recipients, kidney graft acceptance was based on self-tolerance rather than HvG tolerance. Safety issues associated with a protocol that resulted in complete chimerism remain a clinical concern (**Table 1**) and included severe reductions in neutrophils and platelets due to the intensity of conditioning, and the increased risk of severe GVHD. Going forward, and as always, the successful widespread acceptance and adaptation of a cell-based immune suppression drug withdrawal protocol to other centers and to patients in-need considers the balance between the safety of the procedures, associated morbidities, the long-term risks, and the ability to significantly reduce and/or completely withdraw drug.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Transplant Tolerance, Not Only Clonal Deletion

Bruce M. Hall, Nirupama D. Verma, Giang T. Tran and Suzanne J. Hodgkinson*

Immune Tolerance Laboratory, School of Medicine, University of New South Wales (UNSW) Sydney, Ingham Institute, and Renal Service and Multiple Sclerosis Clinic, Liverpool Hospital, Liverpool, NSW, Australia

The quest to understand how allogeneic transplanted tissue is not rejected and how tolerance is induced led to fundamental concepts in immunology. First, we review the research that led to the Clonal Deletion theory in the late 1950s that has since dominated the field of immunology and transplantation. At that time many basic mechanisms of immune response were unknown, including the role of lymphocytes and T cells in rejection. These original observations are reassessed by considering T regulatory cells that are produced by thymus of neonates to prevent autoimmunity. Second, we review "operational tolerance" induced in adult rodents and larger animals such as pigs. This can occur spontaneously especially with liver allografts, but also can develop after short courses of a variety of rejection inhibiting therapies. Over time these animals develop alloantigen specific tolerance to the graft but retain the capacity to reject third-party grafts. These animals have a "split tolerance" as peripheral lymphocytes from these animals respond to donor alloantigen in graft versus host assays and in mixed lymphocyte cultures, indicating there is no clonal deletion. Investigation of this phenomenon excludes many mechanisms, including anti-donor antibody blocking rejection as well as anti-idiotypic responses mediated by antibody or T cells. This split tolerance is transferred to a second immune-depleted host by T cells that retain the capacity to effect rejection of third-party grafts by the same host. Third, we review research on alloantigen specific inhibitory T cells that led to the first identification of the $CD4^+CD25^+$T regulatory cell. The key role of T cell derived cytokines, other than IL-2, in promoting survival and expansion of antigen specific T regulatory cells that mediate transplant tolerance is reviewed. The precise methods for inducing and diagnosing operational tolerance remain to be defined, but antigen specific T regulatory cells are key mediators.

Keywords: clonal deletion, graft versus host disease, transplant tolerance, regulatory T (Treg) cells, chimerism

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Edited by:
Nina Pilat,
Medical University of Vienna, Austria

Reviewed by:
Georgia Fousteri,
San Raffaele Hospital (IRCCS), Italy
Katayoun Ayasoufi,
Mayo Clinic, United States

****Correspondence:***
Bruce M. Hall
b.hall@unsw.edu.au

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Abbreviations: ALS, Anti-lymphocyte serum; CML, Cell mediated lysis; CSA, Cyclosporine A; CTL, Cytotoxic T cell; GVH, Graft versus host; mAb, Monoclonal antibody; MHC, Major histocompatibility complex; MLC, Mixed lymphocyte culture; TDL, Thoracic duct lymphocytes; Treg, T regulatory cell.

THE ORIGIN OF THE CLONAL DELETION THEORY OF TRANSPLANT TOLERANCE

For over 60 years, the concept of clonal deletion has dominated the field of immunology and the quest for acceptance of transplanted tissue without ongoing immunosuppression. The clonal theory for immune cells and the concept that during ontogeny self-reactive clones are deleted, was made at a time when the function of lymphocytes and the existence of T cells was not appreciated. Because of this, most clinical attempts to induce transplant tolerance aim to delete specific alloreactive cells and the establishment of lympho-haemopoetic chimerism.

Transplant tolerance can be induced in the presence of clones reactive to the graft and in the absence of lympho-haemopoietic chimerism, however. There are many animal models of operational tolerance, where grafts continue to function without immunosuppressive therapy. *Ex vivo* expanded Treg promote tolerance induction (1). In most there is no deletion of alloreactive clones.

This review revisits the findings that led to the theory of clonal deletion and transplant tolerance and describes innumerable mechanisms that control the rejection of allografts without deletion of alloreactive clones. A variety of models of operational tolerance are described, including the spontaneous acceptance of liver grafts and the induction of specific unresponsiveness in murine and swine models by short-term therapy to minimize early rejection. These models do not produce clonal deletion. This review focuses on the induction of alloantigen specific T regulatory cells (Treg) and their role in the generation of "Operational Tolerance" to allografts. These forms of operational tolerance raise the possibility that attempts at clonal deletion have confused the field and may be misguided.

Self and Non Self

In 1949, Burnet and Fenner sought to explain why antibody was not generated against self-antigens (2) and how foreign antigen was recognized as non self. They proposed that "self" was defined during embryonic development. The key observations that led Burnet to propose the clonal deletion hypothesis are summarized in **Figure 1** and **Supplementary Table 1**. First was Owen's observations in dizygotic bovine twin calves who share a placenta *in utero* causing cross circulation of blood. These twins throughout life share each other's red cell groups (3, 4). These twins produce red cells of their twin, as well as their own red cells, and are haemopoietic chimeras. The Clonal Deletion theory was also supported by an earlier observation by Taube who reported that viral infections acquired *in utero* did not induce antibodies to the virus, whereas mice infected postpartum eliminated the virus (2, 5).

Until the 1960s, immunity was only considered in the context of an antibody response. Jerne in 1955 (6) proposed small amounts of antibody to antigen circulates in blood and when bound to antigen, the complex induces cells to produce more antibody to the antigen. Burnet modified Jerne's theory to hypothesise that cells had pre-formed antibody to only one antigen, and that antigen activated these clones to produce antibody to the antigen (7, 8). That B cells produce only one specific antibody, was demonstrated by Burnet's student Gus Nossal, together with Nobel Laureate Joshua Lederberg, in experiments using an assay of flagella immobilization after immunization with two bacteria with different flagella antigens (9, 10).

At that time, the fate of lymphocytes was unknown. There were two hypotheses; one that lymphocytes could differentiate into many different cell types, and the second that they were terminally differentiated cells that could not divide (11). The central role of lymphocytes in immunity was not appreciated until Gowan's work on thoracic duct lymphocytes (TDL) in the early 1960s (12, 13). Thus, the clonal deletion theory was

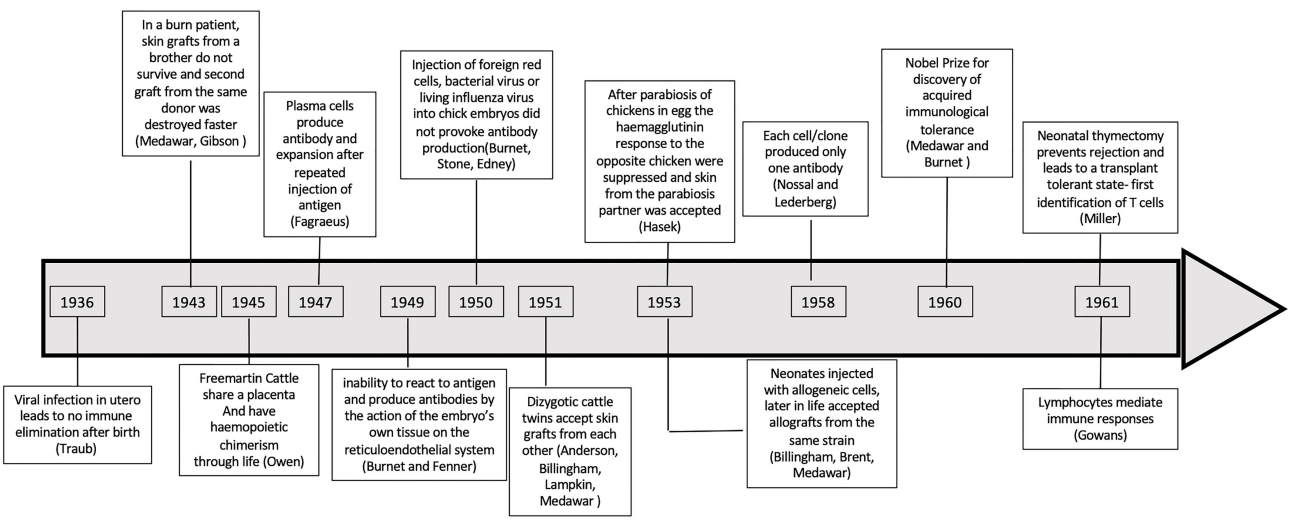

FIGURE 1 | Timeline of observations that supported clonal deletion *in utero* and in neonates. A chronological representation of studies that led to establishment of clonal deletion to explain tolerance.

accepted before the role of lymphocytes in immunity was known or recognized.

Transplant Rejection

The quest for transplant tolerance arose from work to examine if allogeneic tissue could be accepted, so making tissue transplantation clinically possible. To understand why skin grafts failed in burns patients (14), Peter Medawar went from bedside to bench. He observed rabbits that had rejected a skin graft, had accelerated second-set rejection of a subsequent graft from the same donor but not of third party grafts (15). This suggested rejection was an immune process. Prior studies on transplanted tissue had not supported an immune mediated response (16), but others did (17). The inflammation causing loss of a skin graft was associated with a lymphocyte, not a granulocyte infiltration (18). At that time antibodies, not lymphocytes, were considered the mediator of rejection (19).

Medawar's group was asked by the Animal Breeding Research Organization to perform skin grafts between cattle twins as a means of distinguishing fraternal and identical twins. Fraternal twins accepted the other's skin grafts but rejected third party grafts (18, 20, 21). The twin calves shared red cells and the possibility they were chimeras was raised, as female twins had male cells (18).

Work of Gorer and Snell, starting in the late 1930s with breeding of congenic strains of mice, identified the Major Histocompatibility Complex (MHC) as the genes that promoted rejection of transplanted tissue and tumours (22–24). Highly inbred strains provided models with known MHC incompatibility, that were used to define the mechanisms of rejection and transplant tolerance.

Induction of Transplant Tolerance *In Utero* and in Neonates in Murine Models

In 1953 Billingham, Brent and Medawar injected newborn mice with donor cells, and found that later in life these mice accepted specific donor skin grafts and normally rejected third party grafts (25–27). Woodruff replicated these findings in neonatal rats (28, 29). Skin grafts applied to newborn rats were accepted to varying degrees and second skin grafts from the same donor strain were delayed in rejection, as were donor strain thyroid grafts (30).

Transfer of normal unsensitised recipient strain cells to mice with tolerance induced slow rejection of some but not all grafts (31). A second donor strain skin graft often was slowly rejected without affecting the original graft, suggesting some anti-donor immune reactivity was present. It was proposed that there is incomplete clonal deletion (28). Some mice induced to become tolerant at birth developed runt disease (26) and autoimmunity (32) indicating an aberrant interaction of the tolerizing process and immune responses to self. These unexpected findings were not explained at that time. They suggest a loss of autoimmunity control mechanisms.

Induction of Transplant Tolerance in Developing Chickens

In the early 1950s, skin grafts in chicken eggs and newborn chickens were also studied albeit histocompatibility could not be matched (19). In a small proportion of transplants, skin grafts between newly hatched chickens of different strains had prolonged survival with good feather growth (19, 33, 34). In some chicks, there was delayed loss of feathers from chronic rejection and these had a lymphocytic infiltrate (35). However, chicks with grafts that appeared tolerated rejected a second skin graft from the same donor but usually retained the original graft (36). This suggested graft accommodation and there was no specific systemic transplant tolerance.

Hasek in Czechoslovakia showed parabiosis of chicken eggs suppressed the haemagglutinin response to the paired chicken (37) and a skin graft from the parabiosis partner was accepted (37, 38). This work was published, in a Czech journal of limited circulation, in the same year that Medawar's group reported neonatal induced tolerance in mice. Hasek interpreted his findings in relation to the Stalinist theories enforced by Lysenko and Michurin, which ignores genetics (39). When aware of Billingham, Brent and Medawar's findings in mice, Hasek re-interpreted his experiments significance for transplant tolerance (40).

In birds, embryonic cross transfusion of RBC alone induced graft survival as did bone marrow cells (37). Embryonic cross-transfusion was most effective at 12-18 days post fertilization, suggesting early exposure to alloantigen is required (41).

In 1957, Simonsen reported leukocytes induce reactions on chick membranes (42) as reported by Murphy in 1916 (43). Transfer of white cells to the embryo results in non-antigen specific delay in rejection of skin, as the injected cells induce splenomegaly in a GVH reaction (34). Cross transfusion with blood from chicks of the same strain as the donor, but not the actual donor, prolongs donor skin graft survival, showing the pre-treatment with allogeneic cells is not always alloantigen specific (44). The non-alloantigen specific immune depletion is due to GVH response mediated by transferred immunocompetent cells.

Studies with chicken eggs replicated those with murine models. Both can induce alloantigen specific tolerance, but there also can be non-specific immunosuppression due to GVH response mediated by transferred cells.

In 1960, Medawar and Burnet shared the Nobel Prize for Medicine and Physiology "*For discovery of acquired immunological tolerance*". The key observations leading to the concept of clonal deletion are listed in **Figure 1** and **Supplementary Table 1**. Although, at that time there was evidence for clones of B cells, the thymus was considered irrelevant. The data on antigen specific tolerance in neonates was consistent with immune ignorance that could be due to clonal deletion or specific inhibitory mechanisms.

Induction of Transplant Tolerance *In Utero* and in Neonates in Large Mammals

The experiment of nature in Freemartin cattle demonstrates that Medawar type tolerance induction could occur in large mammals. To examine if Medawar-like transplant tolerance can be induced in larger animals, MHC incompatible bone marrow depleted of T cells were infused *in utero* to miniature swine. Induction of tolerance was evident by induction of chimerism, low reactivity of lymphocytes to donor alloantigen and acceptance of a donor kidney allograft (45, 46).

Combined, these studies support the notion that donor alloantigen during embryonic development induced a state of immune hypo-responsiveness to tissue transplant from the same donor strain. This was interpreted as clonal deletion.

Evidence That Exposure to an Alloantigen *In Utero* or at Birth Does Not Always Induce Clonal Deletion

The mechanism of neonatal tolerance induction is not universal and the reason for the failure to induce tolerance was not considered. Not all strain combinations are susceptible to neonatal transplant tolerance induction (47) and this is due to MHC and non-MHC genes (48). In some animals, second donor strain grafts were rejected, albeit often slowly, showing tolerance and therefore clonal deletion was incomplete. Many studies at the time indicated the process of transplant tolerance is not simply clonal deletion.

If transplant tolerance was solely due to clonal deletion, infusion of normal naïve immune cells would cause allograft rejection. TDL effect rejection of long surviving skin grafts on tolerant animals (49). Transferred syngeneic lymphocytes proliferate in tolerant hosts but later host cells produced by the thymus mediated anti-donor reactivity (50, 51). Transfer of host strain naïve lymphoid cells do not always break tolerance, even when large numbers of cells are transferred (31). Parabiosis of an animal with long-term transplant tolerance with a naïve host does not always break tolerance (52), but in other studies tolerance is broken (31).

Role of Donor Haemopoietic Chimerism in Maintenance of Transplant Tolerance

Persistence of neonatally induced tolerance requires maintenance of lymphoid chimerism (53, 54) including in the thymus (55). The most potent cells for inducing neonatal tolerance are bone marrow (31), although cells from kidney, testes and spleen can also induce tolerance (25). Chimeric cells enter the thymus where they tolerise T cells (55). A skin graft to a neonate can also induce tolerance (30). Persistence of tolerizing antigens is required to maintain tolerance, as treatment with allo-antisera to deplete chimeric cells abolishes tolerance (56, 57). Transfer of neonatal tolerance to irradiated syngeneic hosts, requires transfer of chimeric cells (53).

The Response of Donor Cells Against Recipient- Graft Versus Host Assays

A GVH response by lymphoid cells is usually by transfer to a host that will not react against the transferred cells (42), usually a F1 hybrid of donor x recipient. Lymph node cells and blood cells (26), as well as small TDL mediate GVH (58), described at that time as runt disease (12). TDL also induces runt disease in rats (59, 64). The small lymphocytes transform into large pyroninophilic cells that divide (12). These cells were similar to cells that may produce antibody described by Fagreus (60) and lymph node and spleen cells induced by a skin homograft (61).

Injection of parental strain lymphoid cells into an F1 host, particularly if the host was irradiated, induce a similar reaction (62, 63). Cells from adult homologous lymphoid tissues induce runt disease in embryo chickens (42), newborn mice (26) and newborn rats (64), suggesting lymphoid cells mediate this GVH reaction (26).

Testing of peripheral lymphoid cells from animals with neonatal tolerance in GVH assays (65) and in mixed lymphocyte cultures (MLC) (66) showed lack of reactivity to specific donor alloantigen but have normal response to third party alloantigen. These studies were interpreted to support the clonal deletion hypothesis for neonatal tolerance.

The Role of Lymphocytes in Transplant Tolerance

In 1954 Lord Florey stated "nothing of importance is known regarding the potentialities of lymphocytes other than that they move and that they reproduce themselves" (67). This reflected the conclusion of doctoral studies by Jean Medawar, wife of Peter Medawar, who was a student in Florey's department. She had cultured lymphocytes from TDL and show they did not spontaneously differentiate (11). In the 1950's Gowans, another member of Florey's department, showed thoracic duct lymphocytes in TDL recirculate from blood into lymphoid tissue and then back to lymph (68, 69). Later, Gowans showed small lymphocytes initiate immune responses (13, 58), develop into antibody producing cells (70), promote rejection of allografts (49) and GVH responses (12). He showed TDL include both T and B cells (71).

B Cells

In the mid 1950s, it was shown that bursectomy in chickens impairs antibody production (72) and reduces lymphocyte numbers, but has no effect on rejection of skin allografts (73). In birds, the Bursa of Fabricius was considered similar to the thymus, in that it was a lymphoid organ present in early life that atrophies (74). At that time lymphocytes and the thymus had no known immune function and adult thymectomy had little effect on antibody production (75–77). All immune responses were attributed to antibody, including graft rejection.

Attempts to accelerate graft rejection with antigraft antibody are unsuccessful, whereas sensitised lymphoid cells transfer alloantigen specific rejection (78). In other studies, the presence of anti-donor antibodies delays rejection and enhances survival of the graft, inducing a form of tolerance (77, 79, 80). Preformed alloantibody lead to hyper-acute rejection in man (81), sheep (82) and rats (83), however. The role of B cells in clonal deletion and in the mediation of transplant tolerance is beyond the scope of this review. The central rejection mechanism is a T cell response, both $CD4^+$ and $CD8^+$ T cells (84).

The Thymus

In 1961, Miller reported that mice thymectomized within 16 hours of birth were lymphopenic in blood and lymphoid organs, with deficiency in germinal centres and plasma cells (85–87). The neonatally thymectomized mice, accept allogeneic skin grafts from 41 to over 100 days, whereas sham thymectomized and normal mice reject all grafts in 10-11 days. The animals with surviving skin grafts were described as tolerant, but many died of

runt disease. At that time runt disease was considered due to infection and was not induced in specific pathogen free mice (88). Runt disease resembles GVHD (89) and a form of autoimmunity seen in FoxpP3 deficient mice (88).

In chickens, thymectomy of neonates, led to an inability to reject a skin allograft but preserved antibody responses (8). Neonatally thymectomized rats (90) and nude mice (91–94) also do not reject allografts. Neonatally thymectomized mice that were grafted with a host strain thymus, and mice thymectomized 5 days after birth rejected skin grafts (87). The thymus is required for recovery of lymphocytes after whole body irradiation (95). Involution of the thymus increases susceptibility to autoimmunity (96), suggesting the thymus plays a key role with aging in maintaining immunity.

Burnet and Jerne immediately predicated that the thymus would be a site of clonal deletion of self-reactive cells (97, 98), which turned out to be true (99–101). On the other hand, after Miller described the effects of neonatal thymectomy (86), Sir Peter Medawar stated in 1962; *"we shall come to regard the presence of lymphocytes in the thymus as an evolutionary accident of no very great significance"* (102).

The fact that T cells, like B cells were clonal was established by the identification of T cells that respond to a specific alloantigen (103) and that sensitized hosts have memory T cells for specific sensitizing alloantigen (104). The cloning of an immunoglobulin like molecule as an antigen specific T cell receptor (105, 106) and the generation of T cell clones by repeated stimulation with antigen, reviewed (107), confirmed there were antigen specific T cell clones. The finding that T cells identify antigen presented by MHC molecules (108), further confirmed T cells were antigen specific.

Clonal Deletion in the Thymus

The developing $CD4^+CD8^+$T cells in thymus undergo a complex selection process. This has been extensively investigated over the last 60 years as set out in other reviews (109). This review will be limited to and focus on the thymus and T cells role in transplant tolerance.

The majority of thymocytes have no affinity for MHC and by neglect die by apoptosis (110). Thymocytes with strong affinity to self MHC, also die by over activation (110). In this step, APC activate thymocytes that recognize host and they are deleted by apoptosis (101).

After surviving in the thymic cortex, thymocytes enter the medulla where they contact autoantigens. Here, self-reactive T effector lineage cells are deleted and $FoxP3^+$Treg lineage that recognize autoantigen survive (111). T cell anergy to antigen requires continued exposure to antigen (112).

The AIRE (autoimmune regulator) molecule plays a major role in deletion of autoreactive cells and promotion of auto-antigen protective Treg, as reviewed (113). AIRE is expressed by thymic epithelial cells located in the medulla of the thymus. These thymic epithelial cells also express class II MHC and CD80. Expression of AIRE, Class II MHC and CD80 on thymic epithelial cells can be observed in day 14-15 mice embryos.

AIRE promotes promiscuous gene expression by thymic epithelial cells, which includes hundreds of genes whose expression is normally restricted to peripheral specialized tissues. Effector T cells with TCR recognizing these autoantigens expressed by Class II MHC on thymic epithelial cells, causes their deletion and central tolerance. Similarly host dendritic cells in thymic medulla, also promote deletion or anergy in thymocytes that recognize self- antigen, to prevent autoimmunity. Thymic epithelial cells are more tolerogenic for $CD4^+$T cells than $CD8^+$T cells (100).

Donor cells given to induce neonatal tolerance can enter thymic medulla of the host and promote central tolerance by induction of anergy or apoptosis of T cells recognizing the donor alloantigen. In this latter process, donor alloantigen selects for survival of $CD4^+CD25^+FoxP3^+$Treg, discussed further below. These mechanisms are of relevance to tolerance models where there is chimerism and the thymus is essential (114).

Probably due to murine studies with neonatal thymectomy and the limited consequences of thymectomy in adults, the role of the thymus during life has been underappreciated. During life, the thymus continues to produce naïve T cells, and presumably naïve $CD4^+CD25^+FoxP3^+$Treg cells (115). After deletion of peripheral T cells by irradiation, chemotherapy as in bone marrow transplantation (116) or HIV infection (117), the peripheral T cell pool is re-established by expansion of remaining T cells in the periphery and later in a delayed fashion by generation of naïve T cells in the thymus (116). IL-7 in thymus promotes production of naive T cells that are exported to the periphery. These cells protect against infection and malignancy, as well as autoimmunity (118).

The thymus by deleting new alloreactive naïve T cells and selecting alloreactive Treg probably contributes to tolerance induction in adults as well as *in utero* and newborn.

T Regulatory Cells and the Thymus

Thymocytes are prone to develop to Treg (119). Human babies produce $CD4^+CD25^+$Treg at 13 weeks of gestation (120). Thymectomy in the first month of life, usually for cardiac surgery, later in life results in a higher rate of autoantibodies (121) and a reduced naïve T cell pool (122). Children thymectomized in the first year of life, have reduced numbers of T cells, $CD4^+$ and $CD8^+$T cells and $CD31^+$T cells, with a reduced diversity of their TCR repertoire throughout life (123). CD31 is a marker of T cells recently exported from the thymus (123).

In contradiction to neonatal thymectomy depleting immunity, neonatal thymectomy in mice at day 3, not day 0, resulted in autoantibody production (124–127) and a variety of autoimmune diseases. The organ attacked is determined by host genetic factors (94, 128–130). In mice thymectomized as neonates, autoimmunity is prevented by a thymus graft or injection of naïve adult thymocytes or peripheral lymphocytes (131). Neonatal thymectomy of rats also results in development of autoimmunity (132).

Adult thymectomy and whole body irradiation induces thyroiditis in rats (133), that can be prevented by transfer of normal lymphocytes (134). Rat thymocytes depress autoantibody responses (135). Loss of Treg is considered the cause of experimental autoimmune gastritis (128).

In the 1979-80's, $CD8^+$T cytotoxic cells were considered the main mediators of rejection (136) but also included $CD8^+$I-J^+ suppressor T cells. The first reports of suppressor T cells documented their inhibition of B cell responses (137–141). Tissue specific suppressor cells also were shown to protect against autoimmunity (142). Treatment with anti-donor I-J, but not anti-host I-J, broke neonatal tolerance (143). At that time, I-J was considered a marker of $CD8^+$T suppressor cells (144), until the gene for I-J was not found (145). This error in phenotyping, led to a decade or more delay in the study of regulatory T cells. Later work on adult models of transplant tolerance, led to the rediscovery of suppressor/regulatory cells, which were $CD4^+$T cells not $CD8^+$T cells as a major immune cell (146). The key points related to discovery of Treg are summarized in **Figure 2** and **Supplementary Table 2**.

In a mouse model of oophoritis, induced by thymectomy 2-4 days after birth, $Ly1^+$ T helper cells prevent autoimmunity (147). $Ly1^+$T cells from normal animals were shown to prevent onset of autoimmunity (148). Ly1 is a marker of non CD8 cells, of the helper lineage, now better identified by expression of CD4.

At that time, in 1985, we described that adult transplant tolerance was maintained by $CD4^+$T cells, not $CD8^+$T cells (149). In 1990, we reported that $CD4^+CD25^+$T cells mediate transplant tolerance (150). This was the first description of a regulatory function of $CD4^+CD25^+$ T cells. We also showed $CD8^+$T cells played no role in maintaining transplant tolerance (150). Later in 1995, the Sakaguchis used our finding to show $CD4^+CD25^+$T cells prevented onset of autoimmunity in day 3 thymectomized mice (151).

$CD4^+CD25^+$Treg express the transcription factor FoxP3, which distinguishes them from effector lineage cells (152). During development of thymus, production of $FoxP3^+$Treg is delayed compared to production of effector lineage $CD4^+$T cells (153). $CD4^+CD25^+FoxP3^+$T cells control effector $CD4^+$ and $CD8^+$T cells to prevent induction of autoimmunity (154). Neonatal thymectomy reduces $CD4^+CD25^+FoxP3^+$Treg that prevent autoimmunity (155–159).

$CD4^+CD25^+FoxP3^+$T cells that enter thymic medulla contact thymic epithelial cells and dendritic cells that express host autoantigens induced to be expressed by AIRE. This contact of $CD4^+CD25^+FoxP3^+$T cells with auto-antigen in the thymic medulla promotes their expansion and survival (160, 161). Treg with specificity for an autoantigen prevent autoimmunity.

There is limited information on the role of $CD4^+CD25^+$ $Foxp3^+$Treg in neonatally induced tolerance. The output of Treg from thymus in the neonatal period, makes it possible that Treg specific for the allogeneic cells are activated alongside $CD4^+CD25^+Foxp3^+$Treg that prevent autoimmunity.

Assays of Clonal Deletion of T Cells

Assays of T cell alloreactivity include quantitative GVH (162, 163), MLC (164, 165) and cell mediated lympholysis assays (CML) (166). $CD4^+$T cells responding to Class II MHC are assayed in GVH (65, 167) and MLC (168). There is a weak response to Class I MHC (169), which is assayed by generation of $CD8^+$T cells to CML in MLC.

Tolerant hosts have reduced frequency of alloreactive T helper cells (170–173) and it is loss of this response, rather than CML that is associated with neonatal tolerance (174). On the other hand, in one study 75% of lymphocytes from animals with neonatal tolerance, responded to donor class II MHC and

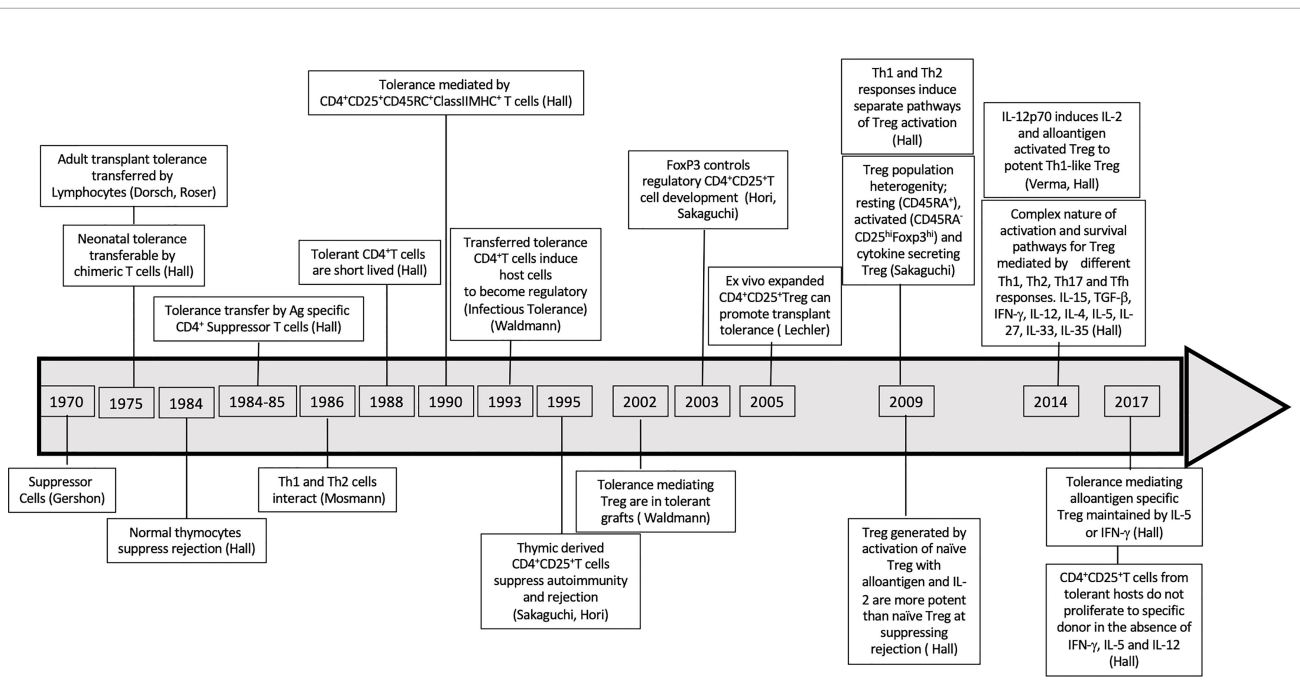

FIGURE 2 | Discoveries Timeline related to suppressor regulatory T cells. Timeline of major discoveries that led to recognition of role of T regulatory cells in tolerance specially in antigen specific tolerance.

produced IL-2 (175). Tolerant class II MHC reactive cells on activation *in vitro* produce IL-2, IFNγ, IL-4 and IL-5 (176). In one neonatal tolerance model, lymphoid cells could mediate GVH to specific donor, suggesting incomplete clonal deletion (177). *In vitro* class II MHC responsive tolerant cells undergo apoptosis when re-exposed to donor alloantigen (178).

Cytotoxic T cells (CTL) precursors to specific donor assessed in limiting dilution assays are reduced in neonatal tolerance which is considered due to clonal deletion (32, 170, 171, 179). Donor alloantigen reactive cells are not active in neonatal tolerance, but reactivity to third party alloantigen is retained (180). Lymphoid cells from animals with neonatal tolerance are less cytotoxic to donor cells consistent with clonal deletion (166, 172, 179). However, complete clonal deletion of cells reactive to donor, in some mice strain combinations does not result in transplant tolerance (181). Absence of MLC and CML responses did not predict the induction of tolerance to an allograft (182).

Not all studies show clonal deletion in neonatal transplant tolerance. Cytotoxic T cells effective against Class I MHC are generated in MLC of lymphocytes from tolerant hosts (103, 183), but have reduced function compared to normal cells (184). Other studies showed lymphoid cells from tolerant hosts were not deleted, and were either anergic or suppressed (146). Inhibitory cells or factors were not found in tolerant hosts (185, 186).

Assay of Tolerant T Cells in Rejection Models

TDL and T cells mediate rejection in whole body irradiated host (187–189), showing antibody and B cells are not essential to the rejection response. Deletion of clones of T cells reactive to donor strain, by passage from blood to lymph in a donor strain host, do not effect rejection but also do not induce tolerance, as recovering host lymphoid cells mount a rejection response (190).

In contrast, TDL from rats with neonatal tolerance do not effect rejection of donor strain skin grafts on irradiated rats, but effect rejection of third party grafts (191). Recirculating T cells from a tolerant host, on adoptive transfer to irradiated hosts, suppresses skin graft rejection (192). This transfer of tolerance is dependent on chimeric donor strain T cells (53, 193). Deletion of chimeric cells breaks tolerance (194). Further adoptive transfer of tolerance requires a donor suppressor T cell (53, 191). Treatment of cells from tolerant hosts with anti-donor sera, removes their ability to transfer tolerance to an adoptive host (53). Thus, the tolerant state depends on the chimeric donor strain cells.

On the other hand, chimerism persists in animals that are not tolerant, and application of the donor skin results in expansion of these chimeric cells, even though the graft is rejected (195).

After neonatal tolerance is broken by transfer of naïve cells, the transferred cells contain all the alloreactivity (51). Later, host thymus derived cells develop and have donor reactivity (50). In these experiments, chimeric donor strain lymphoid cells are lost and cannot promote clonal deletion in the thymus.

The variable results related to GVH, MLC and CML assays, together with the failure of normal cells to effect rejection in tolerant hosts, suggests clonal deletion is not the sole or the essential mechanism for induction and maintenance of transplant tolerance after injection of donor cells in the neonatal period.

Attempts to Induce Medawar Type Transplant Tolerance in Adults

These pre-clinical and clinical models deplete hosts of peripheral lymphoid cells by irradiation and/or myeloablation and transfer donor lympho-haemopoietic cells to try and establish chimerism. The level of chimerism in these models is greater (up to 80%) (197) than in neonatal transplant tolerance (196, 197) where chimerism is only a few percent of peripheral blood and lymphoid cells (53, 54, 193). The presence of mixed chimerism in blood, thymus and bone marrow indicates donor allografts will be tolerated (198).

These protocols required very high doses of irradiation to allow establishment of chimerism and were too toxic for use in humans. This led to assessment of a variety of immunosuppressive protocols to induce bone marrow chimerism (199).

To reduce the side-effects of whole-body irradiation, total lymphoid irradiation (TLI) is used. TLI targets lymphoid tissues including thymus and spleen and minimizes irradiation of non-lymphoid tissues including skull, lungs, limbs and pelvis (200, 201). TLI given before transplant induces tolerance to organ grafts in rats (200), dogs, non-human primates and humans (201, 202).

In rats, infusion of donor bone marrow cells post-transplant induces chimerism, and the rate of chimerism is high in animals where the thymus was protected from irradiation (203). GVH is not induced by the infused allogeneic cells. In this study, early post-transplant there was non-alloantigen specific hypo responsiveness of host lymphoid cells, which after months became alloantigen specific (203). These host accepted long-term fully allogeneic heart allografts. Post TLI transplant tolerance is maintained by a combination of clonal deletion and suppression (197).

Non-myeloablative regime of non-lethal doses of irradiation, thymic irradiation and T cell depletion, can be used to establish myeloid chimerism and the potential of transplant tolerance (204). These chimeric models of transplant tolerance can be due to central and peripheral tolerance.

To overcome the need to give TLI pre-transplant, TLI was tested by use of anti-lymphocyte antibodies and conventional immunosuppression, which is tapered once TLI was administered. In a high responder rat strain, a combination of anti-CD3 mAb and TLI induced tolerance to fully allogeneic heart grafts and this synergized with donor blood transfusion (205). In this model chimerism was not established (205).

Two groups, one at Stanford and the other Medeor Therapeutics are using post-transplant TLI and anti-thymocyte globulin in renal transplant recipients (206). Early post-transplant, these patients receive some conventional immunosuppression, which is later withdrawn. In HLA matched related donor transplant, infusion of $CD34^+$ cells and some T cells, has established chimerism in a large proportion and many are off immunosuppressive treatment. There were no serious infections or engraftment syndromes which are a form

of GVH. Some patients required long-term immunosuppression. With HLA incompatible grafts, there was an engraftment syndrome in some patients, and chimerism was lost (206).

Induction of Bone Marrow Transplants to Induce Transplant Tolerance

Several other protocols have been described, with success. These protocols are discussed in detail in a recent review and will not be described here (206). The details of these protocols and the immunological mechanisms operating are incompletely understood and are beyond the scope of this review.

INDUCTION OF TRANSPLANT TOLERANCE IN THE ADULTS, SPECIFIC UNRESPONSIVENESS WITHOUT CLONAL DELETION

There are several methods of inducing "Operational Tolerance" in adult animals, and many do not induce clonal deletion. These models and the non-clonal deletion mechanisms by which they are induced and maintained will be reviewed. Key mechanisms of induction and maintenance of transplant tolerance in adults are listed in **Table 1**.

Three broad groups of specific unresponsiveness induced in adult animals where there is no "Clonal Deletion "will be reviewed

Spontaneous Acceptance of a Directly Vascularized Organ Allograft Without Immunosuppression Induces Specific Unresponsiveness

The best example is allogeneic liver allografts, which in some hosts are accepted without immunosuppression, reviewed (207, 208). This was first observed with liver transplants in pigs (209), but also occurs in rats (211) and mice (210). In rats, such tolerance is only induced in low responder strains whereas in mice liver transplants induce tolerance in nearly all strain combinations. In miniature swine thymectomy reduces the rate of tolerance induction to liver allografts (88).

In rats, liver allografts rapidly induce systemic donor hypo-reactivity (211) and reverse rejection of other donor strain organs (212). There is partial clonal deletion in peripheral lymphocytes (213) including specific donor memory lymphocytes (214).

Liver transplant tolerance induction depends upon passenger leukocytes in the liver graft (215, 216). Immune activation by donor leukocytes in the graft is a major mechanism (217), that leads to clonal exhaustion (218). Compared to heart allografts, there is more rapid migration of passenger leukocytes to spleen and lymphoid tissue with a more rapid activation of T cells (219). There is activation of Th1 responses with induction of IL-2 and IFN-γ (220). Reduction in Th1 response by administration of corticosteroids (221) or the Th2 cytokine IL-4 (222) prevents development of tolerance and promotes liver allograft rejection. Paradoxically, prior treatment of donor with rIL-4 increases macrophages in the donor liver and induces tolerance to livers in strain combinations where liver allografts are not spontaneously accepted (217).

Passenger leukocytes transplanted within the liver allograft can mount a GVH response and provide a source of donor cells (223). Micro chimerism of donor lympho-haemopoietic cells occurs and promotes tolerance (223, 224). Whether this GVH leads to further clonal deletion is unclear. The thymus is essential for stopping GVH in the liver graft but is not required long-term (225).

The mass of the liver protects it from rejection. Activation of T cells by hepatocytes, rather than antigen presenting cells, leads to incomplete activation and rapid loss of function (208). High alloantigen expression in the liver exhausts alloantigen reactive $CD8^+$T cells (226). Direct contact of alloreactive T cells with liver cells, through fenestrations in the endothelium of hepatics sinusoids, results in their deletion or exhaustion (227). Deletion may also be related to the massive activation of alloreactive T cells (220), which may become anergic or be deleted by apoptosis of T cells (219, 220, 228), including alloreactive T cells (229). Sensitized T cells are deleted in the periphery (214).

In rats, transplantation of a liver immediately stops rejection of a heart graft from the same donor, demonstrating a systemic effect, which may include secretion of MHC molecules from the liver (230, 231) or other immunosuppressive molecules (232, 233).

T suppressor cells have been implicated in tolerance to liver allografts (234). There is limited evidence that $FoxP3^+$T cells mediate tolerance to a liver allograft (235, 236). $CD4^+CD25^+FoxP3^+$T cells are present in rejecting and tolerated liver allografts (210). Therapy with $FoxP3^+$Treg has been trialled (237, 238). A combination of donor dendritic cells and $CD4^+CD25^+$Treg is more effective at inducing tolerance in a strain that does not spontaneously accept liver grafts (239). In

TABLE 1 | Immune Mechanisms described in Transplantation Tolerance.

Mechanism	
Clonal Deletion	$CD4^+$ cells
	$CD8^+$ T cells
	B cells
Clonal Exhaustion	Apoptosis
Clonal Anergy	Systemic donor hypo-reactivity
Specific unresponsiveness	$CD4^+$ cells transfer from tolerant hosts
Regulatory T cells	Naïve Treg
	Activated Treg Ts1, Ts 2, Highly potent
	Th1-like Th2 like
Chimeric Donor Derived haemopoietic and lymphoid cells	Regulatory
	Effect Clonal silencing
Graft Factors	Alloantigen mass
	Inhibitory factors secretion
Donor Dendritic cells	Depletion/graft adaptation
	Stimulation of regulatory cells
	Failure to stimulate effector cells
Immune ignorance	–
Antibodies to Class II MHC	Blocks $CD4^+$T cell activation and effectors
Excessive Immune activation	Dependent on grafts antigen presenting cells

mice, pre-treatment with anti-CD25 mAb prevents tolerance induction, increases the anti-donor T cells response and reduces apoptosis (235, 240).

Current clinical trials of immunosuppression withdrawal from liver allograft recipients have recently been summarized (206). Operational tolerance occurs in patients with liver allografts.

In some mice strain combinations, kidney allografts are spontaneously accepted, and tolerance is induced. This is associated with induction of FoxP3^{+}T cells, not Th1 cell activation (241). With rat kidney allografts, administration of donor leukocytes at the time of transplantation induces donor specific transplant tolerance (242). This increases T cells activation and induction of IL-2 and IFN-γ in the allograft associated with the infusion of donor leukocytes, suggesting overactivation, as occurs with liver allografts, induces tolerance (242, 243).

Transplant Tolerance With Specific Unresponsiveness Without Clonal Deletion in Large Animals With Kidney and Heart Allografts

In studies with inbred miniature swine, a single or double class I MHC incompatible kidney or heart allograft treated with a short course of cyclosporine A (CSA) (244) or tacrolimus (245) therapy develop a form of tolerance. A large proportion of these animals develop tolerance, with no anti-donor antibodies, variable CML to donor, and most accepted a second donor allograft without immunosuppression. In the swine kidney allograft model, 12 days of high dose CSA induces tolerance and the to be tolerated grafts have a cellular infiltrate not dissimilar to rejection in the untreated kidney allografts, with induction of inflammatory cytokines in both tolerated grafts and rejecting grafts (246). This infiltrate spontaneously resolves (247). This suggests the tolerance is central, and not due to graft adaptation (244, 245). Donor antigen presenting cells in the second transplanted graft do not trigger rejection. The maintenance of tolerance requires the presence of the original tolerated renal allograft (248).

Class II mismatched miniature swine kidney grafts with no immunosuppression are rejected but a short course of CSA induces long term graft acceptance (249). These animals with long surviving allografts accept skin and second kidney allograft from the same donor strain indicating tolerance (249). Class II MHC matching is more important in tolerance induction in miniature swine than class I MHC matching (250). For a second test heart graft to be accepted by a swine tolerant to a kidney graft, the second graft must share class II MHC with the original kidney graft (251).

Host thymus is essential for tolerance induction (252) but not-long term maintenance of tolerance (253). Thymectomized miniature swine are resistant to induction of tolerance to cardiac allografts (254–256).

Co-transplantation of thymus and a kidney allograft enhances tolerance induction (244). Vascularized thymus allografts in miniature swine transplanted at the same time as a heart graft, combined with a short course of tacrolimus, induce transplant tolerance (257). Combined thymus/heart grafts have increased survival compared to heart grafts without thymus (258, 259).

In miniature swine, combined heart and kidney allografts are accepted and induce tolerance, whereas single grafts are rejected. This effect of combined heart and kidney allograft is in part due to increased alloantigen load (260). Irradiation of the kidney but not the heart allograft prevents tolerance to both grafts (255).

In pigs, the transfer of the tolerated kidney with cells from the tolerant host induces prolonged survival in a second irradiated host, suggesting the graft and cells from tolerant animals promote tolerance (261). The tolerated kidney allograft when transplanted to a second host, induces tolerance with or without co-transferred tolerant cells, suggesting a peripheral mechanism of tolerance (261). Tolerated kidneys are rejected when retransplanted into a normal host, indicating graft adaptation is not the mechanism of tolerance (262).

Application of donor strain skin to swine tolerant to a kidney allograft induces anti-donor CTL, but the kidney graft is not rejected (263).

Lymphocytes from miniature swine tolerant to a kidney allograft inhibit responses to specific donor but not third party (264). Tolerant hosts have reduced helper and CTL capacity against donor strain (265). Lymphocytes from tolerant hosts, do not generate CML against donor but do to third party (266). CD25^{+} lymphocytes suppress donor specific CML (267).

Prior specific donor blood transfusions increased the rate of induction of tolerance by CSA to heart transplants, suggesting a peripheral mechanism of tolerance induction (268). Prior induction of tolerance by a bone marrow transplant in swine allows acceptance of donor strain kidneys (269).

These studies in miniature swine, performed by a group at the NIH and Massachusetts General Hospital, show specific unresponsiveness to an organ allograft can be induced by methods used in rodent models that are reviewed below. They establish that acceptance of a graft is (i) preceded by a rejection like response that spontaneously resolves, (ii) is facilitated by the thymus, (iii) is alloantigen specific and that peripheral lymphocytes can promote tolerance and are not clonally deleted. The mechanisms of tolerance induction and maintenance may be very similar to those in the rat models, making it possible that such specific unresponsiveness may be induced in all species including man.

Split Tolerance or Specific Unresponsiveness in Adult Murine Models

In adult rodents, a variety of treatments reduced rejection and induce a state where organ allografts are accepted without ongoing immunosuppression. This phenomenon was called specific unresponsiveness (270, 271) and is a form of "operational tolerance" (272), reviewed (273). These models of tolerance have similarities to the swine models, as described above. Like in the swine models, a rejection response is generated but is insufficient to reject the grafts which survive. It then takes weeks for tolerance to fully mature after exposure to alloantigen. Tolerance is associated with a loss or change in dendritic cells

and the development of suppressor cells, which in all cases include $CD4^+$T cells (273).

One of the first such models of transplant tolerance was induced by treatment of mice with donor liver cells and anti-lymphocyte serum, which led to acceptance of fully allogeneic skin (274, 275). The acceptance of these allografts requires induction of suppressor cells now known as Treg (276–279). Overtime, there has been increasing acceptance that Treg contribute to this form of tolerance (280, 281) and that alloantigen from the graft can induce host Treg (282).

Other models of specific unresponsiveness, described in the 1960-70s, were passive or active enhancement (283). In these models, there was no attempt to induce chimerism or clonal deletion. In active enhancement, donor peripheral lymphoid cells or haemopoietic cells are given ivi, either at the time of transplant or 7-10 days prior to transplantation. Class II MHC and B cells promote enhanced allograft survival (284). Passive enhancement is induced by injection of alloantibody to the donor stain (79), particularly alloantibody to Class II MHC (77). Kidney and heart allografts are easier to enhance survival of than skin or lung. Sensitization to donor strain alloantigen prevents induction of enhancement of allograft survival (285). Not all host strains can be induced to develop tolerance of an allograft (286, 287).

It is only after weeks that an enhanced allograft induces a state where a second donor strain graft, usually skin, is accepted while third party grafts are rejected (288, 289).

Long surviving grafts can have pathological lesions of rejection (290) but the graft continues to function.

In part, acceptance of enhanced kidney allografts is due to depletion of donor dendritic cells, so that the graft cannot provoke a rejection response when re-transplanted into a naïve recipient strain host (291–294). The loss of alloantigen stimulation to provoke rejection, is not the sole mechanism as second donor strain allografts, with a normal complement of alloantigen presenting cells, are accepted (288, 289).

Treatment with a short-course of CSA (149, 295–300) is more reliable at inducing specific unresponsiveness to allografts than our enhancement protocol (289, 296). The mechanisms of graft acceptance seems to be similar to those in enhancement models (298), although infiltration of grafts by allospecific CTL is impaired (301). Later, other reliable models of specific unresponsiveness were developed, including therapy with antilymphocyte sera (ALS), blood transfusions (302), anti-CD4 mAb (303–306), a combination of anti-CD4 and anti-CD8 mAb (307), anti-CD3 mAb (308, 309) and anti-CD25 mAb (310). The many models for specific unresponsiveness are reviewed elsewhere (273) and include models of transplant tolerance in adult animals (304).

In these models, specific unresponsiveness takes time to develop and is usually not manifest until after 100 days post-transplant (289, 296). It is only after a period of weeks, that second donor strain grafts are delayed in rejection and after time (usually ten weeks) most are accepted (271, 289). Normal rejection of third-party grafts is retained by these hosts at all times post-transplant (271, 289).

Cells from animals with specific unresponsiveness have normal reactivity in MLC (309, 311–313), CML (309, 311, 313–316), and GVH (317–320). There is no experimental evidence for clonal deletion. Animals with enhanced allograft survival make donor specific alloantibody responses (315, 321).

Early attempts to demonstrate suppressor cells in hosts with specific unresponsiveness were unsuccessful (322), but later suppressive T cells were identified (149, 276, 289, 323, 324) and confirmed by others (325–330). The maintenance of suppressor cells is dependent on alloantigen from the graft (331).

The difficulty in measuring suppressor/regulatory cell activity, led us to develop an assay using limited numbers of alloreactivity of cells, and establishing their ability to mediate rejection or transfer specific unresponsiveness in an immunodeficient irradiated host.

ROLE AND ACTIVATION OF T REGULATORY CELLS IN TRANSPLANT TOLERANCE

An Assay to Assess Ability of Lymphocytes to Mediate Rejection or Transfer Specific Unresponsiveness

We developed a model in which different numbers of peripheral lymphocytes capacity to mediate rejection or inhibit rejection of fully allogeneic directly vascularized heart allografts is tested in adoptive hosts whose own lymphocytes had been depleted by whole body irradiation (104, 188). For these studies, DA recipient and PVG heart grafts are used with Lewis rats as third-party donors.

Using cells from naïve animals, we have shown that the most potent are TDL, then lymph node cells and spleen cells (188). Thymocytes and bone marrow cells do not restore rejection (188). Larger numbers of TDL, spleen or lymph node cells mediate faster rejection (188). Enriched recirculating T cells are effective at mediating rejection. Cells from adult thymectomized animals are not impaired and tend to reject faster than cells from non-thymectomized hosts (188, 332). Injecting thymocytes mixed with normal lymph node cells or spleen cells delays rejection (332), suggesting in normal animals thymus cell and peripheral T cells recently produced by thymus inhibit the rejection response (332). In this model, host thymectomy allows transferred cells to mediate faster rejection, suggesting the hosts' immune reconstitution following irradiation promotes development of tolerance (189).

In this model, enriched $CD4^+$T cells mediate rejection whereas $CD8^+$T cells and B cells do not restore rejection (189). Dilution of $CD4^+$T cells, shows that half a million cells are as effective at restoring rejection as two hundred million cells. This allows studies on tolerant cells to be with very small numbers of naïve $CD4^+$ T cells (149, 333). That allows the effects of the suppression by tolerant cells to be assayed, which is not possible in hosts with a redundant effector T Cell population.

Removal of $CD25^+$ cells from naïve $CD4^+$T cells results in more rapid rejection, consistent with naïve $CD4^+CD25^+$T cells non-alloantigen specific effect on allograft rejection

responses (334). Mixing 5x10^6 CD4$^+$CD25$^+$T cells from naïve animals with 5x10^6 unfractionated CD4$^+$T cells totally suppresses rejection (334). Tolerance was only induced when the mixture was 1:1 (334). Lower ratios of CD4$^+$CD25$^+$T cells to effector CD4$^+$T cells do not supress rejection and at the normal ratio of 1:10 rejection is not suppressed showing that naïve/resting Treg are weak at suppressing rejection (334). Ratios of 1:1 are impossible to achieve long-term in animals.

Cells from syngeneic donors sensitized to specific donor strain by rejection of skin grafts are more potent (104). Compared to naïve cells, these cells from TDL, lymph node and spleen, accelerate rejection of specific donor allografts but not third party grafts, showing an increase in potency and in alloantigen specific memory T cells (335). These memory T cells do not rapidly recirculate from blood to lymph (104, 336), consistent with what is now known as effector memory T cells. Memory CD4$^+$ and CD8$^+$T cells mediate rejection, showing sensitized or memory CD8$^+$T cells mediate rejection without help from CD4$^+$T cells (337).

Transfer of Specific Unresponsiveness by Lymphocytes

This model of rejection was adapted to the study of specific unresponsiveness. We use DA rats as specific unresponsiveness to PVG heart grafts can be induced by a variety of treatments including passive enhancement (289), CSA treatment (296, 317), anti-CD4 mAb treatment (303, 338) and anti-CD3 mAb treatment (309). In this assay, the relative potency of different cell populations can be examined.

Our studies show peripheral lymphoid cells, especially spleen cells and lymph node cells, but not thymocytes transfer alloantigen specific tolerance (288, 289). B cells and an antibody response is not required to transfer tolerance (297). Enriched T cells populations transfer tolerance to specific donor grafts to an adoptive host (289, 296, 297). Peripheral lymphocyte from tolerant hosts, suppress the ability of naïve peripheral lymphoid cells to restore rejection (289, 296, 297, 339). The tolerant CD4$^+$T cells must be at ratios of ≥4:1 to naïve cells. This ratio of specific unresponsive host cells to naïve cells is used in all our subsequent experiments on suppression of rejection. Such ratios of tolerant CD4$^+$T cells to host naïve lymphocytes cannot be achieved in normal adoptive host. Thus, tests of transplant tolerance transfer need to use severely immunocompromised hosts, such as those given whole body irradiation, B rats, Rag and SCID mice.

We prepare T cells that recirculate from blood to lymph, by injecting irradiated recipient strain rats with lymph node and spleen cells from rats with specific unresponsiveness. The T cells that recirculate from blood to lymph, do not suppress rejection in a third adoptive host (289). Thus, suppressor T cells from specific unresponsive hosts migrate to peripheral tissue, not secondary lymphoid tissue, and behave like effector memory T cells (104).

CD4$^+$T Cells, Not CD8$^+$T Cells, Mediate Specific Unresponsiveness

Examination of the role of CD4$^+$T cells and CD8$^+$T cells (340) in specific unresponsiveness, produced what is a very surprising result, reviewed (341). That is the CD4$^+$T cell fraction transfer specific unresponsiveness, whereas the CD8$^+$T cells do not inhibit graft rejection or transfer specific unresponsiveness. A key role of CD4$^+$T cells in maintaining unresponsiveness to an allograft is shown in enhancement (319), after CSA treatment (297), anti-CD3 mAb treatment (309) and anti-CD4 mAb treatment (303) mAb. Up until that time suppressor cells were considered to be CD8$^+$T cells (cytotoxic/suppressor) not CD4$^+$T cells, which were helper/inducer.

Early after transplantation, when there is immunosuppression to induce tolerance, at 8 and 20 days post-transplant, CD4$^+$T cells effect rejection (342). It is only after 50 days that tolerance is transferred by CD4$^+$T cells (342), consistent with the observation that second donor strain graft are only accepted after 50 days post-transplant (342). With regards to CD8$^+$T cells, at 8 and 20 days post-transplant, they effect rejection, much like CD8$^+$T cells from controls where no immunosuppression is given (342). CD8$^+$T cells at 50 and >75 days do not effect rejection, and do not suppress rejection (342). These studies show that during induction of specific unresponsiveness the hosts CD4$^+$ and CD8$^+$T cells have capacity to effect rejection and in the case of CD8$^+$T cells are activated. With time, the CD4$^+$ tolerance mediating cells develop and prevent rejection of a second donor allograft.

Further characterization of the CD4$^+$T cells from tolerant hosts, show they cannot suppress specific donor rejection mediated by sensitized CD4$^+$T cells, but can suppress rejection mediated by specifically sensitized CD8$^+$T cells (150). Depletion of the adoptive host of CD8$^+$T cells by thymectomy or treatment with an anti-CD8 mAb demonstrated that CD8$^+$T cells are not required to re-establish tolerance in the adoptive host, neither was a thymus in the adoptive host (150). These studies in 1985 were the first to show suppressor/regulatory cells are CD4$^+$, not CD8$^+$T cells.

In mice with adult induced transplant tolerance, tolerant CD4$^+$T cells promote induction of tolerance in host T cells, a phenomenon called "infectious tolerance" (282).

In animals with specific unresponsiveness to an allograft, removal of the allograft 50 days post-transplant results in a loss of tolerance transferring CD4$^+$T cells within 8 days and these cells effect rejection in the adoptive host (150). Further, cyclophosphamide treatment of the animals with specific unresponsiveness depletes CD4$^+$T cells with the ability to transfer specific unresponsiveness (150). These experiments show that a subpopulation of cells that suppress within tolerant CD4$^+$T cells, are rapidly dividing and need alloantigen stimulation. Such activated T cells usually require cytokines to promote their survival and activation. This led us to examine which cytokines could promote their survival and proliferation.

Shortly after our description of a CD4$^+$T cell mediated suppression of rejection, Goran Moller in an editorial entitled "Do Suppressor cells Exist?" (145), cited three reasons for doubting the existence of T suppressor cells. First, there was no marker for T suppressor cells to distinguish them from CD8$^+$cytotoxic T cells. Second, the gene for the purported marker of suppressor cells "I-J" was not found in the MHC region of mice (343). Third, there was no evidence that the alpha

and beta chain of TCR are expressed in suppressor cells, making the existence of antigen specific suppressor T cells impossible.

In the late 1980's suppressor cells became unfashionable, and many if not most immunologists considered they did not exist and that the apparent suppression described were random artifacts. Suppressor T cells could not be mentioned in polite immunological circles as manifest by Jan Klein (1990) in a preface to his textbook on immunology (344), stated *"I have attempted to find the fundamental truth in immunology and to separate it from hypothesis, regardless of how fashionable they might have been at the time of writing. Consequently, the reader will not find certain topics (such as specific suppressor T cells) discussed at any length, as they are judged not to be a fundamental truth."* The derision was as blunt as that of Medawar's dismissal of lymphocytes and T cells as mediators of immune responses such as transplant rejection.

Our work in the mid 1980s shows suppression is mediated by $CD4^+$T cells not $CD8^+$T cells. These cells transfer alloantigen specific suppression and are not non-specific. Suppression by thymocytes was natural and not antigen specific. In 1990, the role of $CD4^+CD25^+$T cells as inhibitors in transplant tolerance was first described (150). But work on suppressor cells became so unfashionable, its grant funding was cut.

To address the paradox that $CD4^+$T cells effect rejection and could also maintain transplant tolerance, we looked for other markers of the suppressor T cell subset.

Tolerance Promoting $CD4^+$T Cells Die Without Specific Alloantigen and Cytokines

We observed in studies to characterize the specificity of suppression by $CD4^+$T cells, that culture of $CD4^+$T cells with specific donor antigen presenting cells, led to a loss of capacity to suppress rejection of specific donor and a gain in ability to effect rejection (340). This occurred within three days of culture (150, 340). We then cultured the tolerant $CD4^+$T cells with specific donor stimulator cells and supernatant from ConA activated splenocytes. This cytokine rich media promoted survival of suppressor $CD4^+$T cells, only if specific donor stimulator cells were present.

We found IL-2 partially maintained suppressor function (345) and that depletion of CD25 expressing cells from tolerant $CD4^+$T cells removed their capacity to suppress rejection in our adoptive transfer assay (150).

As an anti-idiotypic response was suggested (346, 347), $CD4^+$T cells from hosts with specific unresponsiveness were cultured with idiotype of donor alloantigen activated T cells from a naïve host. Even in the presence of supernatant from Con A activated splenocytes, suppressor function of tolerant $CD4^+$T cells was lost in culture with idiotype expressing cells (311, 345).

As Con A supernatant is rich in IL-2, we examined if the cells that transfer tolerance expressed the IL-2 receptor, which is now known as CD25. In an attempt to phenotype the $CD4^+$T cells that suppress rejection from the $CD4^+$T cells that can mediate rejection, we deplete $CD25^+$ cells from tolerant $CD4^+$T cell. Depletion of $CD4^+CD25^+$T cells, left a population of $CD4^+CD25^-$T cells that mediate rejection of specific donor grafts (150, 348). This work published in 1990 was the first demonstration of $CD4^+CD25^+$T cells as a regulatory or suppressor cell. This observation, discussed with the Sakaguchis, led them in 1995 to report $CD4^+CD25^+$T cells prevent autoimmunity in mice thymectomized in the neonatal period (151). The identification of $CD4^+CD25^+$T cells as suppressor cells slowly led to rehabilitation of the concept of regulation within the immune system.

There is now widespread acceptance that $CD4^+CD25^+$T cells suppress all immune responses. The naïve Treg described by the Sakaguchi's are very different to the $CD4^+CD25^+$T cells that mediate transplant tolerance. Naïve Treg suppression is not antigen-specific, whereas our tolerant cells transfer donor alloantigen specific suppression.

We also showed tolerance transferring $CD4^+$Treg express CD45RC (150), a marker of an activated Treg whereas naïve Treg express CD45RA (349). Tolerance transferring Treg also express Class II MHC, a marker of activated Treg (150). $CD45RA^-(CD45RC^+)$, Class II MHC^+ remain two key markers of activated Treg, that can be used to distinguish them from naïve Treg (349, 350).

In Our Models of Tolerance, rIL-2 Alone Did Not Sustain Suppressing $CD4^+$T Cells That Transfer Tolerance

Although specific transplant tolerance is transferred by CD25 expressing cells, and survival of these cells in culture requires a cytokine rich supernatant from ConA activated lymphocytes, use of recombinant IL-2 alone in culture does not fully sustain the suppressor capacity of these cells (345). This raises the possibility that the alloantigen activated $CD4^+CD25^+$Treg needed cytokines other than IL-2 to promote their proliferation and survival. We tested other cytokines and found key roles for several.

At that time the description of Th1 and Th2 responses (351) resulted in a hypothesis that deviation to Th2 and reduced Th1 responses may explain specific unresponsiveness. We found specific unresponsiveness could be induced by suppression of either Th1 (338) or Th2 (308) responses. Specific alloactivated $CD4^+$Th2 cells generated *in vitro*, mediate rejection not tolerance (352–354). Thus, alloantigen specific suppression is not mediated by a switch to a Th2 response, albeit Th2 cytokines such as IL-4 (355, 356) and IL-5 (357, 358) inhibit rejection and promote transplant tolerance induction.

Given IL-2 alone does not sustain full suppressor function in $CD4^+$T cells from animals with specific unresponsiveness (345), we examined the possible role of other Th1 and Th2 associated cytokines. **Figure 3** shows the parallel pathway of activation of Th1 and Th1-like Treg that we have described.

To do these studies we obtained clones or cloned a variety of rat T cell cytokine producing cell lines. Treating rats with fully allogeneic neonatal heart allografts, we found IL-12p70 (359, 360), IL-4 (356), IL-5 (357) and IL-13 (361) delay rejection, while rIL-2 promotes rejection. To our knowledge no cytokine therapy induces specific unresponsiveness to an allograft, except IL-5 in a chronic rejection model with only Class I MHC incompatibility (358).

The mechanism by which these cytokines delay rejection is unclear. Thus, we examined the effect of various cytokines on $CD4^+CD25^+$Treg in culture with and without alloantigen. IL-13

inhibits macrophage activation, not Th1 cell activation (361) and to date has no reported effect on Treg.

First, we enriched naïve $CD4^+CD25^+$Treg and cultured them with alloantigen or self-stimulator cells. Different cytokines were assayed for their effects on proliferation. Both rIL-2 and rIL-4 induce proliferation to self and alloantigen. Alloantigen alone induced a small proliferative response (312). rIFN-γ, rIL-12, rIL-5, rIL-13, rTGF-β and rIL-10 do not induce proliferation of naïve $CD4^+CD25^+FoxP3^+$T cells (312).

When $CD4^+CD25^+FoxP3^+$Treg from animals with specific unresponsiveness were assayed in MLC against self, specific donor and third party, we observed a difference in response to that of naïve $CD4^+CD25^+FoxP3^+$T cells. Most interesting is that their response to specific donor is at background levels, that is there is no response (312). Their response to third party remains normal (312). This result is consistent with our earlier observation that the ability of $CD4^+$T cells from specific unresponsive host was lost within days of culture with specific donor alloantigen in the absence of Con A supernatant (340, 345). Again, rIL-2 and rIL-4 increase proliferation of tolerant $CD4^+CD25^+$T cells to self, specific donor or third party. Three cytokines induce increased proliferation to specific donor but not to self and third party (312). These are rIL-5, IL-12 p70 and IFN-γ whereas TGF-β, rIL-10, rIL-13 do not promote proliferation to specific donor (312).

These studies showed tolerance transferring cells may depend on these cytokines. We tested this by culture of tolerance transferring $CD4^+$T cells with specific alloantigen and one of these cytokines. IFNγ (362) and IL-5 (363) sustain their tolerance transferring capacity, whereas cells cultured with rIL-4 cannot transfer tolerance and mediate rejection (352). Further evidence that cytokines other than IL-2 are required to sustain survival and proliferation of tolerance mediating $CD4^+$T cells.

Activation of Naïve $CD4^+CD25^+FoxP3^+$Treg by Alloantigen and T Cell Cytokines Induces Expression of Other T Cell Cytokine Receptors

These observations led us to examine cytokine receptor expression after naïve $CD4^+CD25^+FoxP3^+$Treg are cultured with alloantigen and either rIL-2 or rIL-4 (364). This uncovered pathways whereby naïve $CD4^+CD25^+FoxP3^+$Treg are activated to more potent antigen specific Treg, reviewed (365, 366). Those cultured with rIL-2 and alloantigen or autoantigen are induced to express the receptor for IFN-γ and IL-12 (364, 367), but not the receptor for Th2 cytokines such as IL-5 (364). We call the naïve Treg that had been activated with the Type 1 cytokine IL-2 and express receptors for the Type 1 cytokines, IFNγ and IL-12, Ts1 cells (364, 368). The naïve Treg when activated by the Type 2 cytokine IL-4 and antigens they are induced to express receptor for the Type-2 cytokine IL-5 (364, 369). We call these rIL-4 and alloantigen activated cells Ts2 (364, 369).

In MLC, Ts1 and Ts2 cells suppress responses to specific donor at ratios of 1:32-1:64 (364), whereas naïve $CD4^+CD25^+$T cells only fully suppress MLC at 1:1 -1:2 (168). On adoptive transfer to irradiated hosts restored with $5x10^6$ naïve $CD4^+$T cell, Ts1 or Ts2 cells suppress rejection at 1:10, whereas naïve $CD4^+CD25^+$Treg only suppress at 1:1 to effector $CD4^+$T cells (334).

Ts1 cells are activated to express CD8 as well as CD4 becoming double positive cells (370). The double positive cells are the cells with the increased potency (370). Further, activated Ts1 cells have increased expression of CD62L (370), suggesting they are programmed to migrate to other peripheral lymphoid tissues, not to the site of inflammation in the graft.

Cytokines Other Than IL-2 Activate $CD4^+CD25^+$Treg

We next examined if Ts1 cells could be further activated by culture with rIL-12p70 and specific donor alloantigen. In the absence of rIL-2, rIL-12 induces Th1-like Treg (367). These Th1-like Treg suppress in MLC at 1:1000 and are the most potent Treg described. Small numbers of these cells can inhibit allograft rejection in a normal host. Th1-like Treg express T-bet, the Th1 transcription factor, as well as FoxP3, and express IFN-γ but not IL-2 (367).

Ts2 cells can be further activated by rIL-5 in the absence of IL-4, to develop a Th2-like phenotype, expressing the Type 2 transcription factors GATA3 and IRF4, as well as Type 2 cytokines IL-5 (358). They do not express Type 1 cytokines and transcription factors (358).

In man, there is increasing evidence that in parallel with activation of Th1, Th2, Th17, Tfh responses, there is activation of naïve $CD4^+CD25^+FoxP3^+$Treg to a phenotype similar to the effector lineage (371, 372). That is T-bet and IFNγ with Type-1 cytokines (359) and GATA3, IRF4 and IL-5 with Type-2 cytokines (358).

In humans, $CD4^+CD25^+FoxP3^+$ Treg can be isolated by their lack of expression of the IL-7 receptor CD127 (373). Focussing on the $CD4^+CD25^+FoxP3^+CD127^-$ Treg memory/activated Treg can be distinguished from resting Treg by their low expression of CD45RA (349). Some activated/memory Treg express CXCR3, CCR6 or CCR8, the chemokine receptors respectively expressed by Th1 (371), Th17 (374) and Th2 cells (371). These cells called Th-like Treg respectively express transcription factors T-bet, RORγt and GATA3. CXCR3 promotes cell migration to its ligand CXCL10 expressed at sites of Th1 mediated inflammation (375). CCL20 is induced by IL-17 and produced by Th17 cells, promoting migration to sites of Th17 inflammation (376, 377). Th1 like Treg produce more IFN-γ than other Th-like Treg (372). Th17-like Treg produce more IL17 and Th2-like Treg produce more Th2 cytokines, including IL-4, IL-5 and IL-13 (372). A proportion of activated $CD4^+CD25^{hi}FoxP3^{hi}CD45RA^-$Treg express both CXCR3 and CCR6 and are Th1/17 like Treg. Th2-like Treg express CCR8, the Th2 chemokine receptor.

CCR4 is expressed by all Th-like Treg and promotes migration to its ligands CCL17 and CCL22 produced by dendritic cells in lymphoid tissues (378). Expression of CCR4 by $CD25^+FoxP3^+$T cells is required for induction of tolerance (88).

This activation of potent Treg is a two-step process that produces effector Treg that can migrate to the site of inflammation by expression of the relevant chemokine receptor, such as CXCR3 on Th1-like Treg. These activated Treg do not migrate from blood to lymph, as we observed in the 1980s (289). In the site of immune attack, they can inhibit

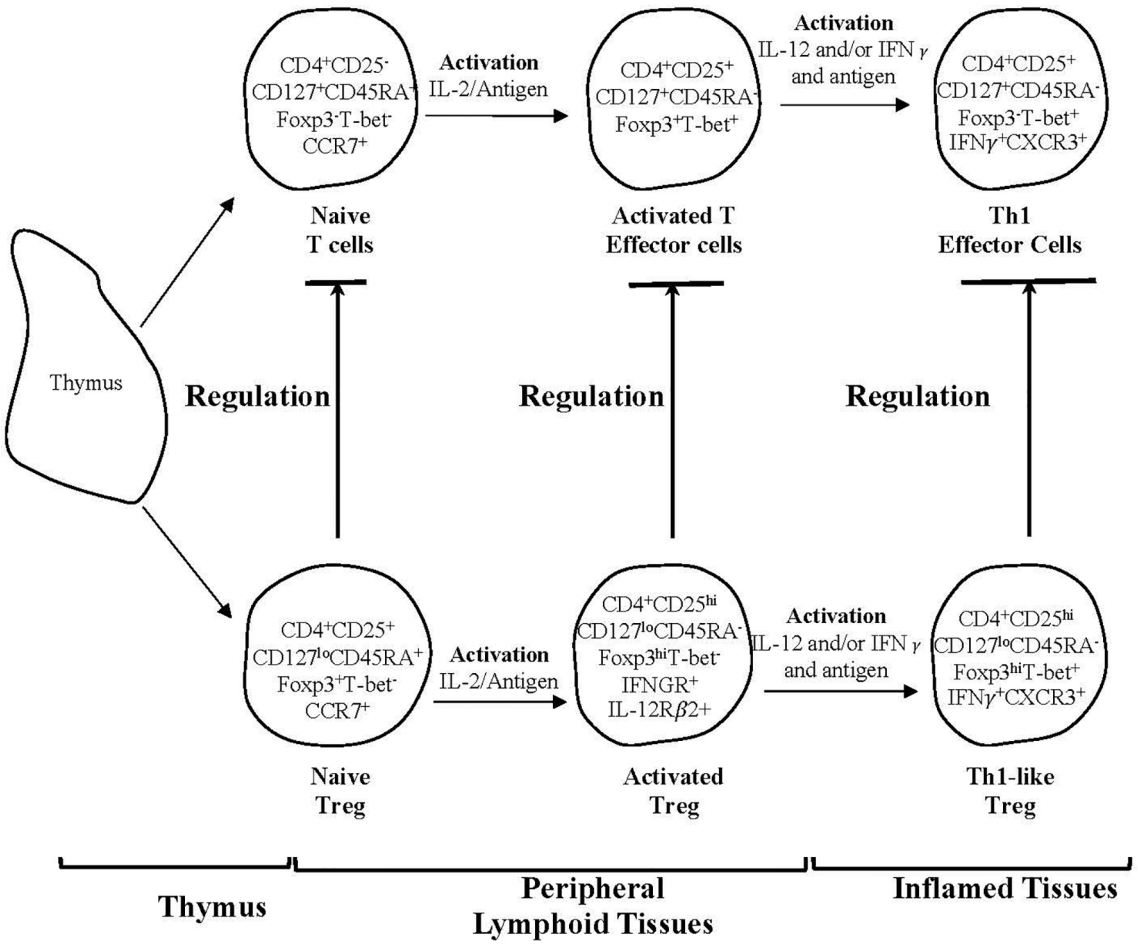

FIGURE 3 | A schematic representation of two subpopulations of $CD4^+$T cells produced by the thymus and one of several pathways for their activation by an antigen and cytokines in peripheral lymphoid tissues and sites of inflammation. The activation by an antigen of effector lineage $CD4^+CD25^-CD127^+CD45RA^+Foxp3^-$ cells induces them to produce cytokines that promotes activation of $CD25^+CD127^{lo}CD45RA^+Foxp3^+$Treg that have been activated by antigen. This figure shows the parallel pathways of activation of effector and regulatory $CD4^+$T cells, when producing and being activated by Type-1 cytokines. The cytokines produced by the effector cells are required for the full activation of Treg. Both lineages of cells have been produced by thymus and have migrated to peripheral lymphoid tissue. Their subsequently recirculation from lymphoid tissue to blood and back to lymphoid tissue, is promoted by expression of CD62L and CCR7. This recirculation increases their chances of recognizing antigens. In peripheral lymphoid tissue upon recognition of an antigen, both effector and regulatory $CD4^+$T cell populations are activated and proliferate. Effector lineage $CD4^+$T cells start producing IL-2 and express IL-2R including CD25 (IL-2Ra chain). Naïve resting Treg expand polyclonally. During an immune response naïve/resting $CD4^+CD25^+CD127^{lo}CD45RA^+Foxp3^+$T-$bet^-CCR7^+$Treg are activated by an antigen and the IL-2 produced by activated T effector cells and are induced to express the receptor for late Th1 cytokines IL-12 and IFN–γ. Naïve $CD4^+CD25^+CD127^+CD45RA^+Foxp3^-$T-$bet^-CCR7^+$T cells also acquire CD25, Foxp3 and T-bet expression but no longer express CD45RA. Transient expression of Foxp3 and CD25 on activated effector T cells blurs the distinction between Treg and effector T cells. In the event of ongoing immune response, activated T effector cells, in the presence of IL-2 and IFN-γ get further activated to express the transcription factor t-bet and the chemokine receptor CXCR3. These activated effector $CD4^+$T cells produce IFN-γ, which together with IL-12 further activate Treg to Th1-like Treg ($CD4^+CD25^{hi}CD127^{lo}CD45RA^-Foxp3^{hi}$T-$bet^+$IFN-$\gamma^+$ $CXCR3^+$). Th1-like Treg express mRNA for Th1 transcription factor T-bet, Th1 cytokine IFN-γ and Th1 chemokine receptor CXCR3. Expression of CXCR3 enables these Treg to migrate to inflamed tissues, where they control immune inflammation as in the graft and promote tolerance. Th-like Treg, such as Th1-like Treg are the mediators of transplant tolerance and are a hundred to a thousand-fold more potent at suppression of rejection than naïve resting Treg. This figure only represents one pathway of activation of Treg and there are others such as Th-2 like Treg promoted by Th2 cells and Type-2 cytokines. The survival of highly activated Treg is dependent on continued antigen stimulation and key cytokines produced by the inflammatory response, IL-2 alone does not sustain these cells and may inhibit them.

effector lineage T cells. This inhibition may include killing effector cells, producing a quasi-clonal deletion in the graft.

Nature of activation and survival pathways for Treg is complex and may involve different Th1, Th2, Th17 and Tfh responses. IL-15 (379), TGF-β (380), IFN-γ (381), IL-12 (360), IL-4 (364) , IL-5 (369), IL-27 (382), IL-33 (383), IL-35 (384).

Activated Treg in Transplant Tolerance Are Different to Naïve $CD4^+CD25^+FoxP3^+$Treg

The precise mechanisms that effect suppression are not fully known but include CD39 on Treg producing adenosine (385), IL-35 inducing Treg (386, 387), or consumption of essential

amino acids (388). Class II MHC expression may contribute to control of inflammation.

It is the activated Treg that maintain immune tolerance, not the resting naïve Treg described by the Sakaguchis. Such highly activated Treg cells have not been generated *in vitro* as a therapy, as most studies use polyclonal expansion of naïve Treg cultured with rIL-2 with anti-CD3 and anti-CD28 mAb (206, 389). Some naïve Treg cultured with rIL-2 and donor alloantigen have been trialled (390). Therapy with Treg is beyond the scope of this review, however the current limited understanding of the processes that activate alloantigen specific Treg of high potency limits these cells full potential when applied to the clinic. To our knowledge no highly activated Th-like T reg have been trialled in the clinic.

Relevant to the key role of antigen activated, inflammation seeking potent $CD4^+CD25^+FoxP3^+$Treg, the main features are:

I. they suppress rejection at ratios of 1:1000 to effector $CD4^+$T cells and are more potent than naïve $CD4^+CD25^+$ $FoxP3^+$Treg (391).
II. they are a small fraction of the $CD4^+CD25^+FoxP3^+$ T cells population and this population remains <5% of $CD4^+$T cells in hosts with transplant tolerance.
III. their survival is key to the maintenance of transplant tolerance (392).

In contrast, naïve $CD4^+CD25^+FoxP3^+$T cells only suppress rejection when ratios of 1:1 are achieved. Such high ratios of naïve $CD4^+CD25^+$T cells has only been achieved with rIL-2/anti-IL-2 complex therapy (393). As homeostatic mechanism prevents Treg exceeding 10% of $CD4^+$T cells. Other differences between naïve and antigen activated $CD4^+CD25^+FoxP3^+$Treg have been summarized elsewhere (341, 366, 394, 395). Naïve Treg are identified as $CD4^+CD25^+FoxP3^+CD127^{lo}CD45RA^+$T cells, and those that are recent migrants from the thymus express CD31 (123, 396).

CONCLUSIONS- THE FULL NATURE OF ALLOANTIGEN SPECIFIC TREG REMAINS TO BE FULLY RESOLVED

For over 60 years the concept of "clonal deletion" has dominated the mechanism of self- non self and transplant tolerance. At the time the theory was proposed, there was no knowledge of T cells or regulatory processes. The prime role of peripheral T cells, not antibody, in allograft rejection was not appreciated.

The study of T cells, led to the discovery of numerous pathways for the activation of effector T cells to distinct functional subtypes including Th1, Th2, Th17, Tfh. Suppressor T cells were described early in the T cell era but the reliance on CD8 and I-J as markers of these cells led to a belief that suppression was an artefact. Suppressor T cells were taboo from the mid 1980s until the early 2000s.

Our work on alloantigen specific T regulatory cells in transplant tolerance identified they were $CD4^+CD25^+$T cells and were alloantigen-specific. FoxP3 expression is essential for functioning Treg (397) and the induction of transplant tolerance (398). Studies of activated alloantigen-specific Treg were difficult as they die rapidly *ex vivo* even if stimulated by specific alloantigen (150, 340, 345). Recently others have reported that activated $CD4^+CD25^{hi}FoxP3^{hi}CD45RA^-$Treg die and are hard to get to proliferate (349, 399). What promotes the survival and function of these activated Treg, is a key question to be resolved to maximize their use in promoting transplant tolerance.

Our studies described above are one of the few to have addressed this question, and identify at least three cytokines (IL-12, IFN−γ and IL-5) produced late in the immune response by effector cells. These cytokines appear after production of early cytokines such as IL-2 and IL-4 wanes. It is in this late chronic phase of the allograft response that the activated effector cells produce cytokines that activate alloantigen specific Treg that mediate transplant tolerance by inhibition of the rejection response at the site of inflammation.

At present and for the last 25 years, studies on Treg have focussed on resting naïve Treg. These cells can be expanded by the presence of IL-2 or IL-4, and possibly other cytokines that are yet to be defined. The Treg that mediate transplant tolerance die without activation by specific alloantigen and cytokines produced by the ongoing effector response to the allograft. They do not mature in the presence of IL-2, and do not need IL-2 to survive.

While cytokines such as IL-2 and IL-4 activate naïve $CD4^+CD25^+Foxp3^+$Treg they cannot sustain the highly potent Treg, which become dependent on cytokines produced in the late stages of activation of effector T cells when production of IL-2 and IL-4 wanes. In Type 1 responses, these are IFN-γ and IL-12p70. In Type 2 responses, IL-5 continues to be produced as does IL-13, both of which are anti-inflammatory (358, 361).

The early studies on neonatal thymectomy unmasked a dual and parallel function of the thymus, first producing effector T cells that were not fully deleted of auto-reactive clones, and a few days later releasing T cells that suppress autoimmunity. We now know these inhibitors of autoimmunity are naïve $CD4^+CD25^+FoxP3^+$Treg. In neonatal tolerance induction, the alloantigen could selectively activate the newly produced Treg to suppress the allograft response. There were early cues that neonatal tolerance was in part maintained by inhibitory forces. It could be argued that tolerance induction in neonates uses the same processes that protect against autoimmunity, where $CD4^+CD25^+FoxP3^+$Treg control the activation of auto reactive cells that are not deleted during ontogeny.

There are many other immune mechanisms that can come into play, including response of the graft, loss of donor antigen presenting cells, and overactivation of the immune response, leading to exhaustion. We are still some way from understanding all these mechanisms, especially the multiple pathways of activation of naïve Treg, We recently reported that naïve $CD4^+CD25^+$Treg cultured with IL-2 and alloantigen are induced to express CD8 as well as CD4, and the $CD4^+CD8^+$T cells are the potent alloantigen specific Treg (370). This finding raises the possibility naïve $CD4^+CD8^-CD25^+FoxP3^+$Treg could produce $CD8^+$Treg. Many other types of regulatory cells have been described but our focus was on alloantigen specific

$CD4^+CD25^+FoxP3^+$Treg as this is the most common and dominant regulatory cell.

It is increasingly apparent that specific alloantigen activated $CD4^+CD25^+FoxP3^+$Treg, not naïve Treg mediate alloantigen specific transplant tolerance. How to induce them and monitor them remains a challenge. Harnessing the potent antigen-specific Treg, may lead to tolerance to grafts in patients. It is also apparent that in many models of Transplant Tolerance, clonal deletion is not present and is not necessary.

Within the heterogenous populations of $CD4^+CD25^+FoxP3^+$ $CD127^{lo}$Treg, the highly activated Treg express more CD25 and FoxP3. These cells die and are thought not to proliferate, leading to the belief they serve little or no function. This has parallels with Medawar and Florey's dismissal of small lymphocytes and thymus derived cells, mentioned above. More intense study of these cells may draw us closer to solving how to induce transplant tolerance.

AUTHOR CONTRIBUTIONS

BH, NV, GT, and SH all contributed to the design, writing, proof reading of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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