Facilitating Cells: A Journey from Bench to Bedside

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Abstract

The development of safe conditioning protocols has reduced the morbidity and mortality of hematopoietic stem cell transplantation (HSCT), allowing broader application for treatment of a variety of non-malignant conditions including autoimmune diseases, hemoglobinopathies, metabolic disorders etc., as well as inducing tolerance to solid organ transplants. One of the most successful clinical trials using a facilitating cell enhanced HSCT paired with a kidney transplant has effectively induced tolerance in the absence of immunosuppressive drugs, maintaining the function of the transplanted kidney, and reconstituting the immunocompetence of the recipient. This novel protocol eliminates the need for immunosuppressive drugs, the key source of kidney and liver toxicity, increased malignancy, and shortened life span. CD8+ TCR facilitating cells (FC) are a population of tolerogenic cell which promote hematopoietic stem cell engraftment across human leukocyte antigen (HLA) barriers. In this review, we discuss the bench to bedside journey of FC, from discovery in mouse models, characterization of the subpopulations of FC, the mechanisms by which FC induce tolerance and clinical application. As a novel personalized medicine, FC may change the approach to overcoming HLA barriers for both HSCT and solid organ transplant recipients.

Keywords: Bone marrow transplantation; Graft versus host disease; Tolerance; Facilitating cell

Inception of Bone Marrow Transplantation

Bone marrow transplantation (BMT) emerged as a specialty during the nuclear experimental phase of the 1940s and 50s after the world first witnessed the devastation caused by the nuclear blasts of World War II. Irradiation of animals was subsequently widely studied to determine the effects of nuclear fallout on humans. It was found that radiation sickness can occur in humans with as little as 30 cGy, while doses of 120 cGy have the potential to cause lasting damage which in some, without treatment, could lead to death. (The Center for Disease Control and Prevention, 2017). While lethal doses were defined, the mechanism of death remained unknown until an experiment by Jacobson and colleagues [1] demonstrated that lethally irradiated mice whose spleens had been shielded recovered from radiation poisoning. Scientists soon determined that bone marrow was the ultimate source of the life-saving cells, as autologous cell transplants could rescue ablated mice that experienced no shielding [2]. They found the bone marrow to be the source of immunogenic cells, red blood cells, and ultimately, cells that had the potential to change how we viewed medicine; hematopoietic stem cells (HSC). The manipulation of bone marrow to benefit people became a focus for the medical community, resulting in what is now known as “cellular therapy.”

During this same period, in 1945, Owen described genetically disparate freemartin cattle twins who shared a common placenta that were red blood cell chimeras. Notably, the mixed red blood cell phenotype was not passed on to progeny. Owen hypothesized that the cattle exchanged cells through the common shared placenta. The term chimera stems from the ancient Greek mythological creature that possessed body parts from several creatures. In a similar fashion, the cattle lived with functional genetically disparate cells in a single body, a phenomenon that had not yet been described [3]. Building on Owen’s foundation, Billingham, et al. in their 1953 study, induced mixed chimerism in mice by inoculating fetuses with live tissue and cell suspensions from a genetically different strain. At eight weeks of age, they transplanted skin from the original donor strain. Normally in untreated mice, skin grafts were rejected within two weeks. The mice who had received the splenocyte cell suspensions in the womb demonstrated donor-specific tolerance to the graft. They concluded that chimerism actively acquired tolerance to the donor. This chimerism, and tolerance, lasted the life-span of the subjects [4,5]. Barns and colleagues found a new method and application for preparing syngenic chimeras by using radiation to rid the body of its natural lymphocytes and replacing them with lymphocytes from a donor mouse of the same strain. The researchers managed to successfully treat leukemia in a mouse model using this method [6].

While these studies demonstrated success in autologous systems (i.e., from the subject's own body) and with syngenic (genetically identical) systems, several barriers remained to be conquered to allow success in allogenic (across genetically disparate individuals) transplants. Insufficient radiation or immunosuppressive conditioning pre-transplant results in graft rejection; the graft is destroyed by the recipient's immune system. However, if the recipient is lethally irradiated to rid the body of all immunogenic cells, or excessive numbers of cells are transplanted to overcome the recipients' immune system, the donor T cells attack the major organs of the recipient's body, a syndrome called graft-versus-host disease (GVHD). These became the primary hurdles facing the medical community to conquer stem cell and, ultimately, tolerance for solid organ transplantation.

Challenges Facing Hematopoietic Stem Cell Transplants (HSCT)

Both rejection and GVHD are linked to the highly polymorphic human leukocyte antigen (HLA) complex encoded by the major histocompatibility complex (MHC). HLA antigens allow
immunocompetent cells to recognize cells as similar (self) or dissimilar (foreign). More than 200 genes are associated with MHC, 40 of which are responsible for the HLA type. HLA antigens can be further divided into three distinct classes depending upon their function and tissue location. These proteins are expressed on the surface of nearly all nucleated cells. Should a cell or tissue be found with an unrecognizable antigen (i.e., transplanted organ or infectious agent), effector T cells will target the incompatible HLA-expressing cell. The effector cells have the potential to attack transplanted organs, and also to cause serious injury throughout the body.

As early as 1972, clinical trials using HLA-matched bone marrow transplants were attempted to treat acute leukemia [7]. Surprisingly, even with a perfect 6 of 6 HLA match between donor and recipient the mean survival time of the patients was only 90 days due to GVHD and associated infections. Modern immunosuppressive therapies have improved success rates but, the recommended clinical match remains 6 of 6 HLA match and ABO compatible with no donor specific antibodies. Even patients with haploidentical related sibling donors are more susceptible to increased rates of GVHD, engraftment failure and higher incidence of infection [8,9].

GVHD is the second most common complication faced by patients who have received HSCT. If the donor cells survive to engraft, they often attack the host. Most often, cytotoxic and helper T cells contained within the HSCT cause GVHD. Several other immune components may contribute to GVHD and graft rejection, including immune cell surface molecules, antibodies, antigen presenting cells, and signaling mechanisms and their associated cytokines [10]. The severity of GVHD is directly correlated with the degree of HLA mismatch between the donor and the recipient. However, even recipients with a perfect HLA match to their donor may experience GVHD [7,11,12].

Acute and chronic GVHD are not mutually exclusive. Traditionally, acute GVHD is defined to occur before 100 days post-transplant while chronic GVHD occurs after this time period [9,13-15]. This temporal definition does not encompass how we define these categories today, as modern therapies may delay acute GVHD until after 100 days. Acute GVHD presents as inflamed tissue across the body. Most often patients first report a maculopapular skin rash as a result of lymphocyte infiltration and apoptosis in the basal layer of the epidermis. Patients will also have gastrointestinal distress including nausea, anorexia, diarrhea, and abdominal pain. The inflammation causes thickening of the bowel wall and histological analysis will reveal ulcerations, apoptotic bodies, and abscesses. Chronic GVHD can cause symptoms similar to those associated with acute GVHD, with the addition of abnormalities in the mouth, eyes, liver, lungs, kidneys and heart. The patterns are similar to those seen in a variety of autoimmune disorders as an environment has been created in which an immune system is recognizing its host body as foreign. Chronic GVHD is the primary late, non-relapse cause of death in HSCT patients [10,16]. Both forms of GVHD can be severe and are often fatal. GVHD therefore constitutes a major challenge faced by clinicians.

The chances of developing GVHD can be reduced by understanding mechanisms that cause GVHD and eliminating them. Preliminary studies reduce the number of immunogenic cells to be transplanted; however, the chance of hematopoietic stem cell transplant (HCT) engraftment is also reduced. Striking a balance is difficult, and differentiating the populations necessary for engraftment versus those that cause GVHD is the next step. Discovering the means to manipulate this balance and improve safety and long-term outcomes of transplants has been a high scientific priority for the past thirty years.

Foundations of HSC and Solid Organ Transplant

In 1990, Joseph E. Murray and E. Donnall Thomas were awarded the Nobel Prize in Medicine for their work in solid organ and bone marrow transplantation, respectively. Murray overcame the barriers of solid organ transplant by treating patients with life-long immunosuppressant treatment including imuran, azaserine, actinomycin C and 6-mercaptopurine [17]. These therapies prevented the host's effector cells from targeting and ultimately destroying the foreign tissue. However, the nonspecific nature of this therapy also exposed the patient to severe risks such as increased risk of infection. A variation of this approach has become standard practice for kidney transplant patients. Current immunosuppressive drug protocols for solid organ transplants usually include lymphodepletion induction with anti-thymocyte globulin, anti-CD52 or anti-CD25, anti-rejection prophylaxis using a calcineurin inhibitor, mycophenolate mofetil (MMF), and/or prednisone, and maintenance on a reduced dose of immunosuppression (IS) [18,19]. Unfortunately, detrimental side effects from these drugs include increased malignancies, increased rates of opportunistic infections, toxicity to the kidneys and liver, and an overall significantly shortened lifespan [20,21]. There is approximately 50% mortality within 10 years following a kidney transplant as a result of the toxicity of the immunosuppressive agents and rejection (OPTN, 2012).

Murray's counterpart, Thomas, used high levels of total body irradiation (TBI), cytotoxic drugs, and immunosuppressants to enable the engraftment of donor bone marrow to treat a series of blood disorders. Since then, the field of bone marrow or hematopoietic stem cell transplants has expanded to include treatments for hematologic malignancies, sickle cell anemia, severe aplastic anemia, other severe non-malignant disorders, as well as to prevent rejection of solid organ transplants (Figure 1) [22]. Along with side effects associated with Murray's work, additional side effects due to high levels of radiation including impaired growth, sterility, cataract formation, and secondary malignancies were observed in Thomas' patients [23].

Fuchs et al. significantly reduced the toxicity of conditioning by establishing less toxic immunosuppressant regimen for patients undergoing haploidentical HSC transplant in order to achieve chimerism [24]. The reduced intensity conditioning consisted of cyclophosphamide (14.5 mg/kg/day; days -5 and -6), fludarabine (30 mg/m²/day; days -6 through -2), and TBI (200 cGy; day -1). The patients then received one more dose of cyclophosphamide (50 mg/kg) on day 3 or 4 followed by MMF and Tacrolimus as postoperative IS maintenance. MMF was discontinued at day 35 and patients were maintained on tacrolimus for two to three years. Patients also received prophylactic anti-microbial therapy starting the day after transplant. Fuchs' protocol has been shown to induce hematopoietic chimerism.
while reducing the incidence of GVHD and addressing both malignant and non-malignant blood disorders, ranging from autoimmune to sickle cell anemia with great success [25,26]. With minor variations, this protocol has proven to be safe for women who plan to become pregnant in the future [27] and for pediatric patients [28]. Of the 68 patients treated in total, nine experienced graft failure, and 34% experienced acute GVHD. Two patients died due to these complications. With a one-year mortality rate of only 15%, this protocol has proven to greatly improve the outcomes with a 20% reduction in risk of an adverse event based on previous haploidentical or mismatched HSCT transplants [29].

Tolerogenic Strategies

The holy grail of transplantation is to achieve life-long tolerance to grafts without the use of long-term IS [30]. Operational tolerance (OT) is a result of either non-compliance or a clinical protocol aimed to gradually wean patients off IS. This term is most often used for those patients who have achieved tolerance that was not induced by chimerism. OT is observed in a limited number of patients- almost exclusively liver transplant patients. It is posited that repopulation of the endothelial cells of the liver vasculature by the recipient cells allows for this phenomenon [31]. The endothelial repopulation is unique as it has not been observed in any other transplanted organs.

Shapiro et al. designed a regimen using induction pretreatment and low-dose postoperative IS that allowed for partial weaning of IS in kidney transplant patients. One hundred and fifty patients were treated with prednisone and rabbit antithymocyte globulin (ATG) before transplant [32]. After the transplant, patients were maintained on tacrolimus monotherapy with the addition of steroids only for rejection episodes. Seventy-three percent of patients developed limited operational tolerance and began the partial weaning protocol. Ninety-four patients were eventually maintained on tacrolimus dosed from every other day to once a week. Although some patients were successfully tapered from IS and remained free from graft rejection, four patients died within the first year and an additional five grafts were lost. In all, 56 patients experienced rejection or mortality using the planned IS withdrawal. Notably, there was no reliable biomarker to predict success vs. failure in individual patients [32]. The study has since been closed because rejection episodes impair long-term allograft survival [32].

Other studies that have attempted to achieve OT have used a wide range of manipulations from length of weaning period and follow-up to differences in immunological background and patient age [33]. Unfortunately, the attempts at clinical induction of OT have been largely unsuccessful due to inability to predict success, and often leads to rejection and permanent graft damage [33-35]. A reliable procedure has yet to be found and a major shortcoming of OT protocols is that progress cannot be tracked except to note damage to the organ upon biopsy or functional impairment of the graft [33]. Because rejection episodes are associated with inferior graft-outcomes, most IS minimization protocols have been abandoned.

Deletional tolerance is most often defined as tolerance that occurs as a result of chimerism induction. In contrast to OT, chimerism may serve as a reliable biomarker allow for a real-time indication of safety for the graft. There may be no need to rely upon biopsies to ensure the status of the transplant as a physician can simply use peripheral blood (PB) samples to measure progress [36]. In animal models, chimerism can be induced by bone marrow transplantation, inoculation of fetuses in the womb with hematopoietic cells, or transplantation of mobilized purified HSC. Transplantation of mobilized HSC has become the most common approach to obtain HSC for HSCT in the clinic [37]. For application of this approach to induce tolerance the challenge remains to find an approach to safely perform transplantation and develop strategies to overcome the associated challenges while ensuring engraftment across HLA barriers.

Early work in improving transplant outcomes

In the 1990s, T cells were identified as the primary effector cell in GVHD. Many investigators attempted to deplete donor T cells and progenitor T cells to prevent GVHD [38-40]. Some clinical trials used matched transplants with T cell depletion and similar protocols to those used historically with limited success [41]. In a modification of traditional protocols, the use of T cell depleted HSC transplants increased significantly. Engraftment was found to be possible over HLA mismatch barriers with related donors using this protocol. Megadose of stem cells derived from the bone marrow, peripheral blood, or both have shown success at reducing GVHD in patients undergoing HSCT to treat leukemia [37]. However, a number of unexpected severe adverse events were associated with TCD [42]. Patients were less likely to experience GVHD, but they experienced a significantly increased incidence of graft rejection, delayed reconstitution, increased rates of relapse and increased post transplantation lymphoproliferative disorders [43]. In the clinical trials, about half of the patients with acute lymphoblastic leukemia relapsed and transplant-associated mortality reached about 40% [44]. Patients receiving T cell depleted grafts avoided GVHD but had a significantly higher graft failure overall [42].

Also, Mesenchymal Stem Cells (MSC) with immunoregulatory properties have been used to induce tolerance and avoid GVHD [45]. In a pilot study by Remuzzi et al. safety and feasibility was demonstrated in two patients with ESRD. These patients received kidneys from living-related donors along with autologous MSC. Both patients demonstrated stable graft function. The tolerance was associated with increase in regulatory T cells and reduction in CD8+ T cell activity [46]. In another clinical trial by Tan J et al. induction therapy with MSC resulted in a lower incidence of acute rejection, decreased infection and better renal function [47]. The immunomodulatory function of MSC includes upregulation of Tregs and secretion of cytokines that induce tolerance by downregulating function of effector T cells [48].

Regulatory T (Treg) cells are a foundational element that acts as checks and balances to control inflammation and exert regulation of the immune response. Tregs are responsible for mediating the immune response to alloantigens and ultimately preventing rejection in vivo. CD4+CD25+FoxP3+ Treg arise naturally from the thymus (natural Treg). A portion of Treg can be induced (inducible Treg) through exposure to TGF-β [49] or IL-10 [50] from CD4+ precursor cells. Treg modulate the immune response with multiple mechanisms. Each of these can impact graft rejection by tampering the immune response to the graft. Treg secrete cytokines including IL-10, TGF-β, and IL-35, which inhibit the effector T cell populations as well as increase the number of Treg, leading to a cycle which gathers strength until the immune response is checked [49,51-53]. They also have the ability to prevent antigen presenting cells (APC) from stimulating T effector cells by downregulating their co-stimulatory molecules CD80 and CD86. Without this stimulation, T effector cells remain anergic in the presence of other inflammatory stimuli [54]. Treg can have a more
direct role by triggering the granzyme A/B and perforin cascade which induces apoptosis in inflammation-inducing CD8 and CD4 effector T cells as well as both immature and mature dendritic cells (DC) [55]. Finally, Treg have the ability to reduce the amount of the IL-2 survival signal in the environment which also ultimately leads to cell death of T effector cells [56-58]. Each of these mechanisms allows for decreased inflammation and decreased activity of CD4+ effector cells which ultimately promotes tolerance in grafts [59]. Regulatory T cells have been used as a means to predict damage to renal transplants, and to detect subclinical rejection [60].

In 2002, Martelli and colleagues determined that alloreactive natural killer (NK) cells prevent relapse of hematologic malignancies and facilitate engraftment using data compiled from multiple clinical trials [61,62]. Tumor cells modulate surface MHC expression and evade immune surveillance. The NK cells act on the malignant cells that have lower expression of MHC preventing immune evasion through MHC class I switching [63]. The transplant success is attributed to the ablative nature of recipient reactive donor NK cells which may reduce the populations of donor-reactive T cells. When high proportions of alloreactive NK cells are infused with bone marrow, less ablative conditioning can be utilized and GVHD is reduced in mice models and humans [62].

Discovery of facilitating cells

Kaufman's 1994 publication in Blood is the first report of a population of CD8+ TCR-cells with facilitating potential for engraftment of HSC across MHC barriers [64]. The authors systematically removed defined populations from bone marrow and transplanted the remainder into allogeneic recipients (B10.BR donors to B10 recipients) to determine which population, when removed, reduced engraftment success. The critical cell populations for engraftment were CD8+, CD45+, CD45R+, and Class IIdim/intermediate. More specific subpopulations with potential facilitating properties were isolated using two- and three-color rare event cell sorting. Using the same allelogenic mouse model, a total of 17 populations were combined with a purified HSC transplant. The addition of CD8+CD45R+Class IIdim/intermediate Thy 1+αTCR-γδ TCR-cells to sorted HSC increased engraftment to 96%-99%. This population, generally identified as CD8+αTCR-γδ TCR-, is the novel population of bone marrow-derived cells that can enhance stem cell engraftment across MHC disparities (Figure 2).

Five years later, Gandy et al. reported on two facilitating populations present within BMC: CD8+ TCR and CD8+ TCR-cells [65]. The former represents a classic effector population while the latter is described as having a granular morphology and sharing more characteristics with lymphoid dendritic cells than T lymphocytes. Gandy and colleagues performed allelogenic bone marrow transplants by combining traditional MiniMacs sorted HSC with various sorted populations from C57Bl/Ka mice. These cell populations were combined and transplanted into BALB/c mice via tail vein injection. The recipients were evaluated at several time points for survival, the percent of donor chimerism, lineages derived from the donor graft, and the resident recipient immune populations. While CD8+ TCR+, CD8+ TCR-, and CD8+ total cells enhanced HSC engraftment, CD8+ TCR+ cells were the most efficient with only 10,000 cells needed to facilitate engraftment. The authors attributed the success of donor CD8+ cells transplanted with HSC as a result of their ability to eliminate residual recipient effector T cells and NK cells, whose numbers decreased after transplantation [65].

The CD8+ TCR+ facilitating cell population (FC) constitutes approximately 0.4% of total bone marrow cells. FC are found in the lymphoid gate. They are morphologically similar to immature dendritic cells. FC are heterogeneous in lineage and morphology and cannot be found in the peripheral blood, but are present bone marrow and spleen of mice (Figure 3) [64]. When FC are transplanted with HSC, ablated recipients maintain either stable full chimerism or durable mixed chimerism with no GVHD. Notably, the FC must be MHC matched to the HSC in order to have a facilitating effect [64].

Transfer of as few as 30,000 sorted FC with 5,000 HSC improves the engraftment in an MHC-disparate allelogenic mouse model [66]. FC also facilitate the engraftment of Sca1+ c-kitLin fetal liver stem cells in an allogenic model. Without FC, 20,000 fetal liver cells are needed to reconstitute lethally irradiated adult mice. With the addition of 30,000 MHC-matched purified bone marrow derived FC, as few as 2,000 fetal liver cells are needed to establish multilineage durable engraftment [67].

Phenotypic characterization of mouse FC revealed that a large subpopulation expresses CD11b and B220, while a smaller subpopulation expresses NK1.1, Gr-1, Sca-1, and CD14. Mouse FC do not express CD11c, CD19, or c-Kit [65]. About 40% of FC are CD4+CD8+, similar to double-positive, immature T cells found in the thymus. This along with the mRNA and protein expression of pre-TCRα, another T cell progenitor marker, indicate that bone-marrow derived FC contain a T-cell progenitor population similar to those found in traditional developing thymocytes [68]. NK1.1 is associated with natural killer (NK) cells, suggesting that there may be a subpopulation of NK-FC. Low populations of NK cells are associated with suppressed GVHD [69] so it is likely that those NK cells previously assumed to be traditional NK cells are a part of the CD8+ TCR+ FC population.
Figure 3: G-CSF mobilized lymphocytes of a healthy patient analyzed by flow cytometry. The lymphocyte gate consists of 3% CD8+ TCR- cells by flow cytometry. The facilitating cells make up 0.4% of the total bone marrow.

Mechanisms of Mouse Facilitating Cells

A study performed by Grimes, et al. examined the origins of FC by transplanting green fluorescent protein (GFP) expressing HSC into donors. The labeled cells were tracked and found to produce CD8+ TCR- FC cells. Although the FC population is derived from stem cells, they are not stem cells themselves. FC are unable to form colonies in vitro in a CFU assay and they lack the ability to rescue ablated mice when transferred alone. This lineage, while similar to traditional T cells, is developmentally independent. Mice that express mutant TCRα, with no functional αTCR, still produce functional FC [70].

When HSC are transplanted alone, large numbers of cells are needed to achieve engraftment. Co-administration of FC allows smaller physiologic numbers of HSC to engraft without GVHD. When cultured with HSC, FC increases the clonogenicity of stem cells and increases the proportion of multipotent progenitor cells [71]. In addition, HSC cultured with FC are less likely to undergo apoptosis. While the FC produce TNFα and upregulate Bcl-3, reducing apoptosis, these cytokine effects are minimal compared to the effects seen with cell-to-cell contact [72]. FC transplanted in conjunction with HSC most likely modulate their survival and function by direct interaction to improve efficiency of homing to the bone marrow niches and provide protection.

To examine further how this heterogeneous population can have such a profound effect, unique protein expression of these cells has been examined. While FC are αTCR- by flow cytometric analysis, they have a TCR analogous protein structure that utilizes the TCR protein which is critical for functionality. FC derived from mice deficient in TCRβ, including TCR-/- or RAG1-/-, are not able to facilitate engraftment in an allogenic model (Table 1). The novel protein structure consists of a surface protein called FCp33 in complex with CD3ε, TCR β, and FCRγ dependent signaling. The presence of this complex directly correlates with enhanced engraftment of HSC in vivo [73]. FC functionally require CD3 ε, however, it is only seen on about 5% of the FC total population [70]. Cell surface FC CD3ε expression is dimmer than conventional T cells on Southern Blot indicating that it may be a slightly different variant than the traditional CD3ε protein. When CD3ε or CD3δ are deleted from the FC, they lose their facilitating function. Thus, both molecules may play a role in the analogous complex described [70]. FCp33 may serve a similar function to that of calnexin which is found on immature thymocytes (Figure 4).

| Flow Cytometry | PreTCR Complex | Mature T cell TCR Complex | FC TCR Complex |
|----------------|----------------|---------------------------|----------------|
| αTCR           | -              | +                         | -              |
| αTCR           | -              | +                         | -              |
| TCR            | +              | +                         | +              |
| CD3ε           | +              | +                         | +              |
| FCp33          | +              | +                         | +              |
| Western Blot   | αTCR           | 85 kDa                    |                |
|                | αTCR           | 40 kDa                    |                |
Table 1: Flow cytometric and western blot analysis of the TCR complex in three T cell populations of B6 mouse origins.

| Protein | Molecular Weight |
|---------|-----------------|
| TCR     | 45 kDa          |
| CD3ε   | 25 kDa          |
| pre-Tα | 33 kDa          |
| FCp33  | 33 kDa          |

Figure 4: Representations of the PreT cell TCR complex compared to a mature CD8$^+$ T cell and a CD8$^+$TCR$^-$ facilitating cell. FC have properties including a 33kDa βTCR-associated molecule (FCp33) and FcR γ dependent signaling that show a similarity to the Pre T cell TCR complex on immature thymocytes.

When FC are transplanted with HSC, there is an increase of anti-inflammatory, Treg-inducing TGFβ expression. In addition, genes associated with CD4$^+$CD25$^+$ T regulatory cells including CTLA-4, GITR and FoxP3 are upregulated in spleens in mice after FC+HSC transplantation. FC are not Tregs nor are they the cells producing these proteins as they do not express those same Treg identifying genes: FoxP3, CTLA4, and GITR [74]. Two subpopulations of FC, CD3ε$^+$ and CD3ε$, induce the generation of Treg cells in vitro. They both express high levels of TLR9 which induces high levels of both TGF and IFN-γ in CD3ε$^+$ cells. Adoptive transfer of FC and HSC into mice in vivo results in development of antigen specific Treg [75]. Notably, these Treg are recipient-derived. Sorted splenocytic recipient-derived Treg that are specific to the donor HLA markers will not facilitate engraftment of HSC from a third party strain. In addition, they do not reach peak functionality until about four weeks post-transplant. Before four weeks, the phenotypic Treg do not display tolerogenic properties. The donor-specific, recipient-derived Tregs enhance engraftment in a dose dependent manner [76].

FC are mobilized by Flt3 ligand (Flt3-L) in mice. Flt3-L increases the number of FC in both the bone marrow and the peripheral blood by up to 100 fold. After mobilization, FC that remain in bone marrow do not maintain their facilitating function. There was a marked absence of NK-FC normally present in the general FC populations, and adhesion molecules including P-selectin, L-selectin, and stromal cell-derived factor-1 (SDF-1) [77]. Unlike FC in the bone marrow, the mobilized FC found in the PB are not impaired. The functional FC found in the PB are associated with increased expression of SDF-1 on HSC and its receptor CXCR4 in the bone marrow, molecules which are integral in the cell adhesion molecule modulation. It is hypothesized that these molecules along with DOCK2 act as chemottractants for HSC within the donor’s body and allow both cell populations to migrate to donor bone marrow in response to an SDF-1 gradient [78].

The majority of mobilized FC cells are phenotypically, morphologically, and functionally similar to plasmacytoid precursor dendritic cells (p-preDCs), a population that allows for HSC engraftment, and donor-specific tolerance to skin transplants [79]. The CD11c$^+$B220$^+$CD11b$^-$ cells induce antigen specific Treg in vivo. Most of the Treg developed after 4 weeks after transplant and are primarily recipient-derived. Even though the Treg did not mature and become functional until after four weeks, they are integral to engraftment as p-preDC depleted FC are unable to facilitate engraftment [75]. However, this population does not provide all of the facilitating properties of FC as p-preDC FC alone will not facilitate HSC engraftment as efficiently as FC total. P-preDC and p-preDC FC are both activated by toll-like receptor (TLR)-9 and expand and mature after Flt-3 stimulation. When activated, both populations produce IFN-α and TNF-α [79].

P-preDC FC are a part of the CD3ε$^+$ population previously discussed. Many distinct populations facilitating function within the FC gate have been described [80]. The diversity of mechanisms and populations involved in engraftment facilitation within the CD8$^+$TCR$^-$ FC gate are encouraging for the future of this population. Even if one mechanism is dysfunctional or absent for in an individual, they may
still be able to have facilitation. In addition, these may be used for different purposes under different circumstances.

In a study examining the FC population in diabetes-prone NOD mice, Huang and colleagues found that p-preDC FC are less efficient than those in the wild type B6 model [81]. NOD FC fail to promote generation of colonies from HSC in vitro in CFC assays. They contain the same phenotypic subpopulations including p-preDC, CD19+ cells, and NK1.1+ DX5+ cells, but are functionally impaired. The CD19 and NK FC cell populations are far scarcer in NOD mice compared to B6 mice. If either of these populations is depleted, there is no difference in FC function. However, if NOD mice are treated with Flt-3, FC from treated mice gain the facilitating function seen in the wildtype model. After mobilization there was an increase in DC and NK subpopulations but the percentage of p-preDCs and CD19+ remained the same. These data indicate that a portion of the NOD faulty mechanism allows for the facilitating function, potentially linking FC to diabetes-pathogenesis [81].

Eighteen proteins were found to have significant differential expression in a gene array study to examine the difference between NOD FC and functional NOR FC. One of the most significant disparities between NOD vs. NOR FC was dedicator of cytokinesis 2 (DOCK2), a hematopoietic cell-specific molecule that plays an important role in the migration of lymphocytes, neutrophils, and pDC through the small GTPase activation pathway [82]. Unlike wildtype B6 FC, FC from DOCK2 knock-out mice cannot convert CD4+CD25+ T cells to Treg or IL-10 producing type 1 regulating T cells [83]. In addition to dysfunctional Treg induction, the DOCK2 -/- FC were also unable to promote HSC survival and engraftment. Finally, enhanced migration of HSC towards stroma-derived factor-1 (SDF-1) in vitro and towards BM in vivo is compromised in these knock-out FC. They fail to migrate to the hematopoietic niche or continue to differentiate, leading to ultimate failure of engraftment. These studies were confirmed using a DOCK2 inhibitor. Overall, DOCK2 is necessary for both the homing and facilitating function of FC [82].

Mechanistic studies of FC in humans

The FC population in humans has been well characterized and shares many similarities with murine FC. The human FC population is functionally similar to the murine FC population and can be found in mobilized peripheral blood, bone marrow, and cord blood [71]. Human FC also does not form colonies in vitro. However, when co-cultured with HSC they induce higher levels of colony formation of granulocytes, erythroids, macrophages, and megakaryocytes than when HSC are cultured alone. FC transplanted into humanized NSG mice also facilitates the homing of human HSC to bone marrow. The human FC population consists primarily of two distinct populations defined by CD56 expression. CD56neg FC promotes early HSC clonogenicity in vitro and in vivo and early homing in vivo. The CD56neg population of FC are of lymphoid morphology and are CXCR4+, CD3ε+, and HLA-DR+. They do not express CD11c and CD123 [84].

In contrast, the CD56bright FC population does not enhance the immediate homing of HSC, but instead enables durable chimerism. This population is responsible for survival after HSC have entered the hematopoietic niche. They more resemble dendritic cells and are CD11c+, CD11b+, CXCR4+, and CD19+. They also have regulatory effects on T and B cells. Together the CD56bright and CD56neg FC populations illustrate the multiple synergistic mechanisms that contribute to successful HSC engraftment. The CD56neg population allows for the immediate homing and function of the graft, while the CD56bright preserves its integrity and allow it to maintain function long-term [84].

Current Clinical Applications

In contrast to operational tolerance, chimerism-induced tolerance can be readily monitored through blood tests and molecular HLA-typing via short tandem repeat (STR) genotyping [85,86]. The monitoring of donor-derived peripheral blood chimerism, especially lymphocytes, allows an inexpensive and reliable biomarker for success [36].

Collection of HSC is no longer performed through the traditional method of aspirating bone marrow from the iliac crest under general anesthesia. Large numbers of viable cells can be obtained by inducing proliferation and exodus of peripheral blood mononuclear cells from the marrow itself into the peripheral blood using treatment of the donor with granulocyte-colony stimulating factor (G-CSF). This is referred to as mobilization and does not require anesthesia for collection of the product. The patient then undergoes apheresis which allows a filter of the blood, removing the target cells and returning all non-target cells to the donor. Mobilized peripheral blood cells have proven to be effective at durable engraftment [37].

To translate the discovery of the FC to the clinic, a method to engineer G-CSF-mobilized HSC product was developed under GMP manufacturing guidelines and approval to proceed obtained from the FDA. The team hypothesized that successful transfants could be performed to induce tolerance to renal allografts using a bioengineered allogeneic cell therapy product from the same donor as the kidney.

Why is tolerance still being pursued?

One could question why tolerance should be pursued in light of current excellent short-term results in renal transplantation [87]. Death-censored graft survival has improved in the past 20 years and early survival outcomes are excellent. However, a sobering observation that has not improved over 20 years is the fact that long-term survival is significantly reduced in kidney transplant recipients: 50% die by 10 years after transplant. The reduced lifespan is primarily attributable to the complications caused by the IS agents and chronic allograft loss. Moreover, especially for our pediatric transplant recipients, the daunting fact of facing at least 4 transplants over a lifetime looms large. It is hypothesized that tolerance would allow one transplant for life by avoiding the toxicities of IS and preventing chronic rejection. In its current state kidney transplants are saving lives but quality of life and long-term outcomes could definitely be improved.

In a very productive collaboration with Northwestern University, the approach was applied to living related and unrelated donor kidney transplant recipients [85,88-92]. This study is currently a phase II clinical trial at Northwestern University and Duke University. The donor is mobilized and undergoes apheresis at least 2 weeks before the kidney transplant is performed. The product is shipped fresh to the University of Louisville where the manufacturing of PCRx is performed. The product is cryopreserved and shipped back to the site after 14 day sterility results are complete. Recipients are treated with reduced-intensity nonmyeloablative conditioning consisting of three doses of fludarabine (30 mg/kg/dose/ days -4, -3, -2), two doses of cyclophosphamide (50 mg/kg/dose/days- and +3), and 200 cGy of TBI on day -1 before receiving a living donor kidney transplant on day 0.
One day after the transplant, the patient receives an infusion of the engineered, facilitating cell enriched, donor HSC (FCRx). Patients are maintained on Tacrolimus and Mycophenolate mofetil (MMF) and are discharged on post-operative day 2 and managed as out patients. After 6 months, if chimerism is stable (>50%), renal biopsy is rejection-free, and renal function is normal, patients are weaned from MMF. At 9 months the tacrolimus is tapered to lower trough levels, and at 12 months IS is completely stopped if the same criteria at month 6 are present [88-92].

As of January 2018, 37 patients have been transplanted with FCRx. The first 31 subjects have reached the >12 month follow up (range 20–96 months) and will be reviewed first. The remaining 6 subjects will be reviewed separately. Subjects ranged in age from 18–65 years. There have been 2 HLA-matched related transplants and the remainder has ranged from 5 of 6 to 0 of 6 matched related and unrelated. Thirteen subjects had unrelated donors and 18 had related. Thirty of 31 subjects had high levels of donor chimerism at one month by STR molecular typing (+5% sensitivity). Durable donor chimerism was established in 23 of 31 subjects. Twenty of these 23 chimeric subjects are >95% donor. Twenty-three of these subjects have been completely weaned off IS and are from 8 months–96 months off IS. None of these subjects have exhibited DSA, rejection, none have lost chimerism, and none have had to resume IS. The one subject who failed to engraft was highly sensitized (PRA>30%). Five subjects developed transient chimerism and are maintained on low dose monotherapy with stable renal function.

There were 2 graft losses. One graft loss was due to viral sepsis and subsequent renal artery thrombosis while still on conventional IS and the second experienced antibiotic-resistant Klebsiella sepsis in his native polycystic kidneys early after transplant that required tapering off IS. Of note, the majority of severe adverse events occurred within the first 6 months when the subjects were still on double-drug IS.

There have been two cases of GvHD. The first occurred in a chimeric subject at the time of conversion from tacrolimus to sirolimus due to CNI-toxicity. The subject presented promptly to the transplant center, was treated with steroids, and promptly responded. He is now off IS with stable renal function but has mild cutaneous donor GvHD. His activity level is excellent.

The second patient experienced multiple episodes of reactivation of CMV while still being followed early after transplant at Northwestern. He returned home to a small town in New York, where he developed severe bloody diarrhea. He presented to a local nontransplant community hospital when the symptoms continued to worsen over a week and was given the diagnosis of GVHD-colitis. He was refractory to steroid treatment. He was then transferred to Northwestern where he underwent a full transplant infectious disease work-up and he was noted to have severe CMV colitis with numerous inclusion bodies on biopsy of his colon. He was started on Gancyclovir but developed sepsis with multiorgan failure and expired. We have modified our approach to patient management to include a weekly call to check on the subjects and encourage them to report any new symptoms to the transplant center. Overall, this represents a 3% incidence of >grade II GVHD in our phase 2 study. There has been no GVHD in the subsequent subjects transplanted.

T cell repertoire is diverse and novel

T cell subsets from chimeric recipients were analyzed using TREC analysis for their clonal diversity, an indication of immunocompetence. 97% of T cells showed novel specificity suggesting that the T cell repertoire was newly developed after transplantation and that the T cell repertoire was not due to homeostatic proliferation of donor or host T cells (Figure 5) [91]. The majority of newly produced T cells was unique and did not resemble the donor or recipient.

One of the four transiently chimeric patients and the patient who never experienced chimerism had a relapse of the autoimmune disease that had originally caused renal failure. No relapse has been noted in any of the durably chimeric patients. Conditions including IgA nephropathy, membranous nephropathy, and FSGS did not have signs of relapse causing kidney damage for at least two years after transplant, even after discontinuation of IS [91]. Although the number of patients is small, the data support previously published reviews and case reports demonstrating that HSCT causes a halt in autoimmune disease progression [93].

![Figure 5: Immune Reconstitution in FCRx patients. CD4 and CD8 T cell subpopulations: T effector memory cells (CD45RO+/CD45RA−/CD62Llow); T central memory cells (CD45RA−/CD45RO+/CD62Lhigh); T effector (CD45RA+/CD45RO−/CD62L−); and naïve T cells (CD45RA−/CD45RO−/CD62L+).](image)

**Conclusion**

The road to safe and effective HSC transplant has been a long journey. HSCT may be the answer to safe solid organ transplantation as well as treatment of a variety of other malignancies and conditions [93]. The induction of tolerance and avoidance of IS will allow a transformative improvement in the quality of life, graft outcomes and survival for hundreds of thousands of patients each year. One could finally realize the goal of one transplant for life. By overcoming the barriers of graft rejection and GVHD, we are close to taking full advantage of the potential of chimerism to induce tolerance for solid organ transplantation. It is important to remember that although short-term (1 year) outcomes with standard of care are very good, long-term outcomes are not: there is a 50% mortality for SOC kidney transplant recipients by 10 years [87]. In addition to solid organ transplantation, safe HSC transplantation may have a significant impact on treatment of blood cancers, sickle cell anemia, inherited metabolic disorders, life-threatening autoimmune diseases, and severe combined immunodeficiency.

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