NOVEL THERAPIES FOR TOLERANCE INDUCTION IN SOLID ORGAN AND BONE MARROW TRANSPLANTATION

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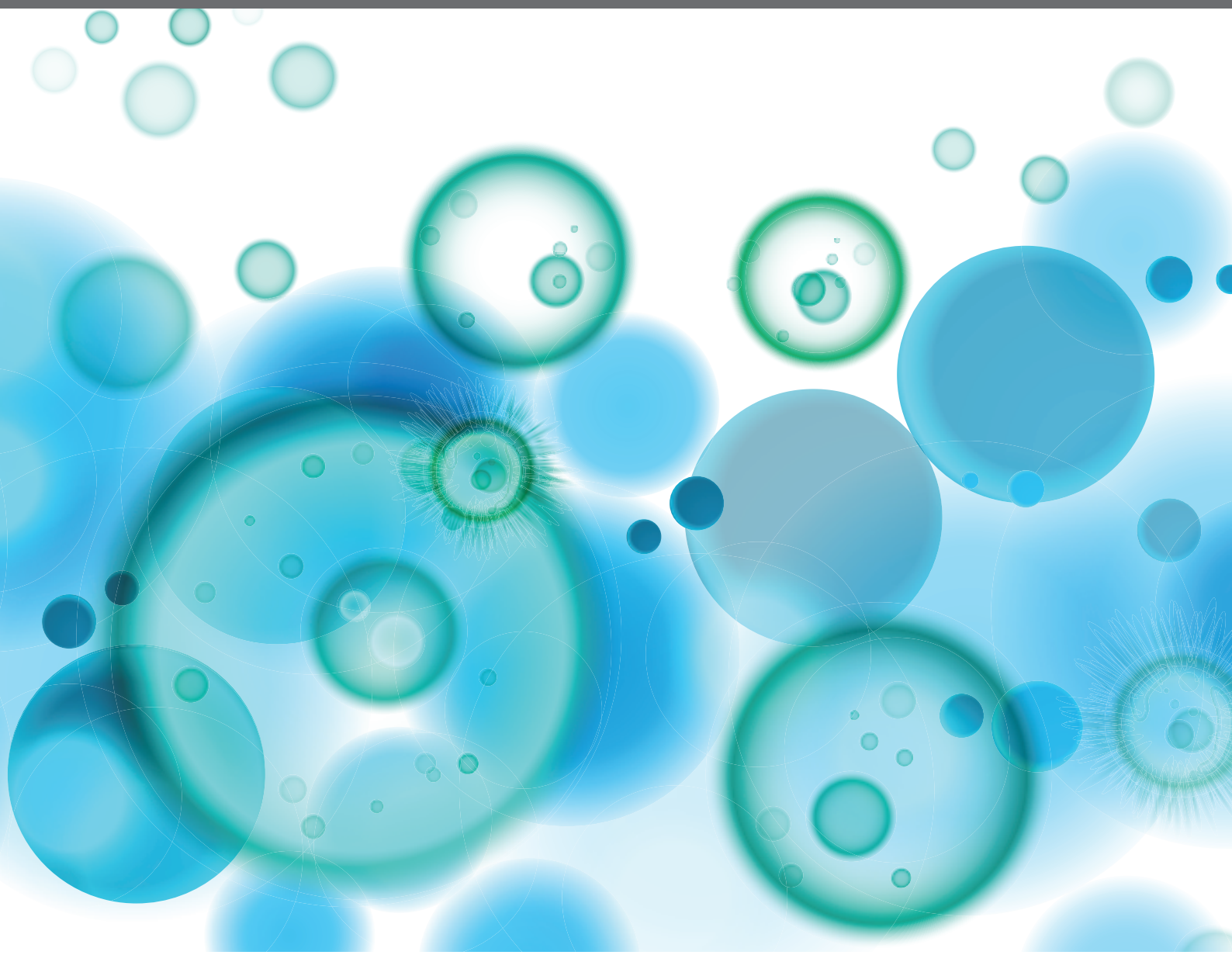

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NOVEL THERAPIES FOR TOLERANCE INDUCTION IN SOLID ORGAN AND BONE MARROW TRANSPLANTATION

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REVIEW
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Tolerogenic Dendritic Cells: The Pearl of Immunotherapy in Organ Transplantation

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Over a half century, organ transplantation has become an effective method for the treatment of end-stage visceral diseases. Although the application of immunosuppressants (IS) minimizes the rate of allograft rejection, the common use of IS bring many adverse effects to transplant patients. Moreover, true transplant tolerance is very rare in clinical practice. Dendritic cells (DCs) are thought to be the most potent antigen-presenting cells, which makes a bridge between innate and adaptive immunity. Among their subsets, a small portion of DCs with immunoregulatory function was known as tolerogenic DC (Tol-DC). Previous reports demonstrated the ability of adoptively transferred Tol-DC to approach transplant tolerance in animal models. In this study, we summarized the properties, ex vivo generation, metabolism, and clinical attempts of Tol-DC. Tol-DC is expected to become a substitute for IS to enable patients to achieve immune tolerance in the future.

Keywords: organ transplantation, dendritic cell, tolerogenic dendritic cell, immune tolerance, metabolism

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INTRODUCTION

Since Dr. Joseph Murray performed the first successful renal operation between identical twins in 1954, organ transplantation has developed extensively (1). However, transplant surgeons and immunologists around the world are always looking for better and safer treatment for severe intra- or post-transplant complications, including rejection, tumor, and infection, which directly or indirectly result from the allograft itself or application of immunosuppressive agents (2). Moreover, traditional immunosuppressants (IS) commonly focus on adaptive immunity (T and B cells); however, once they are activated, stalling the rejection process becomes considerably difficult (3). Therefore, understanding the various factors that activate T and B cells is significant to the therapies for anti-rejection. Dr. Ralph Steinman in 1973 first described dendritic cells (DC) (4). DC are considered to be the most potential antigen-presenting cells (APC), which recognize non-self or even self-antigen and stimulate powerful adaptive-immune cells, such as effector or memory T cells (Teff or Tmem) and indirectly induce plasma cells for antibody production (5). Depleting DC seems to be very effective and technologically advanced for the prevention of organ transplant rejection, which can result in surprising immunodeficiency and lead to some unexpected issues in the body (6). Hence, modifying the DC phenotype and function for inducing transplant tolerance is necessary. A recent study shows prospective strategies to minimize drug treatment, and a reduction in rejection was achieved by combining reduced

amounts of IS with immunoregulatory cell therapy in solid organ transplantation (7). Additionally, many reports focus on cell therapy in organ transplantation, including mesenchymal stem cells (MSC), regulatory macrophages (Mreg), tolerogenic dendritic cells (Tol-DC), and regulatory T (Treg) and B (Breg) cells (8–11). Herein, our attention is focused on Tol-DC, which show immunoregulatory functions in autoimmune diseases (12), infections (13), and cancers (14) as well as organ transplant issues (8). We review their features, ex vivo generation, and clinical applications and discuss their diverse effects on organ transplantation.

THE CHARACTERISTICS AND BIOMARKERS OF TOL-DC

DC, which are the so-called professional APC, characterize the bridge to development of an adaptive immune response (specific cell- and antibody-mediated clearance) from the innate immune response (15). DC were first distinguished in lymphoid tissues from other leukocytes on the basis of this idiosyncratic cell shape and an absence of critical lymphocyte and phagocyte properties (16) and subsequently identified in essentially all other tissues of the body. Immediately after transplantation, pattern recognition receptor (PRR)-mediated danger signals activate DC, leading to APC maturation, upregulation of costimulatory molecules, and secretion of proinflammatory cytokines and cytotoxicity (17). At the present time, four main cell types are generally classified as DC: conventional DC (cDC), plasmacytoid DC (pDC), Langerhans cells, and monocyte-derived DC (mono-DC). Among solid organ transplant models, according to the three allorecognition pathways (direct, indirect, and semidirect pathways), DC either derived from donor or recipient tissues and carrying donor major histocompatibility complex (MHC)-specific antigens could be recognized in the secondary lymphatic tissues of recipients to activate a T cell alloimmune response (18). Nonetheless, in addition to the rejection contribution, DC also play an essential role in allograft tolerance, which shows DC in transplanted models have two sides (19–21). Some DC that are able to suppress immune responses are initially termed as Tol-DC.

Mature DC exhibit the characteristics of high expression of the surface MHC-II and costimulatory molecules (CD80/CD86 and CD40). On the contrary, Tol-DC are often characterized by low expression of MHC-II and CD80/CD86 and CD40, termed as a state of "semi-maturity" (8). Additionally, Tol-DC are also featured with increased expression of anti-inflammatory molecules, such as interleukin-10 (IL-10) and transforming growth factor-beta (TGF-β), and decreased levels of IL-12p70 and other proinflammatory cytokines (22).

Transcriptome and proteome studies illustrate distinctive molecular signatures of Tol-DC. Though it is still difficult to find uniform surface markers to define Tol-DC, it is reported that some genes, such as CYP24A1, MUCL1, MAP7, CCL18, C1QB, C1QC, CYP7B1, and CNGA1, could be considered as possible biomarkers for Tol-DC (23).

There are also other molecules that can be regarded as the biomarkers of Tol-DC. The complement subunit C1q was recently identified as a biomarker for monocyte-derived Tol-DC, which could suppress $CD4^+$ T-cell activation *via* increasing IL-10 secretion (24). Immature DC are a rich source of active C1q, and the expression of C1q is downregulated when DC are approaching the mature state (25). Globular C1q receptors (gC1qR) are one of the receptors expressed in the surface of mono-DC, and C1q could inhibit the differentiation of DC from its precursor *via* combination with gC1qR and DC-specific intercellular-adhesion-molecule-3 grabbing non-integrin (DC-SIGN) (26). In addition, C1q is a functional ligand for leukocyte-associated Ig-like receptor 1 (CD305), which is a transmembrane protein expressed on both myeloid and lymphoid cells, restricting DC differentiation and activation (27). In the immunotherapy of pollen allergic patients, the increased levels of C1q expressed by Tol-DC in peripheral blood mononuclear cells (PBMC) represent a candidate biomarker of early efficacy of allergen immunotherapy (28, 29). Macrophage inhibitor cytokine (MIC-1) is a divergent member of the TGF-β superfamily, and the high expression of MIC-1 has been observed in Tol-DC (30).

Traditionally, the everlasting immaturity of DC is conducive to the tolerant consequence (31). Recent studies, nonetheless, show that, in some cases, mature DC could also display the characteristic of tolerance. For instance, stimulation by recombinant soluble *Schistosoma mansoni* egg antigen (rSm29) could induce mono-DC with high expression of MHC-II and costimulatory molecules while rSm29 could increase IL-10 level and decrease levels of IL-12p40 and interferon-gamma (IFN-γ) in cultured mono-DC, which results in a great therapeutic efficacy on cutaneous leishmaniasis (32).

THE EX VIVO INDUCTION OF TOL-DC

Large amounts of DC can be obtained from monocytes pulsed by granulocyte/macrophage colony-stimulating factor (GM-CSF) and IL-4 (33, 34). In rodents, DC are derived from bone marrow cells; nonetheless, DC are usually derived from peripheral blood mononuclear cell (PBMC) in human. The reason why monocytes are considered as the source of DC is that they are easily obtained and more abundant than other DC precursors. Generally, DC can be induced to immunologic DC and Tol-DC *via* different stimulation *in vitro*. There is currently many a protocol to induce Tol-DC ex vivo. Usually, protocols of Tol-DC induction need diverse stimulators and technology, such as clinically approved drugs, cytokines, experimental inhibitors, and genetic engineering or biological intervention. The process of the generation of Tol-DC is summarized in **Figure 1**.

Clinical Drugs

Vitamin D3 (VitD3) is a fat-soluble hormone that can be acquired from food or be biosynthesized in the skin upon ultraviolet-B radiation and is commonly applied as a drug for rickets, which is considered to be one of the most commonly

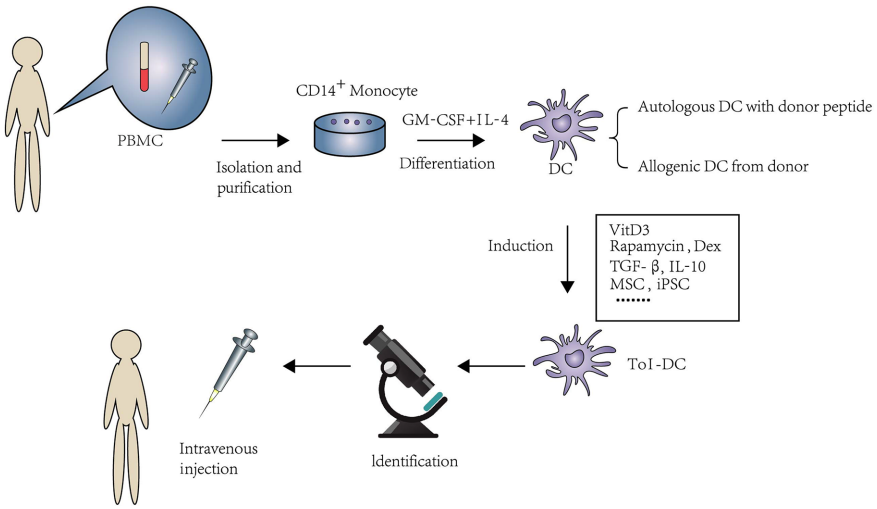

FIGURE 1 | An overview of isolation and expansion procedures of Tol-DC from PBMC and their administration in clinical approaches. PBMC are the source of DC in human. CD14+monocytes were extracted by immunomagnetic separation. Under the stimulation of GM-CSF and IL-4, monocytes were differentiated to DC. The source of DC can be from either donor-derived or autologous DC loaded with donor peptide. Tol-DC can be induced by clinical approved drugs, cytokines, experimental inhibitors, and genetic engineering or biological intervention. After purification and identification. Tol-DC can be transferred to the potential patients through intravenous injection.

used strategies for inducing Tol-DC *in vitro*. VitD3-Tol-DC show low expression of MHC-II, CD80, CD86, and CD40 and high secretion or expression of IL-10, indoleamine-2,3-dioxygenase (IDO), and even immunoglobulin-like transcript 3 (ILT3) (35–38). Several inflammatory pathways are involved in this process, such as extracellular signal-regulated kinase (ERK) 1/2 signaling cascade and specificity protein 1 (SP1) signaling factor and nuclear factor-kappa B (NF-κB) (23). A recent study indicates that high expressions of both MAP7 and MUCL1 genes are observed in VitD3-Tol-DC (39).

Immunosuppressants (IS) are also commonly used to induce Tol-DC *in vitro*. IS, such as rapamycin and dexamethasone (Dex), are proven to be effective for Tol-DC induction *in vitro*. Rapamycin (mTOR inhibitor) could suppress DC maturity with intermediate levels of MHC-II and costimulatory molecules (40). Campos-Acuña et al. transferred Tol-DC conditioned by rapamycin and activated by mono-phosphoryl lipid A to a murine skin graft model, resulting in a longer allograft survival period, more Treg proliferation, and cytokine pattern modification (41). Dex is a steroid widely used for the prevention and treatment of organ rejection. Polymeric nanoparticles containing ovalbumin (OVA) and Dex could change DC to Tol-DC phenotype, which could profoundly suppress OVA-specific immune responses *in vivo* (42). Tol-DC conditioned by Dex with a cocktail of cytokines (IL-1β, IL-6, TNF-α, and prostaglandin E2 (PGE2)) was tested in a clinical trial to evaluate the safety of Tol-DC in the treatment of refractory Crohn's disease (CrD) (43). Human monocyte-derived Tol-DC generated from Dex and VitD3 exhibit a typical tolerogenic phenotype of reduced costimulatory molecules and low production of proinflammatory cytokines (44). This protocol was also used to treat rheumatoid arthritis patients (45).

Cytokines

There are several cytokines used to induce Tol-DC *in vitro*, and most use IL-10 and TGF-β. Under the stimulation of IL-10, the expression of MHC-II and costimulatory molecules in DC could be reduced (46). There are two subpopulations of IL-10-pulsed DC: $CD83^{high}CCR7^{+}HLA\text{-}DR^{high}IL\text{-}10^{+}$ DC and $CD83^{low}CCR7^{+}HLA\text{-}DR^{low}IL\text{-}10^{+}$ DC. The former may become a promising choice for induction or restoration of tolerance *in vivo* because of their stable tolerogenic phenotype, even stimulated by inflammatory molecules, and they could induce highly potent Treg (47). TGF-β increases the expression of programmed death-ligand 1 (PD-L1) on DC, induced T cell apoptosis, and enhanced Treg differentiation (48). Moreover, TGF-β secreted by endothelial stromal cells could induce high expression of Fas-ligand (FasL) in Tol-DC through the ERK pathway (49). Compared to Dex, rapamycin, and TGF-β, IL-10 could induce stronger Tol-DC. Therefore, IL-10 seems to be the optimal inducible therapy for some immune diseases (50). In addition to IL-10 and TGF-β, there are also other cytokines that could induce Tol-DC *in vitro*, such as MIC-1, tumor necrosis factor α (TNF-α)-induced protein 8 like-1 (TIPE1) and PGE2. The expression of malat-1 circular RNA (circ_Malat 1) is the mature signal of DC. When treated with recombinant MIC-1 *in vitro*, the expression of surface molecules CD83, CD86, and HLA-DR is suppressed in DC as a result of the inhibition of circ_Malat 1 and NF-κB pathways. TIPE1, a new member of the TNF-α-induced protein 8 family, could boost PD-L1 expression on DC and restrain the signal transduction to T cell activation (51). Mature DC induced by PGE2 could produce IDO and

promote immunoregulatory capacity (52). Moreover, Tol-DC generated by Dex and a maturation cocktail composed of IL-1β, IL-6, TNF-α, and PGE2 could express more E-type prostanoid (EP) receptors 2 and 3, which, activated by PGE2, can induce IL-10 secretion, exhibiting their tolerant function (53).

Inhibitors/Activators of NF-κB and STAT

NF-κB is a family of dimeric transcription factors (54), and the maturity of DC is related to the activation of NF-κB (55). LF 15-0195 (LF) is a chemically synthesized analog of the immunosuppressant 15 deoxyspergualin, which possesses higher immunosuppressive activity. It is also a blocker of NF-κB. LF-treated DC are characterized by low expression of MHC-II, CD80, CD86, and high expression of anti-inflammatory molecules. These Tol-DC increase $CD4^+CD25^+CTLA4^+$ and $FOXP3^+$Treg levels and improve cardiac graft survival (56). RelB is one of the NF-κB subunits. Tol-DC could be acquired *via* silencing RelB using small interfering RNA, and this kind of Tol-DC also prolongs the survival of the cardiac graft through promoting the induction of Treg (57). NF-κB inhibitors in the induction of Tol-DC has already been applied in clinical trials. In a clinical trial on rheumatoid arthritis, Tol-DC were induced by Bay11-7082, the inhibitor of NF-κB, which irreversibly inhibited NF-κB by preventing phosphorylation of IκBa (58).

Signal transducer and activator of transcription (STAT) is essential in the development and maturation of DC. A total of seven STAT proteins have been identified (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, STAT6) (59). The inhibition or activation of different STAT signals may regulate the phenotype of DC. STAT1 and STAT2 are important in the activation of DC. STAT1 is required for the increased expression of costimulatory molecules in DC (60). Following the stimulation by IFN-γ, the activation of STAT1 could promote the maturation of DC. However, to inhibit the activation of STAT1 *in vitro* by flavonoids, the expression of PD-L1 is decreased in DC, and DC are tend more to an immature phenotype (61). When STAT1 is silenced in inflammation-stimulated DC by siRNA, the expression of CD83 and CD86 are also decreased, and the expression of anti-inflammatory molecules are increased (62). Similarly to STAT1, STAT2 is required for the activation and cross-presentation of DC under the stimulation of toll-like receptor (TLR) ligands (63).

Compared to STAT1 and STAT2, STAT3 is considered to be the negative inhibitor of DC. The activation of STAT3 induces the tolerogenicity in DC, whereas the inhibition of STAT3 induces matures DC. Human DC treated with IFN-α are characterized by high expression of PD-L1 and decreased production of IL-12. However, IFN-α-induced PD-L1 expression is downregulated by inhibitors of p38, Jak, and STAT3 (64). STAT3-deficient DC could enhance immune activity, including increased proinflammatory cytokine production, antigen (Ag)-dependent T cell activation, and resistance to IL-10–mediated suppression (65). The tolerogenicity of DC is correlated to the activation of STAT3. Thymic stromal lymphopoietin (TSLP) can induce the activation of DC with high expression of costimulatory and proinflammatory molecules. STAT5 is required for TSLP-dependent activation, which is a critical component for the promotion of Th2 response immunity during airway inflammation (66). JQ1 is an inhibitor of STAT5. When LPS-activated DC are treated with JQ1, STAT5 phosphorylation and nuclear accumulation is inhibited. As a result of the prevention of STAT5, the expression of CD83 in LPS-DC and the level of IL-12p70 released by DC are decreased (67). The activation of STAT5 may have connection with the maturation of DC followed by external stimulus.

In conclusion, the expression of NF-κB And STAT is critical in the induction of Tol-DC. The inhibition of STAT1, STAT2, and STAT5, but not the activation of STAT3, contribute to the induction of Tol-DC.

Genetic Engineering and Biological Intervention

There are also other protocols that can induce Tol-DC *in vitro*. Strategies of genetic engineering have also been used to induce Tol-DC, including gene knockout, knockdown, and transgenic over-expression of dominant active or negative mutants of molecules (68). For example, promoting the expression of IL-10-related genes in DC could attenuate liver fibrosis in mice *via* increasing Treg induction. This kind of $IL\text{-}10^+$DC is characterized by low expression of costimulatory molecules (69). Nuclear paraspeckle assembly transcript 1 (NEAT1) is proven to use NACHT, LRR, and PYD domain-containing protein 3 (NLRP3) inflammasomes as molecular decoys for miR-3076-3p, so knockdown NEAT1 could facilitate the tolerogenic phenotype in DC, which prevents progression of experimental autoimmune myocarditis and induces immune tolerance in a heart transplantation model (70). The metastasis associated in lung adenocarcinoma transcript 1 (MALAT1) overexpression promotes DC-SIGN expression by functioning as an miR155-5p sponge in the DC cytoplasm, which derives DC to Tol-DC with low expression of costimulatory molecules and high IL-10 secretion, protecting mice from acute rejection after cardiac transplantation (71). Apart from these, some biological interventions have also been used to induce Tol-DC, such as mesenchymal stem cells (MSCs) (72), induced pluripotent stem cells (iPSCs) (73), and recombinant *Schistosoma mansoni* antigens (32). Cai et al. generated Tol-DC from murine iPSCs and injected these Tol-DC 7 days before transplantation into the recipients, resulting in a decreased expression of perforin/granzyme B, increased secretion of TGF-β, and proliferation of $CTLA4^+GITR^+$Treg in mice with prolonged cardiac graft survival (73).

THE FUNCTION OF TOL-DC

The reason why Tol-DC could become a replacement for IS in future organ transplantation is their ability to decrease T cell proliferation and lead to T cell apoptosis, anergy and hyporesponsiveness. Meanwhile, they also can promote Treg induction to induce the tolerance. These two processes could be summarized as contact-dependent and -independent mechanisms. The contact-dependent mechanism means direct

contact between lymphocytes and Tol-DC, which contained surface receptors, such as PD-L1, Fas-L, ILT3, and ILT4. In addition, the contact-independent mechanism means Tol-DC could exert their immunosuppressive ability *via* immunomodulatory molecule release, including immunomodulatory cytokines, such as IL-10 and TGF-β, or enzymes, such as IDO, heme-oxygenase-1 (HO-1), and others. The function of Tol-DC is elucidated in **Figure 2**, and the experimental details are shown in **Table 1**.

Contact-Dependent Mechanism

PD-1 is an important inhibitory molecule expressed on T cells, and PD-L1 is its ligand expressed on DC. The interaction between PD-1 and PD-L1 delivers inhibitory signals to T cells and contributes to the anergy of T cells (90). According to recent literature, cross-dressed DC in the graft are characterized by high expression of PD-L1 after murine liver transplantation, and these cross-dressed DC failed to stimulate proliferation of allogeneic T cells but markedly suppressed antidonor host T cell proliferation *in vitro* (91). DC transfected with PD-L1 recombinant adenovirus could prolong the survival in rat renal transplantation. The effect is correlated with the suppression of CD8+T cell and the decreased secretion of proinflammatory cytokines (92). Fas and Fas-L belong to the TNF receptor and ligand family, respectively. Fas-L expressed on DC can induce T cell apoptosis by combining with Fas expressed on T cells (93). Mono-DC cotransfected with TGF-β1/Fas-L could prolong the survival time in murine liver transplantation. The increased level of Fas-L could induce T cell apoptosis (84). Immature DC transduced by lentiviral vectors expressing human IL-10 and FasL genes could significantly reduce the expression of costimulatory molecules and T cell proliferation and extend the survival period of rat liver allografts (94). Tol-DC have a unique subset: $CD11b^{high}Ia^{low}$ Tol-DC. They can express Fas and inhibit T-cell proliferation in a negative feedback manner through increased IL-10 levels (49). ILT3 and ILT4 belong to inhibitory receptors, which can modulate IκB phosphorylation and degradation through SH2 domain-containing protein tyrosine (SHP) phosphatases, inhibit the activation of NF-κB, and induce Tol-DC phenotype (95). The number of $ILT3/ILT4^+$ DC in patients who received long-term rapamycin after renal transplantation is significant increased. The increased $ILT3/ILT4^+$ DC contributed to Treg induction and expansion of $CD8^+CD28^-$T cell (96).

Contact-Independent Mechanism

IL-10 has always been considered a powerful anti-inflammatory molecule in different diseases (97). IL-10 not only inhibits T cell proliferation, but also shows the ability to induce Treg. Tol-DC induced by IL-10 could also release high levels of IL-10. Prolonged xenograft survival of rat islets in diabetic mice was observed after an autologous IL-10-pulsed DC administration without any immunosuppressive treatment. The injection of IL-10-pulsed DC enriches graft infiltrating regulatory $CD8^+$T cells and tolerogenic myeloid cells with suppression-associated phenotypes (80). DC cotransfected Fas-L and IL-10 have more

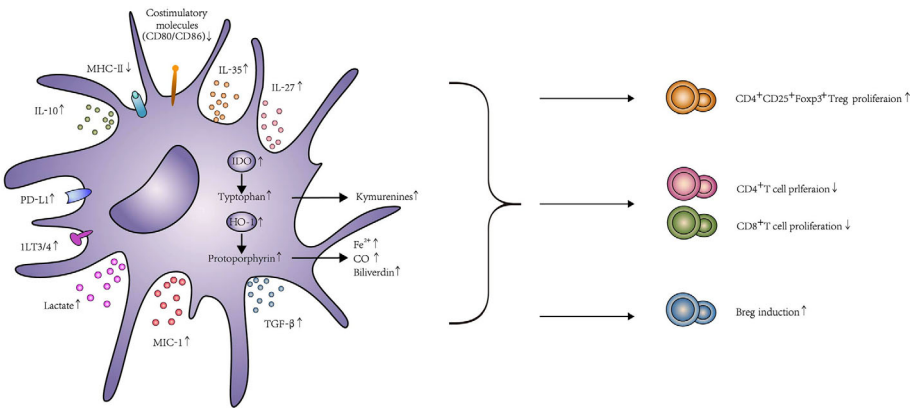

FIGURE 2 | The function and effects of Tol-DC. Tol-DC are characterized by low expression of costimulatory molecules CD80, CD86, and MHC-II. Tol-DC decrease the proliferation of T cells through apoptosis, anergy, and hyporesponsiveness. Meanwhile, they can promote Treg and Breg induction. The mechanism of this process, including contact-dependent and contact-independent mechanisms. Contact-dependent mechanisms include PD-L1, Fas-L, and ILT3/4. Contact-independent mechanisms include the expression of anti-inflammatory molecules, such as IL-10, TGF-β, IL-35, IL-27, and MIC-1. Tol-DC can also exhibit their function through the expression of IDO, HO-1, and lactate. The interaction between PD-1 and PD-L1 delivers inhibitory signals to T cells and contributes to the anergy of T cells. Fas-L expressed on DC can induce T cell apoptosis by combining with Fas expressed on T cells. The increased expression of ILT3/ILT4 in DC contributes to Treg induction. Similarly, IL-10 and TGF-β can broadly inhibit T cell activation by interfering with T cell receptor signaling and eventually promote Treg induction by IDO production. In addition, both IL-27 and IL-35 are considered as important regulators of adaptive immune responses. The high expression of IL-27 was correlated with the induction of IL-10 expressing $CD4^+$ T cells, and IL-35 overexpressed DC could increase Treg. DC transfected with MIC expression adenovirus could enhance T cell exhaustion and Treg proliferation. IDO catalyzes tryptophan degradation to form kynurenines, which consequently, impairs T cell proliferation and promotes Treg differentiation. HO-1 catalyzes the conversion of protoporphyrin to biliverdin, Fe^{2+}, and CO. HO-1 could promote Treg differentiation and prevent T cell–mediated inflammatory diseases because of the increased CO level. Tol-DC could produce high levels of lactate that shape T cell responses toward tolerance, including declines of glycolysis and activation and proliferation in T cell. In addition to T cell modification, Tol-DC can conditionally induce Breg proliferation, too.

TABLE 1 | Experimental details of Tol-DC transfer in animal transplant models.

Induction strategy	Phenotype of Tol-DC	Intervention	Transplanted model	Mechanism	Reference No.
DC pretreated with Cobalt Protoporphyrin (COPP)	HO-1^{high}MHC-II^{low}CD40^{low}CD80^{low}CD86^{low}	Donor-derived Tol-DC(day -7, 5 × 10^6 i.v.)	Allogeneic mouse cardiac	IFN-γ$^+$ T cell↓, alloantibody production↓	(74)
DC infected with Recombinant human growth differentiation factor 15(GDF15) expression adenovirus	GDF15^{high}CD40^{low}CD80^{low}	Autologous Tol-DC (day -7, 1 × 10^6 i.v.) + Rapamycin (day 0-7,1mg/kg, i.p.)	Allogeneic mouse cardiac	T cell exhaustion↑, CD4$^+$ FOXP3$^+$Treg↑	(75)
DC treated with recombinant IL-35/Ebi3	MHC-II^{low}CD86^{low}CD80^{low}	Donor-derived Tol-DC (day -1, 1 × 10^5 i.v.)	Allogeneic mouse cardiac	CD4$^+$CD25$^+$ FOXP3$^+$Treg↑	(76)
DC cultured with urine induced pluripotent stem cells	CD11b^{high}CD11c^{high}MHC-II^{low}CD86^{low}CD80^{low}	Donor-derived TolDC (day -7, 1 × 10^6 i.v.)	Allogeneic mouse cardiac	CD4$^+$CD25$^+$ FOXP3$^+$Treg↑, cytotoxic T cell↓, TNF-α↓, IL-1β↓, IL-6↓	(77)
DC2.4 cells transduced with pAd5/F35-GFP-Jagged-1 viruses	Jagged-1^{high}MHC-II^{intermediate}CD80^{intermediate}CD86^{intermediate}	Exogenous Tol-DC (day -1, 5 × 10^6 i.v.)+ anti-CD40L mAb (day 0, 2, 4 and 6, 0.25mg, i.p.)	Allogeneic mouse cardiac	CD4$^+$CD25$^+$ FOXP3$^+$Treg↑, TGF-β↑, IFN-γ↓	(78)
DC infected with Relb shRNA expressing lentivirus, activated by LPS	Relb^{low}MHC-II^{low}CD86^{low}CD80^{low}CD83^{low}	Donor-derived Tol-DC (day -7, 5 × 10^6 i.v.)	Allogeneic mouse cardiac	CD4$^+$CD25$^+$ FOXP3$^+$Treg↑	(57)
DC pretreated with LF 15-0195	MHC-II^{low}CD86^{low}CD40^{low}	Exogenous Tol-DC (day -7, 5 × 10^6 i.v.)	Allogeneic mouse cardiac	CD4$^+$CD25$^+$CTLA4$^+$T cell↑, CD4$^+$CD25$^+$ FOXP3$^+$Treg↑	(56)
DC treated with 0.1ng/ml GM-CSF	CD11c^{high}MHC-II^{low}CD80^{low}CD86^{low}	Autologous Tol-DC (day -1, 1 × 10^6 i.v.) + anti-CD3 Ab (day -1, 300mg, i.v.)	Allogeneic mouse islet	T cell activation↓, alloantibody production↓, CD4$^+$ FOXP3$^+$Tregs↑	(79)
DCs treated with IL-10	MHC-II^{low}CD40^{low}CD86^{low}CD205^{low}IL-12p70^{low}TNF-α^{low}IL-6^{low}IL-10^{high}	Autologous Tol-DC (day -1, 2 × 10^6 i.v.)	Xenogeneic (rat-mouse) islet	Graft-infiltrating CD8$^+$CD28$^-$ and CD8$^+$PD1$^+$ suppressor T cell↑	(80)
DCs conditioned with TGF-β, activated by LPS	MHC-II^{intermediate}CD80^{low}CD86^{low}IL-12p70^{low}	Donor-derived Tol-DC (day 0, 5× 10^5 i.v.)	Syngeneic mouse islet	FOXP3$^+$Treg ↑	(81)
DCs conditioned with TNF-α and α1-Antitrypsin	MHC-II^{low}CD86^{low}CD80^{low}IL-6^{low}IL-12^{low}IL-10^{high}	Autologous Tol-DC (day 0, 2 × 10^6 i.v.)	Allogeneic rat kidney	FOXP3$^+$Treg↑, TGF-β↑, IL-6↓, IFN-γ↓	(82)
DCs treated with 0.4ng/ml GM-CSF	CD11c^{high}MHC-II^{low}CD80^{low}CD86^{low}	Autologous Tol-DC (day -1, 1 × 10^6 i.v.) + anti-CD3Abs (day –1, 1, 3, 5 and 7, 1mg, i.p.)	Syngeneic mouse skin	CD8$^+$ FOXP3$^+$Treg↑	(83)
DC cotransfected with plasmids encoding EGFP-hTGF-β1 and EGFP-hFasL	TGF-β^{high}Fas-L^{high}CD85^{low}CD80^{low}	Exogenous Tol-DC (day -5, 2× 10^6 i.v.)	Allogeneic rat liver	IL-10↑, IL-1↓, IL-12↓	(84)
DC treated by GM-CSF, IL-10 and FLT3L	MHC-II^{low}CD86^{low}CD40^{low}CD80^{low}	Donor-derived Tol-DC (day -7, 2× 10^6 i.v.)+Penicillin (day 0, 500u/10g, subcutaneous)	Allogeneic rat kidney	IL-2↓, IFN-γ↓, IL-4↑, IL-10↑, CD4$^+$CD25$^+$ FOXP3$^+$Treg↑	(85)
DCs stimulated by VitD3 and IL-10	CD14^{high}MHC-II^{low}CD86^{low}CD83^{low}CD80^{low}PD-L1^{high}	Donor-derived Tol-DC (day -7 and 3, 5-10× 10^6 i.v.)+CTLA4 Ig (day -7 and -4, 12.5 mg/kg, day –1, 0, 2, 4, 7 and 10, 20mg/kg i.v.) + Tapered rapamycin maintenance	Allogeneic monkey kidney	CD4$^+$CD95$^+$Tmem↓, CD8$^+$CD95$^+$Tmem↓ CTLA4 and PD-1 expressed on Tmem↑	(86)
DCs stimulated by VitD3 and IL-10	CD14^{high}MHC-II^{low}CD86^{low}CD83^{low}CD80^{low}PD-L1^{high}	Donor-derived Tol-DC (day -7 and 3, 5-10× 10^6 i.v.)+CTLA4 Ig (day -7 and -4, 12.5 mg/kg, day –1, 0, 2, 4, 7 and 10, 20mg/kg i.v.) + Tapered rapamycin maintenance	Allogeneic monkey kidney	Donor-specific Eomes^{low}CTLA4^{high}CD8$^+$ central Tmem↑	(87)
DCs stimulated by VitD3 and IL-10	CD14^{high}MHC-II^{low}CD86^{low}CD83^{low}CD80^{low}PD-L1^{high}	Donor-derived Tol-DC (day -7 and 3, 5-10× 10^6 i.v.)+CTLA4 Ig (day -7 and -4, 12.5 mg/kg, day –1, 0, 2, 4, 7 and 10,	Allogeneic monkey kidney	Donor-specific Eomes^{low}CTLA4^{high}CD8+ T cell↑, IL-17↓	(88)

(Continued)

TABLE 1 | Continued

Induction strategy	Phenotype of Tol-DC	Intervention	Transplanted model	Mechanism	Reference No.
		20mg/kg i.v.) + Tapered rapamycin maintenance			
DCs stimulated by VitD3 and IL-10	$CD14^{high}$MHC-$II^{low}CD86^{low}CD83^{low}CD80^{low}$	Donor-derived Tol-DC (day -7 and 3, 5-10× 10^6 i.v.)+CTLA4 Ig (day -7 and -4, 12.5 mg/kg, day –1, 0, 2, 4, 7 and 10, 20mg/kg i.v.) + Tapered rapamycin maintenance	Allogeneic monkey kidney	Donor-Specific $CD4^+CTLA4^{high}$ T Cell proliferation	(89)

capacity to inhibit T cell activation and prolong the survival period of allografts than Fas-L alone (94).

TGF-β plays a pivotal role in transplant tolerance, which broadly inhibits T cell activation by interfering with T cell receptor signaling and eventually promotes Treg induction by IDO production (98). If there is a decrease of the expression of TGF-β2 receptors on DC, both T and B cell activation and reduction of the expression of Foxp3 in Treg would occur (99). Smad7 is a potent negative regulator of TGF-β signaling. The presence of Smad7 could prevent the binding of Smad2 and Smad3 to the TGF-β2 receptor, and this inhibitory effect is essential for TGF-β signal transduction. Rodent DC derived from Smad7 deficiency are resistant to the development of experimental autoimmune encephalomyelitis (EAE) due to an increase of protective Treg and inhibition of encephalitogenic effector T cells in the central nervous system (100). TGF-β gene modified DC exhibit the immature phenotype with low expression of MHC-II, CD80, CD86, and CD40, which could downregulate antigen presentation of bone morrow-derived immature DC. The high expression of TGF-β inhibits T cell proliferation and delays the progress of murine inflammatory bowel disease (IBD) (101). Tol-DC generated from TGF-β increase the frequency of Tregs in islet graft and shows long-term graft survival (102). DC cotransfected with plasmids encoding TGF-β and FasL show low expression of CD85 and CD80. These Tol-DC decrease Banff rejection activity index and allow graft function recovery in rat liver grafts, which is correlated to the increased expression of IL-10 and decreased expression of IL-1 and IL-12 (103).

In addition to classical immunomodulatory molecules, such as IL-10 and TGF-β, there are also other cytokines released from Tol-DC, which could regulate T cell activation and Treg proliferation. IL-35 and IL-27 are the members of IL-12 family. Both IL-27 and IL-35 are considered important regulators of adaptive immune responses (104). Under LPS stimulation, mono-DC secrete high levels of IL-35 to prevent the maturation of DC. IL-35 could activate STAT3 and STAT4 signal pathways in DC. On day 1 prior to transplantation, IL-35 overexpressed DC could increase IL-10 and Treg levels in cardiac recipients and lead to prolonged allograft survival (76). IL-27 is mainly produced by DC stimulated by microbial products or other immune stimuli. IL-27 could promote the differentiation of Th1 and type 1 regulatory (Tr1) cells but inhibit Th2 and Th17 cells (105). Overexpression of IL-27 combined with the application of rapamycin could definitely improve cardiac allograft acceptance. The high expression of IL-27 is also correlated with the induction of IL-10 expressing $CD4^+$T cells (106). Moreover, DC transfected with MIC expression adenovirus could enhance T cell exhaustion and Treg proliferation and consequently promote the survival of cardiac allograft (100, 107).

IDO is known to act as a bridge between DC and Treg. IDO catalyzes tryptophan degradation to form kynurenines and consequently impairs T cell proliferation and promotes Treg differentiation (108). Most Tol-DC are characterized by high expression of IDO. Before rat renal transplantation, recipient rats were preinjected with autologous Tol-DC treated with donor alloantigens. The renal allograft exhibited a lighter rejection response and longer graft survival time. This remission was thought to be correlated with increased Treg. However, when IDO is silenced by siRNA in rats, the rejection response is aggravated (85). α1-Antitrypsin is a circulating glycoprotein. α1-Antitrypsin-pulsed DC are characterized by decreased expression of MHC-II, CD80, and CD86 and high expression of IDO. After transferring these IDO^+ Tol-DC, the kidney allograft survival period is prolonged and Treg increase (87). Human soluble CD83 (hsCD83) is able to inhibit DC maturation and cause the anergy of Teff. In both heart and renal transplant models, the injection of hsCD83 down-modulates the expression of costimulatory molecules and up-modulates IDO in DC, which can prolong the allograft survival period (109, 110).

HO-1 is an enzyme that catalyzes the conversion of Fe-Protoporphyrin-IX (Heme group) to biliverdin, ferrous ion, and carbon monoxide (CO). HO-1 could promote Treg differentiation (111) and prevent T cell–mediated inflammatory diseases because of the increased CO level (112). CO can reduce both mitochondrial membrane potential and ATP production, which results in mitochondrial dysfunction in DC. The high expression of HO-1 in DC can resist LPS-induced maturation and release high levels of IL-10. HO-1 expressing DC could modulate the severity of lung inflammatory responses in murine models of airway inflammation with increased Treg (111). Cobalt protoporphyrin (CoPP) is the agonist of HO-1, and DC treated with CoPP are characterized by high expression of HO-1. Adoptively transferring donor-derived high HO-1 expressing immature DC 7 days before transplantation effectively blocks the activation of both T and B cells in cardiac allograft mice (74).

In addition to IDO and HO-1, NO, PGE2, and adenosine also exhibit great capacity to induce Tol-DC. Chloroquine (CQ), an antimalarial drug, also induces Tol-DC and, consequently, promotes the expression of NO synthase and, finally, results in the inhibition of T cell activation (113). After transferring CQ-

pulsed DC to EAE mice, a decline of glial reactivity in the central nervous system is observed (114).

Breg Induction

In addition to T cell modification, Tol-DC can conditionally induce Breg proliferation. When Tol-DC are administered to nonobese diabetic (NOD) mice, two tolerogenic B-cell subsets, $CD19^{+}B220^{+}CD11c^{-}IL\text{-}10^{+}$ B cell and B10 cell proliferate (115). Breg could proliferate through the retinoic acid receptor, which combines with retinoic acid released from Tol-DC (115, 116). The remission of IBD in the mouse model after administrating monocyte derived Tol-DC is correlated with the induction of IL-10-Bregs. However, whether Breg could be induced by Tol-DC in transplantation models or not remains to be further explored.

THE METABOLISM MODIFICATION OF TOL-DC

General Metabolism in Tol-DC

Glycolysis is an indispensable metabolic process in our body, which can rapidly decompose glucose into ATP and supply energy (117). LPS, an agonist of TLR4, is widely used to induce functional DC. However, during this process, DC activation relies on glycolysis for abundant ATP (118). Citrate is a tricarboxylic acid (TCA) cycle intermediate, which plays an important role in LPS-induced DC activation. LPS activates TLR and, consequently, causes glycolysis inside DC through the generation of citrate and the synthesis of fatty acids *in vivo*, which could promote the expansion of endoplasmic reticulum and Golgi networks required for DC activation (119). Complement component C1q subcomponent-binding protein (c1qbp), a multifunctional chaperone protein, plays an important role in mitochondrial function and supports mitochondrial metabolism and DC maturation. The production of citrate regulates DC maturity *via* c1qbp-dependent pyruvate dehydrogenase activity (120). 2-deoxyglucose impairs glycolysis in DC, which contributes to the decreased expression of CD40, CD86, and MHC-II and production of IL-6, IL-12p70, and TNF and causes a Tol-DC phenotype (119). The decline of glycolysis in DC could contribute to impaired maintenance of dendritic shape, motility, CC-chemokine receptor (CCR)7 oligomerization, and migration to draining lymph nodes (121). In malignant melanoma, paracrine-derived Wnt5a protein can alter the metabolic pathway of DC by stimulating β-catenin signaling pathway, which can shift local DC populations from a glycolytic state to oxidative phosphorylation (OXPHOS) and fatty acid oxidation (FAO) *via* peroxisome proliferator-activated receptor (PPAR)-γ-carnitine palmitoyl transferase-1 (CPTIA) axis (122). In a recent first-in-human clinical trial of kidney transplantation, Marin et al. report that autologous Tol-DC could produce high levels of lactate that shape T cell responses toward tolerance, including declines of glycolysis, activation, and proliferation in T cell (123).

During the induction of VitD3-pulsed Tol-DC, genes related to OXPHOS and the protein O-linked glycosylation pathway are overexpressed (23). 1,25-dihydroxy vitamin D_3 is the active form of vitamin D, which can induce human monocyte-derived Tol-DC by metabolic reprogramming and upregulate several genes directly correlated to glucose metabolism, TCA, and OXPHOS (124). As discussed above, Dex has already been applied to induce Tol-DC in clinical practice. García-González et al. studied the transcriptional profile of mono-DC from healthy donors modulated with Dex and activated with monophosphorylate lipid A (MPLA), demonstrating that genes related to FAO are strongly enriched, predicting the activation of alternative metabolic processes than those driven by the counterpart DC (125). Increased expression of inducible nitric oxide synthase (iNOS) (126) and inhibition of 5' adenosine monophosphate-activated protein kinase (AMPK) (127) decreased OXPHOS and FAO in immunologic DC. The activation of nuclear factor E2-related factor 2 (Nrf2) can inhibit the production of iNOS, thereby restoring OXPHOS as the energy source in Tol-DC (128). Compared to immunologic DC, Tol-DC possess a steady OXPHOS program and favors FAO (129). FAO has a regulatory effect on OXPHO. Fatty acids can suppress the TLR-induced hexokinase activity and perturb tricarboxylic acid cycle metabolism, which enhances the production of mitochondrial reactive oxygen species (ROS) (130). miR-142 is central to metabolic reprogramming. Sun et al. demonstrated that miR-142 directly targets carnitine palmitoyltransferase-1a, a key regulator of the fatty acid pathway to regulate FAO. In miR-142 deficient mice, DC fail to shift from OXPHOS to glycolysis and show reduced production of proinflammatory cytokines and ability to activate T cells *in vitro* and *in vivo* models of sepsis and allogeneic immunity (131).

The production of ROS is proven to be more likely related with the immunogenic DC. α-Glucans in *Mycobacterium tuberculosis* can induce ROS production and lead to DC maturation and lymphocyte proliferation, which is partly related to the induction of spleen tyrosine kinase (Syk) (132). The reduction in mitochondrial ROS production dramatically decreases the cross-presentation capacity of pDC and strongly impairs their ability to trigger $CD8^{+}$T cell responses (133). Mogilenko et al. also report that reducing mitochondrial ROS production in DC ameliorates the disease in an IL-23-dependent model of psoriasis because of the reduction in IL-23 and skin inflammation (130).

In summary, Tol-DC is usually characterized by increased OXPHOS and FAO but decreased ROS levels. The phenotype and metabolism of Tol-DC are summarized in **Figure 3**.

mTOR and Tol-DC Metabolism

mTOR is known to be divided into two complexes: mTOR complex 1 (mTORC1) and mTORC2. The differentiation of DC induced by GM-CSF and IL-4 from human monocytes relies on the mammalian target of mTORC1 activation *via* phosphoinositide 3-kinase (134). mTORC1 pathway has a central role in the pathogenesis of some autoimmune diseases and is a mediator of the Warburg effect that allows cell survival under hypoxia (135). Rapamycin, an mTOR blocker, has been widely used to prevent rejection after organ

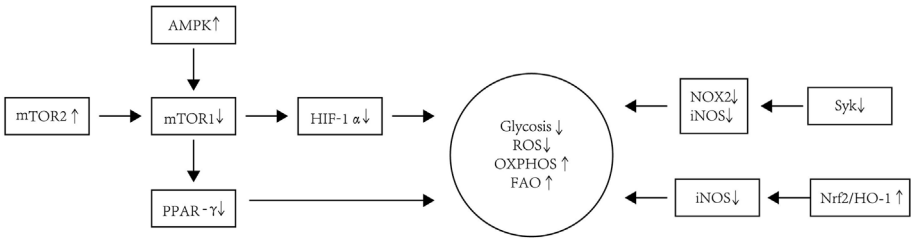

FIGURE 3 | The metabolism modification of Tol-DC. Tol-DC are usually characterized by increased OXPHOS and FAO but decreased ROS and glycolysis. The inhibition of mTOR is correlated with the tolerogenic metabolism in DC. AMPK is one of the main protein kinases regulating glucose metabolism and is located upstream of the mTOR1. The increased expression and activation of AMPK decrease the expression and activation of AMPK downstream kinase mTOR1. The PPAR-γ is the downstream target of mTOR1. The inhibition of mTOR1 can also decrease expression of PPAR-γ, which is a response to lipid metabolism in DC. HIF-1α is responsible for sustained glycolytic reprogramming in DC. The blockage of mTOR1 can influence the expression of HIF-1α. mTOR2 can inhibit mTOR1-regulated metabolic function in DC. Additionally, the blockade of Syk signaling leads to a decrease in levels of iNOS and NOX2, which contributes to the decreased glycolysis and ROS in DC. Nrf2/HO-1 can inhibit the production of iNOS, thereby restoring OXPHOS as the energy source in Tol-DC.

transplantation. Rapamycin-induced DC administration is shown to play an immunosuppressive role in skin transplantation (41). Polymerized allergoids conjugated to mannan (PM), which can induce the tolerance of DC, are thought to be vigorous vaccines for allergen-specific immunotherapy. However, when PM-pulsed DC are adsorbed to alum, their capacity to generate Treg is impaired. This phenomenon is related to the inhibition of mTOR by alum, which alters metabolic reprogramming by transforming glycolytic pathways and inhibiting ROS production in PM-pulsed DC (136). PPAR-γ is the downstream target of mTORC1, which is upregulated early in mono-DC differentiation, affecting mono-DC maturation and function largely through control of lipid metabolism (137). The relationship between the mTOR signaling pathway and metabolism may involve multiple mechanisms. Activation of the mTOR signaling pathway can stimulate hypoxia-inducible factor-1α (HIF-1α) (138), which is responsible for sustained glycolytic reprogramming in DC (121). In HIF-1α knockout mice, APC express lower levels of MHC-II and costimulatory molecules and are less able to induce T-cell proliferation (139). Graphene quantum dots (GQD) are atom-thick nano-dimensional carbon sheets with excellent physico-chemical and biological properties. GQD promote tolerogenic functions in mono-DC, which prevent the pathologies caused by inflammatory T cells. This process is mediated by the reduced activity of mTOR by GQD, which is correlated to the increase in transcription of autophagy genes and autophagic flux in DC (140). AMPK is one of the main protein kinases regulating glucose metabolism and is located upstream of the mTOR. Polyphenol resveratrol is an antitumor drug that has been used in clinical trials and can increase the expression and activation of AMPK and caspase-3 and decrease the expression and activation of AMPK downstream kinase mTOR (141).

Moreover, mTOR1 and mTOR2 can also affect each other. mTORC2 can inhibit mTORC1-regulated metabolic function in DC. mTORC2 knockout DC improves mTORC1 metabolic activity, which is biased toward glycolytic metabolism to generate ATP, increased lipid content, and higher viability stimulated by LPS. Enhanced integrin alpha IIb (Itga2b) and protein kinase 2 (Ptk2)/focal adhesion kinase (FAK) expression can activate hematopoietic cell signal transducer expression and enhance mTORC1 activity (142).

In conclusion, mTOR is important in the metabolism modification of Tol-DC. The inhibition of mTOR could induce the tolerogenicity in DC. The prevention of mTOR activation could contribute to the transformation of OXPHOS and decreased production of ROS. In the mTOR signaling pathway, HIF-1α is responsible for sustained glycolytic reprogramming, and PPAR-γ controls lipid metabolism in DC.

Syk and Tol-DC Metabolism

In addition to the mTOR signaling pathways, Syk can also play an important role in DC metabolism. The activation of Syk contributed to the sustained glycolytic reprogramming in DC. Other than TLRs, C-type lectin receptors (CLRs) are also expressed on DC as PRR to recognize pathogen-associated stimuli, such as dectin-1/2. Fungal-associated β-glucan ligands react with dectin1/2 and induce glycolytic reprogramming in DC *via* a Syk-dependent way, which contributes to the production of IL-1β (143). Dectin-1 binding with annexins which is expressed on apoptotic cells induce a tolerogenic DC phenotype. This is a distinct mechanism from that of the interaction site of pathogen-derived β-glucans and induces selective phosphorylation of Syk, causes activation of nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase-2 (NOX2), moderates production of ROS, (144). The blockade of Syk signaling leads to the improvement of sepsis-induced acute kidney injury in mice as suggested by the attenuation of creatinine/blood urea nitrogen in serum, renal myeloperoxidase activity, and repair of tubular structures in the kidney. This can be correlated to a decrease in levels of IL-6/MCP-1 in $CD11c^{+}$DC and iNOS, NOX2, and nitrotyrosine in neutrophils (145). Syk signaling may serve as an effective therapeutic target in innate immune cells to limit inflammatory cascade, and the inhibition of Syk might prevent glycolysis in DC and lead to the tolerogenicity of DC.

PRECLINICAL AND CLINICAL ATTEMPTS OF TOL-DC IN ORGAN TRANSPLANTATION

Autoimmune disease is a series of dysfunctions and tissue damage caused by the loss of tolerance to self-antigen. Clinical trials are currently carried out to explore the efficacy and safety of transferred Tol-DC to treat autoimmune diseases such as type 1 diabetes mellitus (T1DM), multiple sclerosis (MS), rheumatoid arthritis (RA), and CrD (146). Currently, clinical trials on Tol-DC are in a preliminary stage. What has been proven so far is the safety of Tol-DC to the human body. The efficacy of Tol-DC has a close association with the increase in Treg levels. Further studies are needed to explore the optimal strategies of Tol-DC application in clinical practices. However, there is still no study reporting the efficacy of Tol-DC in human transplantation, so we would like to discuss the efficacy of Tol-DC in nonhuman primate renal transplantation and the current registered clinical trials of Tol-DC related to organ transplantation in clinicaltrials.gov.

Tol-DC in Nonhuman Primate Kidney Transplantation

In recent years, the study of Tol-DC for kidney transplantation has improved. The first preclinical trial of Tol-DC in renal transplantation showed that donor-derived Tol-DC induced by VitD3 and IL-10 were characterized by low expression of CD80 and CD86 and high levels of PD-L1. Tol-DC were cotransferred into rhesus monkey recipients before renal transplantation, with a combined application of CTAL4Ig [blocker of CD80/86 (147, 148)] and rapamycin and without CNI and steroids, which showed Tol-DC injection could prolong the survival of grafts in monkeys (86). CD95 (Fas)$^+$ T cells are considered to have memory capacity, which includes central (CD28$^+$) and effector memory (CD28$^-$) T cells in rhesus monkey. Both PD-1 and CTLA4 are considered markers of exhaustion and expressed on rhesus CD95$^+$ T cell. The administration of Tol-DC could shift CD95$^+$ Tmem to an immunosuppressive phonotype with increased expression of PD-1 and CTLA4 (86). Thereafter, they further explored the mechanism of prolonged graft survival after administration of donor-derived Tol-DC. Eomesodermin (Eomes), a key transcription factor in CD8$^+$ Tmem (149), play a critical role in long-term survival of antigen-specific central Tmem. The prolonged survival of renal allografts after both CTAL4Ig and donor-derived Tol-DC therapy might be related to the maintenance of donor-reactive Eomes^{low}CTLA4^{high} central Tmem, which displayed a regulatory phenotype *in vivo* (87). Compared to CNI, CTLA4Ig may preserve renal function and improve long-term outcomes in kidney transplantation (150). The same research team found the infusion of CTLA4Ig and Tol-DC together could maintain the expression of CTLA4 in CD4$^+$ T cells in another similar preclinical trial. The exposure of CTLA4-expressed CD4$^+$ T cells to donor antigens is essential for the prevention of Teff responses and the promotion of transplant tolerance (89). In addition to donor-derived Tol-DC, the effect of autologous Tol-DC is also evaluated in a preclinical trial of rhesus monkey. Autologous Tol-DC are incubated with vesicles generated from prospective transplant donor PBMC, and these Tol-DC could effectively capture vesicles without changing their own phenotype (88). IL-17 is a proinflammatory cytokine, which plays an important role in organ rejection. The deficiency or neutralization of IL-17 is protective against the development of kidney allograft rejection (151). After transplantation, there was an increased absolute number of donor-reactive CD4$^+$IL-17$^+$ T cells in the renal allograft of rhesus monkey in nondonor antigen-pulsed autologous Tol-DC treated group. However, the number of donor-reactive CTLA4$^+$IL-17$^+$ T cells did not change pre- and post-transplantation in the donor antigen-pulsed autologous Tol-DC treated group. In addition to the inhibition of donor-reactive CTLA4$^+$IL-17$^+$ T cells, donor antigen-pulsed autologous Tol-DC also modulated the expression of PD-1 and CTLA4 in donor reactive T cells (88). In conclusion, the efficacy of Tol-DC in preclinical trials of kidney transplantation has been proven. By administrating either the donor-derived Tol-DC or donor-antigen pulsed autologous Tol-DC, the survival time of the grafts is prolonged. The prolonged survival of the graft is correlated with the increased expression of PD-1 and CTLA4 and the decreased expression of Eomes in donor-reactive T cells. Meanwhile, the administration of Tol-DC can modulate IL-17-mediated inflammation in renal transplantation.

Tol-DC induced by VitD3 and IL-10 could maintain a stable state both *in vivo* and *in vitro*. Even stimulated by inflammatory molecules, Tol-DC are fully resistant to phenotypic maturation *in vitro* (152). Rhesus T cells stimulated initially with Tol-DC failed to proliferate following restimulation with donor alloreactive antigen in a secondary mixed leukocyte reaction, which ensures the stability of Tol-DC injection *in vivo* (86). Compared to non-Tol-DC treated group, the administration of donor-derived Tol-DC significantly prolonged the graft survival period ranging from 50 to 300 days (median=113.5). Graft median survival time of donor-antigen-pulsed autologous Tol-DC was 56 days (88). Additionally, there was no adverse effect observed in these preclinical trials. Meanwhile, the injection of Tol-DC could not induce the circulating donor-specific allogenic antibody, which indicated that Tol-DC could function stably for a long time in the body (86). However, further clinical studies are needed to address the safety, stability, and feasibility of Tol-DC transfusion in human transplantation.

Administration Route and Migration of Tol-DC in Organ Transplantation

Although Tol-DC has been proven effective in rodent and rhesus monkey organ transplantation, it is also important to explore the best administration route. The administration route not only influences the effect of Tol-DC but also the migration of Tol-DC *in vivo*. In an experimental autoimmune encephalomyelitis (EAE) model, intraperitoneal (i.p.) administration of Tol-DC could effectively suppress clinical manifestation of ongoing experimental autoimmune myasthenia gravis more than intravenous (i.v.) administration by regulating T and B cell responses (153). In clinical trials of Tol-DC that have been reported so far,

administration routes of intradermal (i.d.) (154, 155), i.p. (156) and i.v. (157) were all proven to be safe and well tolerated in human. However, i.v. administration of autologous Tol-DC was proven to have better immune tolerance than i.d. in rhesus monkeys (158). Another report demonstrated that 1 day after i.v. injection of Tol-DC in rat liver transplantation, the number of administrated Tol-DC was the highest in the liver graft and also detected in other second lymphoid organs. However, when it came to i.p. administration, the number of Tol-DC was the highest in abdominal lymph nodes 24–48 h after injection, but there were few in the rat liver graft (84). The result implicates that i.v. injection of Tol-DC is preferred to migrate to the graft than i.p. In **Table 1**, we find i.v. injection is the most commonly used route for Tol-DC administration in animal transplant models. In addition, the i.v. route is more readily operated in the clinical practice. Taken together, we recommended i.v. to be the best administration route of Tol-DC injection in future human clinical attempts at transplantation.

The migration of Tol-DC is not only influenced by administration routes, but also by the expression of chemokine and its receptors. Immature DC are characterized by high expression of CCR2, CCR5, and CCR6 and access to nonlymphoid tissues through attraction of CC-chemokine ligand (CCL)2, CCL5, and CCL21, whereas mature DC are characterized by high expression of CCR7, which allows DC to recognize the lymph node-directing chemokines CCL19 and CCL21 (159). Tol-DC tend more to a semimature state. Tol-DC induced by Dex and Vitd3 express chemokine receptors characteristic of an immature phenotype, such as CCR2, CCR5, CXCR1, and CXCR2. However, under stimulation by LPS, Tol-DC downregulates the expression of these chemokine receptors and upregulates the expression of CCR7 although the level of expression is lower than activated DC. The stimulation of LPS induces Tol-DC to migrate in response to CCL19 and move to the lymph nodes (160). Although using a model of allotolerance induction, Liu et al. show a striking failure to tolerate cardiac allografts in CCR7-deficient recipients. The deficiency of CCR7 contributed to a significantly reduced number of pDC in peripheral as well as mesenteric lymph nodes. After single transfer of syngeneic wild-type pDC, the result of cardiac transplantation in CCR7-deficient recipients has significantly improved in a dose-dependent manner (161). This report demonstrates pDC with high expression of CCR7 is considered as a kind of Tol-DC in transplant models. Additionally, α-1 antitrypsin (AAT) is reported to induce the tolerance of DC, and the upregulation of CCR7 is observed in AAT-induced Tol-DC stimulated by inflammatory molecules. The expression of CCR7 induced Tol-DC to migrate to draining lymph nodes in an islet transplantation model (162). In conclusion, Tol-DC expressed a relatively low level of CCR7. However, under the external stimulus, Tol-DC could upregulate the expression of CCR7 and migrate to the second lymphatic organ to induce the anergy of T cells.

Registered Clinical Trials of Tol-DC in Human Organ Transplantation

The first Tol-DC clinical trial in living-donor renal transplantation has been performed to evaluate the safety of administering autologous Tol-DC (NCT02252055) and is still ongoing. Another phase I clinical trial on Tol-DC in living-donor renal transplantation is recruiting (NCT03726307) currently. Its purpose is to evaluate the safety and feasibility of a single infusion of donor-derived Tol-DC administration 7 days before transplantation and explore the best injection dose. Participants will be maintained on a triple immunosuppressant scheme with mycophenolate mofetil, tacrolimus, and prednisone. Additionally, a Tol-DC clinical trial for liver transplantation is being enrolled (NCT04208919). Tol-DC in living donor liver transplantation phase I/II will be evaluated for safety and therapeutic effect a week after Tol-DC infusion, and immunosuppression weaning will be initiated. The levels of donor special antigen and the change in renal function, quality of life, and cardiovascular risk factors will be used as indicators of evaluation. The effect of Tol-DC-based treatment on the prognosis of organ transplantation is still being evaluated, and the clinical attempts of Tol-DC therapy are still in Phase I and II clinical trials.

CONCLUSION AND FUTURE PROSPECTIVE

Organ transplantation is thought to be the most commonly used treatment for end-stage visceral diseases. However, the rejection after operation seriously affects the prognosis of patients. Although the application of IS effectively prolongs the survival of patients, the side effects of IS also influence the life quality of patients. Tol-DC are a small part of DC. They are characterized by low expression of costimulatory moles and proinflammatory factor. Tol-DC induce immune tolerance by inhibiting the activation of T cells and inducing Treg proliferation. There are various agents that can induce the tolerance of DC. These agents include anti-inflammatory cytokines, antisense oligonucleotides targeting costimulatory molecules, IS drugs, VitD3, and PGE2, and so forth. However, there is still no consensus as to the optimal protocol to be used for generation of clinical-grade Tol-DC. More efficient induction protocols remain to be explored in the future. There is growing evidence proving that distinct metabolic reprogramming acts as a regulatory switch in determining the diversity of DC. Tol-DC possess a prominent and stable OXPHOS program and favor FAO but decreased ROS. The targets for the metabolism of Tol-DC are promising tools for tolerogenic vaccination in the future clinical practice. At present, several clinical trials of Tol-DC have been reported. The safety and effectiveness of Tol-DC have been evaluated. However, clinical trials of Tol-DC have stayed in the elementary stage. Future studies are required to identify the optimal dose of Tol-DC and the mechanism of the efficacy. There is still no published report on clinical trials using tolerogenic DC vaccines in organ transplantation. However, the preclinical trials of Tol-DC have been reported. The effect of Tol-DC in organ transplantation is associated with the induction of Treg in rhesus monkey. A phase 1 clinical trial for Tol-DC in organ

transplantation is still under recruitment. It will provide valuable insights into the value of these regulatory immune cells for improved prognosis in organ transplantation.

AUTHOR CONTRIBUTIONS

QZ and HC collected the literatures and drafted the initial manuscript. YM and QZ revised the manuscript and edited the language. YM conceptualized and guaranteed the review. QC and ZL designed the figures and tables. SL formatted the references and whole manuscript. All authors contributed to the article and approved the submitted version. QZ and HC contributed to this paper equally.

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IL-Y Aggravates Murine Chronic Graft-*Versus*-Host Disease by Enhancing T and B Cell Responses

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IL-Y, a synthetic member of IL-12 cytokine family, was found to exert potent immunosuppressive effects by inhibiting the differentiation and activation of Th1 and Th17 cells. However, the role of IL-Y in the development of chronic graft-*versus*-host disease (cGVHD) remains unknown. Here, using murine models of scleroderma-like and lupus-like cGVHD, we examined the function of IL-Y in the pathogenesis of cGVHD by hydrodynamically injecting minicircle-IL-Y expressing plasmids (MC IL-Y). In contrast with the reported immune suppressive function of IL-Y, administration of MC IL-Y enhanced cGVHD severity reflected by deteriorated multi-organ pathologic damages. In lupus-like cGVHD model, urine protein and the serum anti-dsDNA antibody (IgG) were significantly upregulated by IL-Y treatment. Further study demonstrated that IL-Y impacts both donor T and B cell response. In T cells, IL-Y inhibited the generation of $CD4^+Foxp3^+$ regulator T (Treg) cells during the development of cGVHD. IL-Y may also increase the infiltration of pathogenic TNF-α producing $CD4^+$ and $CD8^+$ T cells through IL-27Rα in recipient spleens, as this effect was diminished in IL-27Rα deficient T cells. Moreover, IL-Y enhanced the differentiation of $ICOS^+$ T follicular helper (Tfh) cells. In B cells, the percentage of germinal center (GC) B cells in recipient spleens was significantly upregulated by MC IL-Y plasmid administration. The levels of co-stimulatory molecules, MHC-II and CD86, on B cells were also enhanced by IL-Y expression. Taken together, our data indicated that IL-Y promoted the process of cGVHD by activating pathogenic T and B cells.

Keywords: IL-Y, T cell response, B cell response, Tfh cell, Treg cells, chronic graft-*versus*-host disease

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (Allo-HSCT) remains a cornerstone curative therapy for hematological malignancy. Chronic graft-*versus*-host disease (cGVHD) continues to be a common cause of non-relapse morbidity and mortality after allo-HSCT (1–4). With standard prophylaxis based on a calcineurin inhibitor and methotrexate or mycophenolate mofetil in most regimens, fewer patients have developed acute GVHD (aGVHD) in recent years. However, the

incidence and clinical therapy of cGVHD have not been improved due to the poor understanding of its pathogenesis. Paradoxically, cGVHD prophylaxis and treatment with a calcineurin inhibitor may promote the development of cGVHD by blocking thymic central tolerance and peripheral Treg-cell function (5, 6). More seriously, it is closely associated with increased risk of infection and malignancy recurrence (2, 7). Therefore, new therapeutic strategies are urgently needed to improve curative effect of cGVHD.

cGVHD is a multi-system autoimmune-like syndrome caused by the interactions of donor T and B cells and antibody production, with clinical manifestations including skin and cutaneous sclerosis, bronchiolitis obliterans as well as salivary and lacrimal gland pathology (2–4, 8). The pathogenic auto-reactive and allo-reactive $CD4^+$ T cells escape immune regulation by thymic selection and peripheral mechanisms and differentiate into type 1, type 2, and type 17 helper T (Th1, Th2, and Th17) cells, which maintain inflammation (9–12). It is evident that donor B cells also contribute to the immune pathology and tissue damage characteristic of cGVHD (4, 8, 12, 13). Activated follicular helper T cells expressing the transcription factor Bcl6 and high levels of the chemokine receptor CXCR5 support the generation of germinal center (GC) B cells by providing signaling through IL-21, ICOS, and CXCL13 (11, 14, 15). Interaction of T follicular helper (Tfh) and B cells results in somatic hypermutation, production of high affinity IgG, and formation of long-lived plasma cells, which exacerbate the development of cGVHD (11, 12, 16). Regulatory T (Treg) cells, follicular regulatory T (Tfr) cells, regulatory B (Breg) cells represent peripheral immune tolerance, which also plays a critical regulatory role in the pathogenesis of cGVHD (14, 17–20).

Previous studies using western blot followed by immune-precipitation revealed that a stable association between p28 and p40 was formed possibly *via* disulfide bond (21). Injection of p28/p40 protein suppressed experimental autoimmune uveitis by inhibiting the differentiation and inflammatory responses of Th1 and Th17 cells. These suppressive effects seemed to be ascribed to antagonizing the activation of STAT1 and STAT3 pathways induced by IL-27 and IL-6, both of which signal through the gp130 receptor (21). Moreover, recent studies using adenovirus vector expressing p28/p40 (IL-Y) suggested that treatment of pre-diabetic non-obese mice prevented the onset of hyperglycemia with reduced expression of inflammatory mediators such as IFN-γ (22). Interestingly, their work also demonstrated that IL-Y could activate antigen-presenting cells (APCs) by significantly upregulating both CD86 and MHC-II expression on myeloid derived-suppressor cells (MDSCs) (22). Therefore, these studies implicated that IL-Y might play a dual role in immune regulation.

Given that cGVHD has a wide spectrum of presentations in humans, individual mouse models do not reproduce all features of cGVHD. We investigated how IL-Y regulated T and B cells differentiation and function during cGVHD development in two mouse models of cGVHD, scleroderma-like cGVHD model and lupus-like cGVHD model. We observed that IL-Y aggravated the development of autoimmune manifestations of cGVHD. Furthermore, we found that IL-Y administration increased $ICOS^+$ Tfh cells, promoted the production of TNF-α, inhibited Treg generation, and enhanced the differentiation of B cells to GC B cell. Although the detailed mechanisms of IL-Y promoting cGVHD require further exploration, our results provide a new insight in the role of IL-Y in cGVHD and possible therapeutic strategies targeting p40 (a component of IL-Y) and IL-27Rα signaling.

MATERIALS AND METHODS

Mice

8–10-week-old female DBA/2 ($H2K^d$) mice were purchased from Charles River Laboratories (Beijing, China). 6–8-week-old female C57BL/6 (B6; $H2K^b$) and BALB/c ($H2K^d$) mice were purchased from SLAC Animal Laboratory (Shanghai, China). Experimental animals were maintained in specific pathogen-free conditions. All animal protocols were approved by the Soochow University Institutional Animal Care and Use Committee.

Establishment of cGVHD in BALB/c Mice

Recipient BALB/c mice were conditioned with total body irradiation (TBI) at 650 cGy using an RAD 320 X-ray Irradiator 6–8 h prior to transplant. Irradiated recipients (BALB/c) were intravenously injected with 1×10^7 bone marrow (BM) cells and 1×10^6 whole splenocytes (C57BL/6J→BALB/c) to establish scleroderma-like cGVHD model. 5×10^6 BM cells and 4×10^7 $CD4^+CD25^-$ splenocytes were injected intravenously to irradiated recipients (BALB/c) (DBA/2→BALB/c) to establish lupus-like cGVHD model. CD25 depletion in the spleens was accomplished using biotin-conjugated anti-CD25 mAb (eBioscience, San Diego, California) and anti-biotin micromagnetic beads (Miltenyi Biotec, German), followed by passage through a MACS cell sorter (Miltenyi Biotec, German). The efficiency of depletion was >98%. For hydrodynamic gene transfer (HGT), the recipient mice (BALB/c) were injected intravenously with 120 μg of empty vectors (MC) or minicircle-IL-Y (MC IL-Y) plasmids in a total of 2 ml phosphate buffered saline (PBS) within 5 s using a 23-gauge needle 3 days before transplantation.

Plasmid Construction

The cDNA encoding mouse IL-27p28 and IL-12p40 were amplified by PCR from the total RNA extracted from spleen cells of C57BL/6 mice stimulated with LPS. IL-27p28 and IL-12p40 genes were fused *via* a hydrophobic polypeptide linker (Gly4Ser). The IL-Y expression construct was generated by fusing the nucleotide sequence-encoding Igκ signal sequence to the 5′ end of IL-Y sequence and flag tag to the 3′ end of IL-Y sequence, and then inserted between sites of Nhe I (5′) and Sal I (3′) into minicircle (MC) plasmid (pMC.EF1; SBI, Palo Alto, CA). Positive recombinant clone was analyzed by digestion of restriction endonuclease and DNA sequencing.

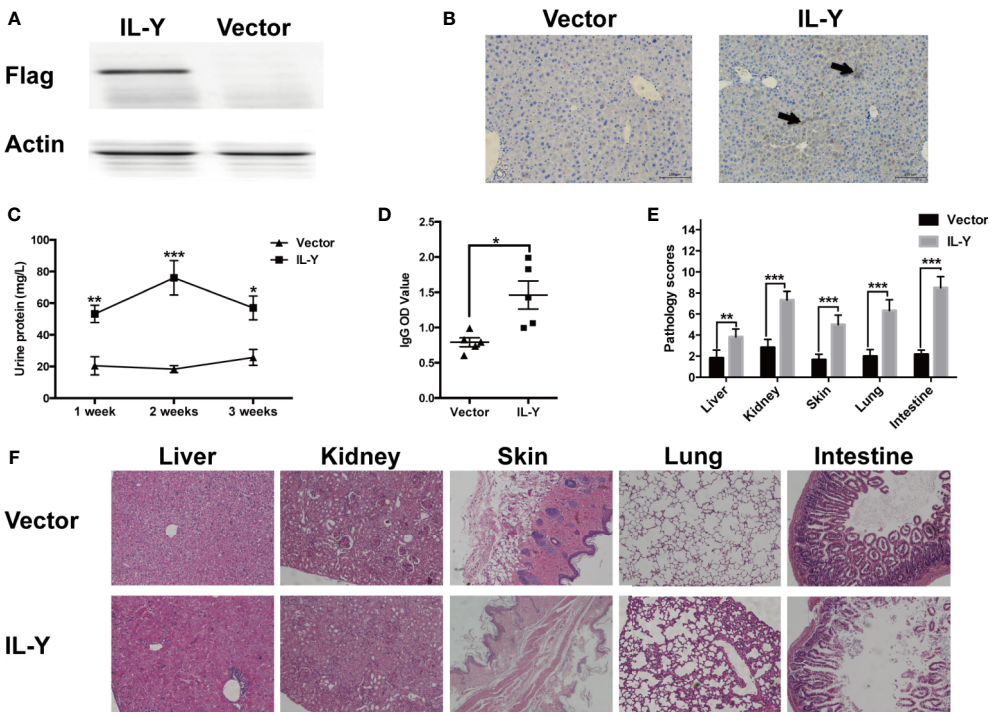

FIGURE 1 | IL-Y promotes the development of murine lupus-like cGVHD. **(A, B)** Mice were hydrodynamically injected with empty plasmids or IL-Y MC plasmids. IL-Y expression was detected by western blot **(A)** and immunohistochemistry **(B)** (original magnification ×40) in liver 7 days after plasmid injection by HGT. BALB/c mice were lethally irradiated (6.5 Gy) and transferred with 5×10^6 BM cells and 4×10^7 $CD4^+CD25^-$ splenocytes 3 days after plasmid injection by HGT. **(C)** Urine protein was detected by BCA Protein Assay Kit. **(D)** Serum level of anti-dsDNA IgG was determined. **(E)** Pathology scores are shown 8 weeks after donor cell transfer. **(F)** Representative histopathology photos of liver, kidney, skin, lung, and intestine are shown. Data are representatives of at least three independent experiments. Values are presented as means ± SD. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Serum Anti-dsDNA Antibody Detection

We made double-stranded DNA (dsDNA) from calf thymus (Sigma, D1501). High-binding ELISA plates (Costar, 3369) were coated with a mixture containing 50 μg/ml dsDNA 2 h at 37°C and then incubated at 4°C overnight. The plates were then blocked with $NaCO_3/NaHCO_3$ buffer solution containing 5% goat serum for 1 h at 37°C. Following blocking, plates were washed several times with 0.05% tween-20 PBS (PBST). Serum samples were added at 1:100 ratio in PBST containing 10% new bovine serum (NBS) and 5% goat serum. Plates were incubated at 37°C for more than 2 h and then washed with PBST for three times. The HRP-conjugated secondary antibody (HRP-IgG or HRP-IgG1 or HRP-IgG2a) (Southern Biotech, Birmingham, Alabama) was then added at a 1:1,000 ratio in PBST containing 10% NBS and 5% goat serum and incubated for 1 h at 37°C. Plates were then washed four times, and 50 μl of TMB Substrate (eBioscience, San Diego, CA) was added to each well. After 15–30 min, the reaction was stopped using 50 μl of 1 mol phosphoric acid, and the plate was read at 450 nm. Wells with no serum were used as negative controls. Plates were read by a SYNERGY-HTX ELISA plate reader (BioTek, Vermont).

Assessment of cGVHD and Histopathology

Recipient mice were monitored for survival, weight loss, and clinical scores of cGVHD. Urine protein was detected by BCA Protein Assay Kit (Beyotime Biotechnology, China). In order to quantify the histopathologic parameters of GVHD target organs, salivary, kidney, skin, lung, liver, thymus, and small intestine of recipient mice were collected 56 days post BM transplantation. Tissues were fixed with 10% formalin and made into slices with hematoxylin and eosin (HE) staining and observed under optical microscope (Nikon, Japan). Tissue damage was blindly assessed on a scoring system described previously (23). In particular, a numeric value was attributed to the changes observed in the kidney (loss of glomeruli, architecture disruption, immune complex deposition, lymphocytes infiltration), in the skin (dermal fibrosis, fat loss, epidermal thickening, follicular loss, and inflammation), in the lung (perivascular and peribronchiolar infiltration, pneumonitis alveolar/interstitial), in the liver (number of involved tracts, lymphocytic infiltration, liver cell necrosis), in the small intestine (mucosal, lamina propria, muscular, serosal). Collagen deposition was quantified by measuring percent of blue area in ImageJ.

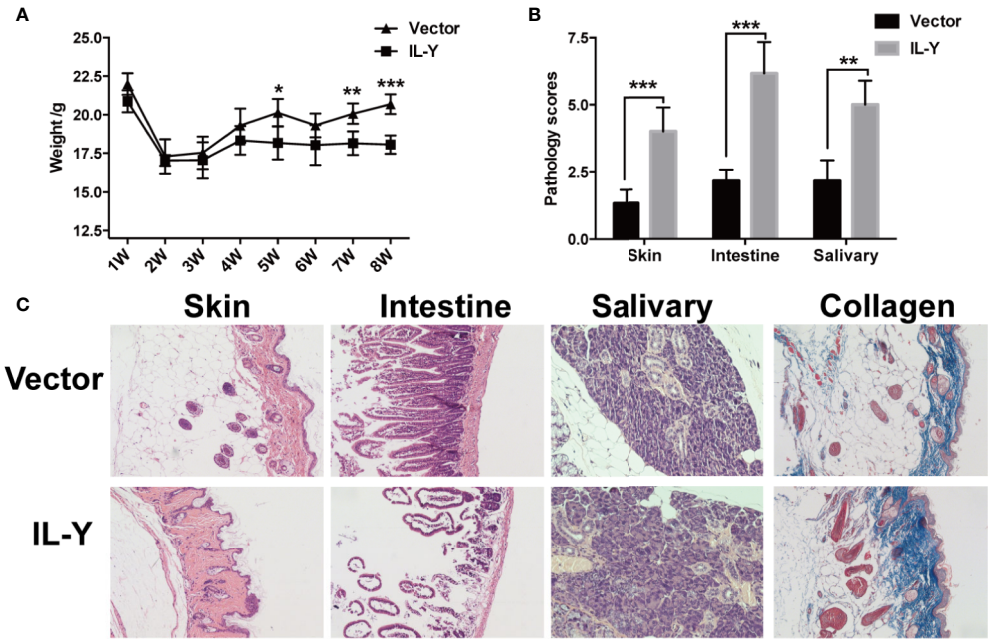

FIGURE 2 | IL-Y promotes the development of murine scleroderma-like cGVHD. BALB/c ($H2K^d$) mice (n = 6 each group) were lethally irradiated and transferred with 1×10^7 BM cells and 1×10^6 splenocytes of C57BL/6 ($H2K^b$) mice 3 days after plasmid injection by HGT. **(A)** Mice body weight is shown. **(B)** Pathology scores are shown 8 weeks after donor cell transfer. **(C)** Representative histopathological pictures of skin, intestine, and salivary gland, as well as Masson's trichrome staining are shown. Data are representative of at least three independent experiments. Values are presented as means ± SD. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Flow Cytometry

Single cell suspensions were obtained according to the methods previously described and stained for surface receptors and intracellular cytokines. The antibodies and reagents used for flow cytometry analysis were listed as below: antibodies to mouse, PE-CF594-CD3e (145-2C11), PE/Cy7-B220 (RA3-6B2), Allophycocyanin-CD138 (281-2), BV421-Gl-7 (Gl-7), rat BV421-IgG2a isotype control (R35-95) BV510-CD95 (Fas, Jo2), PE-CD278 (ICOS, 7E.17G9), PE-IL-27Rα (2918), PE-IL-12Rβ1 (3C9) were purchased from BD Bioscience (San Diego, CA); Allophycocyanin-CD185 (CXCR5, L138D7), Pacific Blue-CD8 (Gl-1), Allophycocyanin/Cy7-IL-17A (TC11-18H10.1), PE-CD21 (7E9), PE/Cy7-CD44 (IM7), PE-IFN-γ (XMG1.2), Allophycocyanin/Cy7-CD23 (B3B4), PE/Cy7-TNF-α (MP6-XT22), PE-Foxp3 (MF-14) Allophycocyanin-IL-4 (11B11), purified anti-mouse IL-12/IL-23 p40 and purified CD16/32 were purchased from Biolegend (San Diego, CA). Foxp3 staining kit was purchased from eBioscience (San Diego, CA). Flow cytometric analysis was performed using a FACS NovoCyte (ACEA Biosciences, San Diego, CA) and the Flowjo software (Tree Star, Ashland, OR).

Serum Cytokine Analysis

The levels of IL-2, IL-4, IL-17A, IFN-γ, TNF-α and IL-21 in serum were quantified by Cytometric Beads Array (CBA) kit (BD Bioscience, San Diego, CA).

T Cell Activation Assay

Naive T cells were sorted from splenocytes of C57BL/6 mice by Mouse Pan-Naive T Cell Isolation Kit according to the manufacturer's protocol (StemCell Technologies, Vancouver, Canada). Plates were coated with 1 μg/ml anti-CD3 and 0.2 μg/ml anti-CD28 Abs (BioLegend) overnight. A total of 1×10^5 naive T cells were cultured for 48 h alone or with 10 μg/ml rIL-Y protein (DETAIBIO, China). T cells were then analyzed by flow cytometry to determine the TNF-α production of $CD4^+$ T cells and $CD8^+$ T cells.

Statistics

Statistical analyses and data presentation were performed using GraphPad Prism 5 software for Mac (Graphpad Software, San Diego, CA). Unpaired Student tests were used to determine statistically significant differences between two experimental groups. Data are expressed as mean ± SD. P value < 0.05 was considered statistically significant (*), less than 0.01 or 0.001 was shown as ** or ***, respectively.

RESULTS

IL-Y Promotes the Development of Murine Lupus-Like and Scleroderma-Like cGVHD

Murine IL-Y expression construct was generated by fusing IL-27p28 and IL-12p40 *via* a hydrophobic polypeptide linker (Gly4Ser).

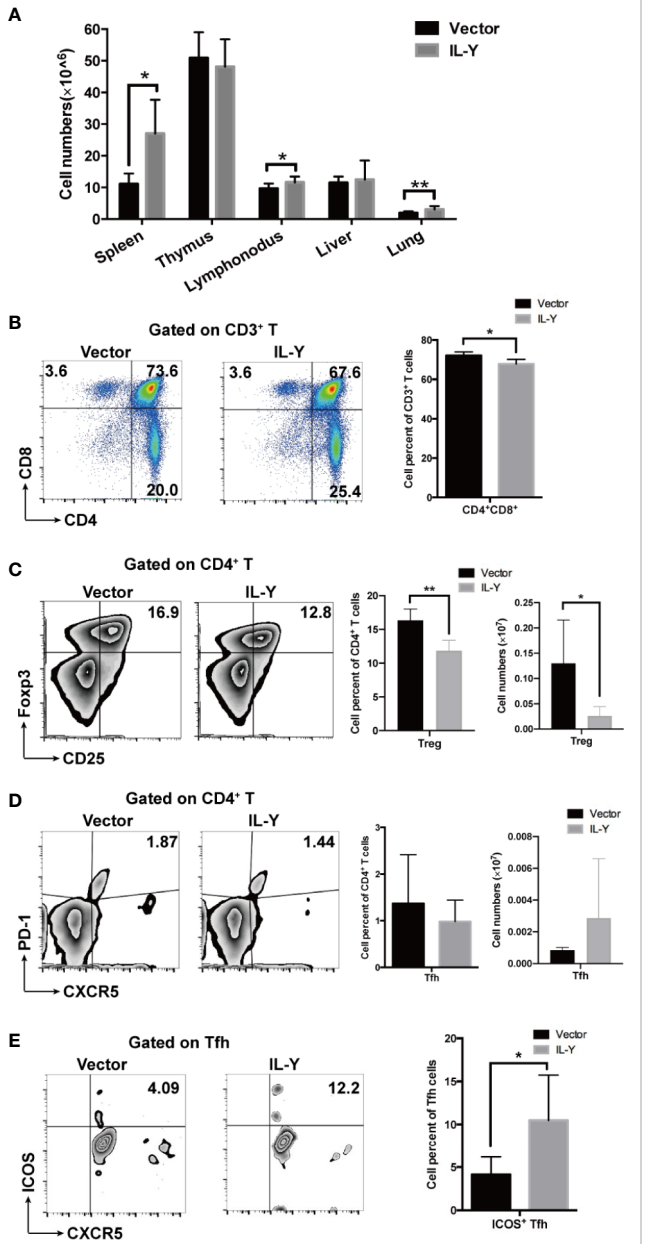

FIGURE 3 | IL-Y suppresses Treg differentiation and facilitates Tfh activation. Splenocytes (n = 6 each group) were collected and stained for FACS analysis 8 weeks after donor cell transfer. **(A)** Numbers of lymphocytes infiltration in different organs are shown. **(B)** Percentage of $CD4^+$ $CD8^+$ T cells in thymus is shown. **(C)** Percentage and number of $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells are shown. **(D)** Percentage and number of Tfh cells ($CD4^+$ $PD\text{-}1^+$ $CXCR5^+$) are shown. **(E)** Percentage of $ICOS^+$ Tfh cells is shown. Data are representatives of at least three independent experiments. Values are presented as means ± SD. *$P < 0.05$; **$P < 0.01$; $P < 0.001$.

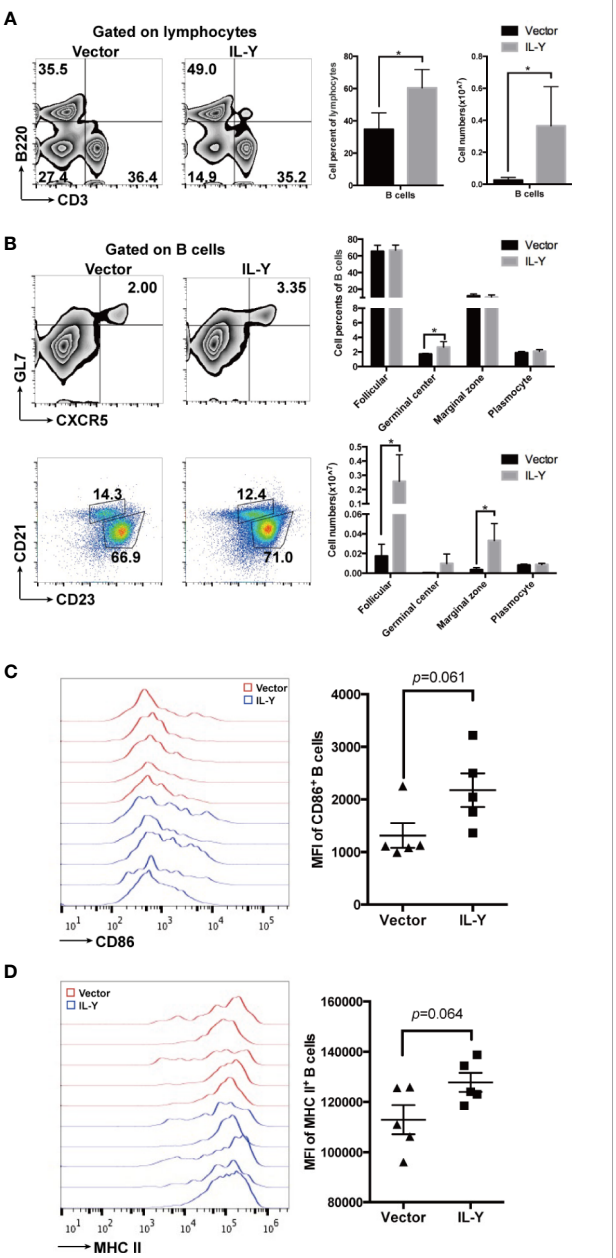

FIGURE 4 | IL-Y increases germinal center B responses and B cell function. Splenocytes (n = 6 each group) were collected and stained for FACS analysis 8 weeks after donor cell transfer. **(A)** Percentage and number of splenic B cells ($CD3^-$ $B220^+$) are shown. **(B)** Percentage and number of follicular ($CD21^+$ $CD23^+$), marginal zone ($CD21^{hi}$ $CD23^{low}$), $GL7^+$ $CXCR5^+$ germinal center B cells and plasmocytes ($CD138^+$) gated on $CD3^-$ $B220^+$ B cells are shown. Further analysis of B cell phenotypes. **(C, D)** Mean fluorescence intensity (MFI) of CD86 and MHC-II is shown. Data are representatives of at least three independent experiments. Values are presented as means ± SD. *P < 0.05.

The nucleotide sequence-encoding Igκ signal sequence was fused to the 5′ end of IL-Y sequence and flag tag was inserted to the 3′ end of IL-Y sequence, then full-length IL-Y was inserted between sites of Nhe I (5′) and Sal I (3′) into minicircle (MC) plasmid. IL-Y release in the liver was achieved by hydrodynamically injecting MC IL-Y plasmids. IL-Y expression in the liver was confirmed by immunohistochemistry and western blot 7 d after plasmid injection (**Figures 1A, B**).

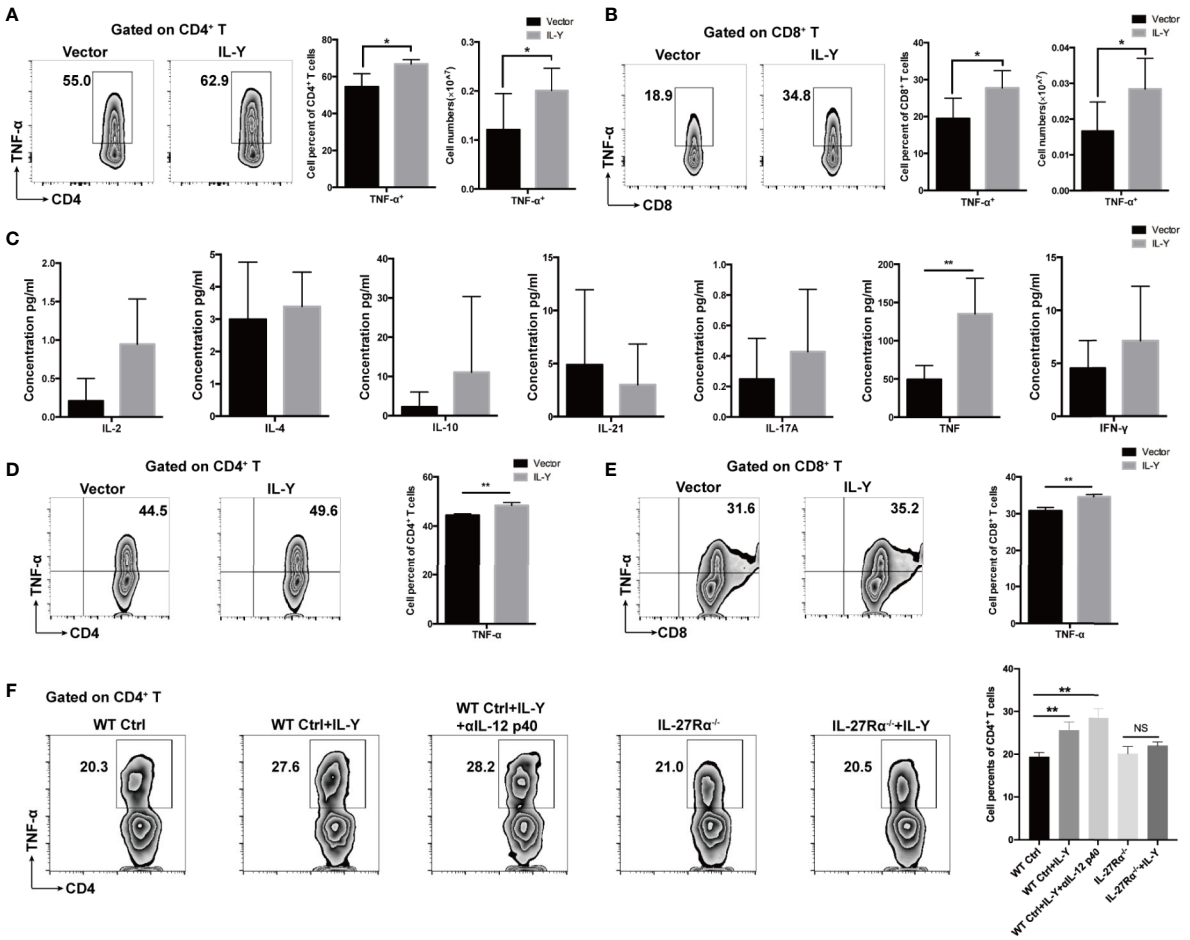

FIGURE 5 | IL-Y promotes generation of TNF-α by $CD4^+$ and $CD8^+$ T cells *in vivo* and *in vitro*. **(A, B)** Splenocytes (n = 6 each group) were collected 8 weeks after donor cell transfer. Splenocytes and intrahepatic leukocytes were stimulated and then analyzed for intracellular cytokine production. Percentages and numbers of TNF-α-producing $CD4^+$ T cells and $CD8^+$ T cells in spleens are shown. **(C)** Serum levels of IL-2, IL-4, IL-10, IL-21, IL-17A, TNF-α, and IFN-γ were measured by CBA assays. **(D, E)** Naïve T cells were sorted from spleens of mice and cultured in plates coated with anti-CD3 and anti-CD28 antibodies and with 100 ng/ml rIL-Y or PBS for 48 h. Percentages of TNF-α-producing $CD4^+$ T cells and $CD8^+$ T cells are shown. **(F)** Naïve T cells from WT and IL-27Rα$^{-/-}$ mice spleens were sorted and cultured in plates coated with anti-CD3 and anti-CD28 antibodies and with 100 ng/ml rIL-Y or PBS or 1 μg/ml anti-IL-12 p40 for 48 h. Percentage of TNF-α-producing $CD4^+$ T cells is shown. Data are representatives of at least three independent experiments. Values are presented as means ± SD. NS indicates no significant difference. *$P < 0.05$; **$P < 0.01$.

To examine the role of IL-Y in the development of cGVHD in mice, we established a lupus-like cGVHD model that is featured with autoimmune manifestations including autoantibody production, glomerulonephritis, proteinuria and ascites. IL-Y MC plasmids were hydrodynamically injected 3 days before irradiation. Bone marrow cells and $CD4^+CD25^-$ splenocytes isolated from DBA/2 mice were injected intravenously into lethally irradiated (6.5 Gy) Balb/c recipients. IL-Y significantly increased the level of urine protein at 1 week, 2 weeks and 3 weeks post bone marrow transplantation (BMT) (**Figure 1C**). In addition, mice administrated with MC IL-Y plasmids displayed significantly higher level of serum IgG autoantibodies (**Figure 1D**). Histologic assessment revealed more severe tissue damage in the liver, kidney, skin, lung, as well as small intestine in recipients with IL-Y MC plasmids administration (**Figures 1E, F**). To further exclude the model specific phenomenon, we established scleroderma-like cGVHD model to confirm the pathogenic role of IL-Y in the development of cGVHD. Specifically, Balb/c mice were injected with 1×10^6 spleen cells and 1×10^7 bone marrow cells from C57BL/6 mice after irradiation (6.5 Gy) 3 days after IL-Y MC plasmids injection. In the late stages of cGVHD, mice in the MC IL-Y group showed more weight loss (**Figure 2A**). Consistently, IL-Y also significantly aggravated the histopathology damage compared with empty vector control in this scleroderma-like cGVHD model (**Figures 2B, C**). As sclerosis is an important feature of cutaneous cGVHD, collagen deposition was found to be significantly increased in the recipient skin in MC IL-Y

plasmids group (**Figure 2C**). Altogether, these results indicated that IL-Y promoted the development of cGVHD.

IL-Y Suppresses Treg Differentiation and Facilitates Tfh Activation

To further explore the underlying mechanism how IL-Y exacerbates the development of cGVHD, we examined the splenic immune cell phenotypes 8 weeks after lupus-like cGVHD model establishment. Lymphocytes infiltration was significantly increased in spleens, lymph nodes and lungs in IL-Y group (**Figure 3A**). As a direct target organ, thymus is heavily involved in cGVHD pathogenesis by inducing auto-reactive emigrants and impairing Treg generation (12). Consistent with the aggravated clinical manifestations, MC IL-Y plasmids treated mice displayed significantly lower percentage of $CD4^+$ $CD8^+$ T cells in thymus (**Figure 3B**). Previous studies pointed to pro-inflammatory cytokines produced by pathogenic $CD4^+$ T cells, Th1 and Th17 cells, as the driving force for the initiation of cGVHD (11, 17, 24). In addition, donor $CD8^+$ T cells preferentially damaged recipient medullary thymic epithelial cells and impaired negative selection, resulting in production of auto-reactive $CD4^+$ T cells that perpetuated the damage to the thymus and augmented the development of cGVHD (10). However, we did not observe significant increase of activated and effector $CD4^+$ and $CD8^+$ T cells in MC IL-Y group (data not shown).

Treg cells play an important role in maintaining immune-tolerance, preventing autoimmune diseases and limiting inflammatory diseases, including cGVHD. Treg population at early time points is crucial for modulating GVHD. In our cGVHD model, percentage and number of Treg cells were significantly decreased in MC IL-Y plasmids group 2 weeks after cGVHD model establishment (**Figure 3C**). At 8 weeks post transplantation, the percentage of Treg cells was also decreased in the IL-Y group (**Figure S1A**). Studies have shown that Tfh cells also play an extremely important role in the pathogenesis of cGVHD by promoting GC B cell formation and B cell activation (12, 15, 16, 25). Interestingly, patients and murine model with active cGVHD have decreased numbers of Tfh cells compared with no or mild cGVHD, but Tfh cells expressed high levels of ICOS and secreted higher levels of CXCL13 in plasma to facilitate contact between Tfh and B cells (11, 16). Indeed, we found that there was no difference in the percentage and number of Tfh cells in spleens (**Figure 3D**). However, the percentage of $ICOS^+$ Tfh cells was significantly increased in spleens by IL-Y expression (**Figure 3E**), which indicated that IL-Y might promote GC formation and B cell activation *via* promoting $ICOS^+$ Tfh differentiation. T follicular Regulatory (Tfr) cells restrain GC responses by inhibiting Tfh and B cell function (18, 20). However, there was no difference in the subgroup of Tfr cells (data not shown). These results suggested that IL-Y selectively inhibited Treg cell differentiation and promoted Tfh activation to facilitate the development of cGVHD.

IL-Y Increases Germinal Center B Cell Responses and B Cell Function

B cells exacerbated the development of cGVHD through GC B cell formation, antibody production, and antigen presentation to T cells. Previous studies indicated that the administration of B cell-depleting anti-CD20 could ameliorate cGVHD in some patients (26, 27). We found that MC IL-Y plasmid treatment significantly increased both percentage and number of splenic B cells (**Figure 4A**). Further analysis of B cell phenotypes showed that there was increased percentage of GC B cells in MC IL-Y plasmids group (**Figure 4B**). In addition, the numbers of follicular B cells and marginal zone B cells were upregulated by IL-Y expression (**Figure 4B**). Levels of co-stimulatory molecules, including CD86 and MHC-II, were found upregulated on donor B cells in MC IL-Y plasmids group (**Figures 4C, D**), suggesting that IL-Y may affect B cell activation and antigen presenting function. Taken together, these data indicated that IL-Y exacerbated lupus-like cGVHD by promoting B cells activation and function.

IL-Y Promotes TNF-α Production by $CD4^+$ and $CD8^+$T Cells *In Vivo* and *Vitro*

We then examined the impacts of IL-Y on the shifting of cytokine balance in the splenocytes of recipients. Both percentage and number of TNF-α-producing $CD4^+$ and $CD8^+$ T cells were markedly elevated by IL-Y expression (**Figures 5A, B**). Moreover, TNF-α secretion by $CD4^+$ T cells and $CD8^+$ T cells were also significantly upregulated in livers (data not shown). We did not observe significant changes in IL-17, IL-10 and IFN-γ (data not shown). Serum levels of IL-2, IL-4, IL-10, IL-17A, IFN-γ, TNF-α and IL-21 were determined by CBA assays. Consistently, serum TNF-α level was elevated by IL-Y expression (**Figure 5C**). To investigate whether IL-Y can directly promote TNF-α production by $CD4^+$ and $CD8^+$ T cell, we stimulated naive T cells from the spleen with anti-CD3/anti-CD28 *in vitro* and analyzed the TNF-α-producing $CD4^+$ and $CD8^+$ T cells subsets in the presence or absence of rIL-Y. The percentages of both TNF-α-producing $CD4^+$ and $CD8^+$ T cells were significantly increased (**Figures 5D, E**). Flores et al. suggested IL-Y exerted its suppressive effect through IL-27Rα (22); this effect was proved diminished in IL-27Rα deficient mice (**Figure 5F**). In addition, the role of IL-12Rβ1 signaling cannot be ignored because IL-12/23 p40 was found to play critical roles in the development of GVHD. However, blockade of IL-12 p40 had no effect on the secretion of TNF-α by $CD4^+$ T cells (**Figure 5F**). The role of IL-27Rα signaling in GVHD is still not clear. It has been demonstrated that IL-27Rα signaling on T cells deteriorates GVHD severity by promoting Th1 responses (28) and IL-27Rα signaling blockade reduced GVHD (29), while Le et al. suggested that IL-27 stimulation enhanced Treg functions to prevent GVHD (30). Thus, differential expression of IL-27Rα on different T cell subsets may paly contrary role during GVHD. We detected the expression of IL-27Rα on $CD3^+$ T cells and Treg cells 14 days post-transplant. The results showed no difference

between the IL-Y and the control group (**Figures S1B, C**). Collectively, these data indicated that IL-Y could selectively promote TNF-α production by $CD4^+$ and $CD8^+$ T cells, which probably signals through IL-27Rα and presumably contributed to the progression of cGVHD.

DISCUSSION

Chronic graft-*versus*-host disease (cGVHD) is a major complication in the late stage of allo-HSCT. With the decrease of mortality in the early stage of transplantation, the increase of the upper limit of the recipients' age, the application of unrelated donors and peripheral blood hematopoietic stem cells, the incidence of cGVHD gradually increases, which adversely affects the life quality of allo-HSCT patients. cGVHD has become an important cause of non-recurrent death in the late stage of transplantation (7, 31). In the current study, we constructed a MC IL-Y eukaryotic expression plasmid and examined the pathogenic role of this novel cytokine in the development of cGVHD. We demonstrated that IL-Y played a critical role in the pathogenesis of cGVHD *via* activating T and B cell responses, and subsequent occurrence of scleroderma and antibody deposition in murine models of cGVHD. Mechanistically, IL-Y could accelerate the initiation of cGVHD by enhancing pro-inflammatory cytokine TNF-α production by pathogenic T cell. In addition, IL-Y perpetuates the pathogenesis of cGVHD by enhancing GC cell responses and antibody production.

Sakoda et al. found that self-reactive donor T cells played an important role in the development of cGVHD and improvement in the thymic function may have a potential to reduce cGVHD (32). Research of Wu et al. showed that donor $CD8^+$ T cells preferentially damaged recipient medullary thymic epithelial cells and impaired negative selection, resulting in production of autoreactive $CD4^+$ T cells, which perpetuated damage to the thymus and augmented the development of cGVHD (10). As a direct target organ, thymus was heavily involved in cGVHD pathogenesis, and its damage induces autoreactive emigrants and impairs Treg generation (10, 32). Consistent with the aggravated clinical manifestations observed, significant lower percentages and numbers of $CD4^+$ $CD8^+$ T cells in thymus were found in the recipients of IL-Y administrated mice. This further suggests that IL-Y may aggravate cGVHD by impairing thymus development.

It has traditionally been assumed that the predominant cytokines produced during cGVHD are Th2 cytokines, which can stimulate host B cell autoantibody production (33, 34). Recent studies have suggested that cGVHD could be caused by cytokines secreted by Th1 and Th17 cells (9, 11, 24). Previous prospective studies have found that levels of serum TNF-α in cGVHD patients were associated with disease severity (35). Similarly, high levels of TNF-α can be detected in patients with systemic sclerosis (36). Several TNF-α inhibitors have been shown to significantly improve the condition of patients with systemic sclerosis (37). TNF-α produced by T cells was involved in promoting the migration and differentiation of $Ly6C^{lo}$ monocytes into pathogenic M2 macrophages, which may contribute to the activation of fibroblast and production of collagen, leading to tissue fibrosis (38). TNF-α released in the GI tract induced epithelial cell alterations and promoted the inflammatory reaction (39). In addition, TNF played a critical role in GVHD, as increased levels of TNF-α before HSCT was significantly correlated with severe GVHD. Several clinical studies have demonstrated that TNF-α blockade exerted promising activity in patients with GI-GVHD (40). In the current study, we demonstrated that IL-Y aggravated the progression of cGVHD by activating T and B cells, and increasing TNF-α secretion by $CD4^+$ and $CD8^+$ T cells in scleroderma-like and lupus-like cGVHD models. This is inconsistent with previous studies that IL-Y can exert an immunosuppressive effect by inhibiting the differentiation of Th1 and Th17 cells (21). It may be due to the different function of IL-Y in different animal models. Treg cells are critical mediators of immune tolerance and are required to prevent fatal autoimmunity in healthy individuals. Treg cell impairment is associated with loss of tolerance, autoimmunity and cGVHD (20, 41). In preclinical models of allo-HSCT, adoptive transfer of Treg cells can ameliorate GVHD without impairing therapeutic GVL responses (42). Impaired Treg cells reconstitution appears predictive for subsequent cGVHD studies (43). Indeed, we observed a significant down-regulation of splenic Treg cells in MC IL-Y group. Therefore, treatment strategies attempting to enhance Treg numbers by blocking the signaling pathway of IL-Y are attractive for cGVHD therapy, offering the possibility of therapeutic immune modulation without generalized immunosuppression.

Stimulation of $CD4^+$ T cells and their interactions with autoantibody producing B cells have been proved to play critical roles in the pathology of cGVHD (9). Accordingly, 8 weeks after establishment of cGVHD, we observed that the frequencies of donor derived activated $CD4^+$ T cells and $ICOS^+$ Tfh cells were obviously increased in spleens of the recipients treated with IL-Y. Tfh cells are necessary for GC B cell formation and maintenance, which were shown to be required for the pathogenesis of cGVHD (44). Additionally, expression of ICOS was demonstrated to play critical roles in Tfh cells to mediate GC B cell reactions (45). In our study, although Tfh cells were not significantly increased, percentage of $ICOS^+$ Tfh cells was significantly upregulated in the recipients given MC IL-Y plasmids, which was consistent with recent clinical reports (16). Furthermore, we found that IL-Y administration promoted the differentiation of GC B cells in recipient spleens. More importantly, higher levels of MHC-II and CD86 were expressed on donor B cells in recipient mice with MC IL-Y plasmids treatment, suggesting that IL-Y may affect B cell activation and antigen-presenting function through regulating T cell activation and differentiation.

IL-Y is a novel cytokine found to be involved in cGVHD pathogenesis in murine model of cGVHD. We have demonstrated that IL-Y could aggravate cGVHD and play pleiotropic roles in regulating the differentiation and function of multiple immune cells involved in the pathogenesis of

cGVHD. IL-Y may selectively promoted TNF-α production by $CD4^+$ and $CD8^+$ T cells through IL-27Rα, leading to the progression of cGVHD. Further studies are needed to reveal whether p40 (a component of IL-Y) could be involved in the diagnosis or prognosis of patients with allo-HCT who developed cGVHD. Taken together, our results provide evidence that targeting p40 (46) and IL-27Rα signaling can be effective therapeutic strategies for cGVHD treatment.

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 animal study was reviewed and approved by Jiangsu Institute of Hematology, The First Affiliated Hospital of Soochow University, Institute of Blood and Marrow Transplantation, Collaborative Innovation Center of Hematology, Soochow University, Suzhou, China.

AUTHOR CONTRIBUTIONS

HL, DW, and YuL designed the study. LW, ZJ, and BH performed the research. KL, LL YoL, YS, YZ, HG, MX, YD, and YX contributed to the experiments. LW and ZJ analyzed the data. LW, ZJ, and YuL 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.2020.559740/full#supplementary-material

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REVIEW
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Tolerance to Bone Marrow Transplantation: Do Mesenchymal Stromal Cells Still Have a Future for Acute or Chronic GvHD?

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Mesenchymal Stromal Cells (MSCs) are fibroblast-like cells of mesodermal origin present in many tissues and which have the potential to differentiate to osteoblasts, adipocytes and chondroblasts. They also have a clear immunosuppressive and tissue regeneration potential. Indeed, the initial classification of MSCs as pluripotent stem cells, has turned into their identification as stromal progenitors. Due to the relatively simple procedures available to expand *in vitro* large numbers of GMP grade MSCs from a variety of different tissues, many clinical trials have tested their therapeutic potential *in vivo*. One pathological condition where MSCs have been quite extensively tested is steroid resistant (SR) graft versus host disease (GvHD), a devastating condition that may occur in acute or chronic form following allogeneic hematopoietic stem cell transplantation. The clinical and experimental results obtained have outlined a possible efficacy of MSCs, but unfortunately statistical significance in clinical studies has only rarely been reached and effects have been relatively limited in most cases. Nonetheless, the extremely complex pathogenetic mechanisms at the basis of GvHD, the fact that studies have been conducted often in patients who had been previously treated with multiple lines of therapy, the variable MSC doses and schedules administered in different trials, the lack of validated potency assays and clear biomarkers, the difference in MSC sources and production methods may have been major factors for this lack of clear efficacy *in vivo*. The heterogeneity of MSCs and their different stromal differentiation potential and biological activity may be better understood through more refined single cell sequencing and proteomic studies, where either an "anti-inflammatory" or a more "immunosuppressive" profile can be identified. We summarize the pathogenic mechanisms of acute and chronic GvHD and the role for MSCs. We suggest that systematic controlled clinical trials still need to be conducted in the most promising clinical settings, using better characterized cells and measuring efficacy with specific biomarkers, before strong conclusions can be drawn about the therapeutic potential of these cells in this context. The same analysis should be applied to other inflammatory, immune or degenerative diseases where MSCs may have a therapeutic potential.

Keywords: mesenchymal stromal cell, graft versus host disease, hematopoietic stem cell transplantation, immunosuppressive drugs, inflammation

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INTRODUCTION

The transplantation of hematopoietic stem cells (HSCT) from a normal donor to a "genetically matched" recipient is a current therapeutic option in onco-hematology. The most common toxicities of the procedure are rejection, disease relapse and acute and/or chronic graft versus host disease (aGvHD and cGvHD, respectively), even though these have been substantially reduced by the introduction of innovative transplantation procedures, wider donor availability with better donor selection, as well as the use of new drugs or new schedules of treatment and prophylaxis. The best example of an innovative prophylaxis treatment is the administration of high doses cyclophosphamide (Cy) post-transplant in order to promote tolerance, reviewed in (1, 2). Indeed, such treatment appears efficacious in reducing allo-reactive donor conventional T cells, while preserving the T regulatory compartment, possibly due to the high content in these cells of aldehyde dehydrogenase, an enzyme that favors chemoresistance (3).

In spite of the more recent reduction in the incidence rate of aGvHD and cGvHD during allogeneic HSCT, these conditions remain a difficult problem, since, in the most severe and resistant forms and after failure of steroid treatment, well defined and clearly effective second- or third-line treatments are not yet available. Fortunately this last statement may not be completely true anymore, since the JAK1/JAK2 inhibitor ruxolitinib has been rapidly approved by the FDA in October 2019 for the treatment of steroid refractory (SR) aGvHD, on the basis of significant results from a multi-center phase III trial (4) (and see below). Furthermore, this drug appears to show promising activity in cGvHD. Nonetheless, many other potential drugs are also being investigated for GvHD treatment, including Mesenchymal Stromal Cells (MSCs). We would therefore like to briefly summarize in this review the knowledge that has accumulated about the pathogenetic and immunological mechanisms behind acute and chronic GvHD (which underlie the lack of tolerance of the donor immune system to the host tissues, i.e. GvH tolerance), and concentrate our discussion specifically on the state of the art with regard to administration of human MSCs as a treatment strategy for such devastating diseases. We will treat the topics of aGvHD and cGvHD separately. The abbreviations used throughout the text are listed in **Table 1**.

THE BIOLOGY AND IMMUNOLOGY OF aGVHD

Only to briefly recall the inflammatory context that underlines aGvHD, we will schematically summarize the main pathogenetic steps which take place in this condition.

The earliest pathophysiological event in the disease process (phase 1) is a diffuse endothelial damage, occurring as a consequence of the conditioning chemo-radiotherapy, which induces neo-angiogenesis as well as the infiltration of innate myeloid cells, neutrophils and monocytes into the intestinal tract. The release of superoxide radicals and other reactive oxygen species (ROS) by neutrophils is an essential physiological element of the innate immune response against invading pathogens. Inflammatory stimuli include sterile damage associated molecular pattern (DAMP) molecules (nucleic acids, intracellular proteins such as high mobility group box 1 (HMGB1), heat shock proteins, histones, actin, ATP and reactive oxygen species (ROS) and extracellular proteins such as hyaluronic acid and biglycan), alarmins released by cellular degranulation (constitutively expressed endogeneous molecules, e.g. IL-1α, IL-33), as well as inflammatory cytokines (e.g. IL-6, TNF). These promote the translocation across the impaired mucosal barrier to the underlying tissue layers of microbiota associated molecular pattern molecules (i.e. Pathogen Associated Molecular Pattern or PAMP, which include LPS, lipoproteins, peptidoglycans, flagellin, fungal components, viral nucleic acids). Bacterial colonization of the classical GvHD target organs, skin, and intestinal tract, as well as liver, has led to the hypothesis that bacterial transmigration is essential for the disease. Both DAMP and PAMP act on specific cellular receptors (5), PAMP being particularly engaged in activation of host antigen presenting cells (APCs) and subsequent priming of T cells to enhance alloantigen presentation. In both cases, Toll like receptors (TLR) pathways are triggered through receptors on the plasma membrane (TLR2, TLR4) and in endosomes (TLR3, TLR7/8, TLR9). TLR pathway activation induces IFNα production *via* transcriptional interferon response factors (IRFs). Particularly important, at this step, is the activation of the inflammasome multi-protein intracellular complexes, such as NLRP1 and NLRP3, which are able to rapidly activate the caspase family proteases, that generate the mature forms of IL-1β and IL-18 from inactive intracellular precursors and then release them into the extracellular milieu (during a process known as pyroptosis of monocytes, i.e. an inflammatory form of cell death) (6, 7). Pyroptosis is considered a mechanism to release DAMP molecules, such as IL-1α, HMGB1, and ATP (6). Activated cells secrete further cytokines, in particular TNF, IL-1α, IL-6, IL-33, IL-12, IL-23, type I IFN, and chemokines (e.g. CCL5), which enhance alloantigen presentation and expression of co-stimulatory molecules and cytokines by host APC. Host dendritic cells (DCs), inflammatory monocytes and neutrophils migrate from the damaged intestinal epithelium towards mesenteric lymph nodes, where donor T cells are activated. Moreover, IFNα and IFNγ can induce chemokines (CXCL9, CXCL10, and CXCL11) that recruit helper T cells 1 (Th1) and cytotoxic T cells 1 (Tc1) and NK cells, all expressing CXCR3 (5, 7–10).

During the second phase, allogeneic peptides presented by major histocompatibility complex (MHC) molecules are recognized by the T cell receptor (TCR) on conventional donor T cells (signal 1) in conjunction with many possible co-stimulatory molecules (CD40, OX40L, CD155/112, ICOSL) on recipient APCs (signal 2) which, together with cytokines such as IL-2, IL-12, IL-6, IL-23 (all signaling *via* JAK1/2)(signal 3), drive the differentiation of naïve T cells into mature helper and cytotoxic Th1/Tc1 and Th17/Tc17 effector cells (third phase). While the Th1/Th2 paradigm (Th1 being most important for aGvHD and Th2 for cGvHD) has been challenged and refined, the role of CD4 Th17 and CD8 Tc17 appears more relevant for both conditions and requires TGFβ/IL-6 and IL-1β/TNFα, respectively. Downstream effector cytokines (IL-2 and IFN-γ,

TABLE 1 | List of major abbreviations used in the text.

Abbreviation	Full name	Abbreviation	Full name
APC	Antigen presenting cell	LIF	Leukaemia inhibitory factor
ATG	Anti-thymocyte globulin	LN	Lymph node
BCR	B cell receptor	LPS	Lipopolysaccharide
BM	Bone marrow	NK	Natural killer cell
Breg	Regulatory B cell	NLRP	Nucleotide binding oligomerisation domain, leucine rich repeat and pyrin domain containing
CR	Complete response	MHC	Major histocompatibility complex
CsA	Cyclosporin A	MMF	Mycophenolate mofetil
Cy	Cyclophosphamide	MMPs	Matrix metalloproteases
DAMP	Damage associated molecular pattern	MSC	Mesenchymal stromal cell
DC	Dendritic cells	MTX	Methotrexate
ECP	Extracorporeal photopheresis	MØ	Macrophage
EV	extracellular vesicles	PAMP	Pathogen associated molecular pattern
FcγR	Fcgamma receptor	PGE2	Prostaglandin-E2
FDA	Federal Drug Agency (US)	PDGF	Platelet derived growth factor
GC	Germinal center	PR	Partial response
GM-CSF	Granulocyte-monocyte colony stimulating factor	ROS	Reactive oxygen species
GMP	Good manufacturing practice	SR	Steroid resistant
GvHD	Graft versus host disease (a: acute; c: chronic)	TCR	T cell receptor
HMGB1	High mobility group box 1	Tc	T cytotoxic (cytotoxic T cell)
HSCT	Hematopoietic stem cell transplantation	TGF	Tumour growth factor
IBMIR	Instant blood mediated inflammatory reaction	Th	T helper (helper T cell)
IDO	Indoleamine 2,3 dioxygenase	TIMPs	Tissue inhibitor of metalloprotease
ISCT	International Society of Cell Therapy	TLR	Toll like receptor
IFN	Interferon	TNF	Tumor necrosis factor
IL-	Interleukin-	Treg	Regulatory T cell
ISCT	International Society of Cell Therapy	Tr1	Regulatory Type 1 T cells

secreted by Th1 and Tc1, respectively, and IL-17 produced by Th17 and Tc17, together with TNF and GM-CSF) cooperate with each other for the recruitment and activation of effector cells that induce target tissue apoptosis *via* FAS ligand and release of granzyme B and perforin (5, 7).

It has to be noted that, at the same time, the donor's T cells may also be engaged in inhibitory interactions *via* other surface APC molecules such as CD86, CD80, Galectin 9, PDL-1/2 and, additionally, that the entire scenario is counterbalanced by the presence of the donor's regulatory T cells: Tregs ($CD4^+CD25^{high}$ IL-2Rα$^+$ FoxP3$^+$ T cells, which require IL-2 for homeostatic proliferation) and Tr1, which bear inhibitory receptors such as TIGIT, CTLA-4, CD28, LAG3, ST2, produce inhibitory IL-10 and TGF-β cytokines and are activated mainly by IL-33, released by damaged cells *via* ST2, the IL-33 receptor. APCs also express inhibitory molecules that can down-modulate the immune response. Generally speaking, these "inhibitory" mechanisms can be viewed as the effort of the damaged tissue to repair and counteract the tissue damage, by inhibiting T cell responses and by the production and release of tissue repair factors such as keratinocyte growth factor (KGF) by fibroblasts, amphiregulin by Tregs, IL-22 by innate lymphoid cells type 3 and R spondin by fibroblasts (5, 7, 8).

As is clear from the above summary, the immune activation and tissue damage that are involved in the triggering and establishment of aGvHD and cGvHD are complex and therefore offer a plethora of molecules/pathways that can be potentially modified by drug treatment. These elements are also the targets of drugs used to try and control GvHD in the clinic.

Figure 1 presents a very schematic and simplified view of the mechanisms of aGvHD induction.

TREATMENT OF aGVHD WITH CONSOLIDATED AND INNOVATIVE DRUGS

The recommended first-line treatment for aGvHD is systemic steroid therapy (aiming to inhibit immune cells activation and switch off the transcription of pro-inflammatory genes); however, about 35–50% of patients become refractory to steroid therapy. SR-aGvHD is generally defined as a clear progression after 3 to 5 days of treatment or no response after 5 to 7 days.

There has been up to very recently no accepted standard-of-care treatment for SR-aGvHD. This is due to the fact that in most cases clinical studies of SR-aGvHD are retrospective, single-arm, phase II studies, and cannot be easily compared with current patient populations due to the significant changes that have been introduced in recent years, not only in terms of supportive care, but also prophylaxis of aGvHD. Indeed this was a very recent conclusion made by the European Bone Marrow Transplantation GvHD management recommendation expert panel, which stated that "not enough data from well-designed studies are available to be able to compare the efficacies of the different second-line treatment options" (11). During the last several years, nonetheless, several drugs have been used as second-line therapy of SR-aGvHD, based empirically on the mechanisms of action described above and on the

idea that blocking the donor's T cells mediated attack on host tissues and associated acute inflammation would be beneficial and these are reported in **Table 2**. The most interesting is the already mentioned JAK1/2 inhibitor ruxolitinib, which has been approved in October 2019 by the FDA for the treatment of SR-aGvHD in adult and pediatric patients above 12 years old, based on the very recently published phase III clinical data showing an overall response rate at days 28 and 56 significantly higher in the 154 ruxolitinib treated patients compared to the 155 control group (4). Ruxolitinib finds a strong rationale in several aspects of the pathogenetic mechanism discussed above: it should be able to inhibit the activity of IFNγ, IL-6, IL-11, IL-12, IL-23, and IL-27 (which signal through JAK1/JAK2) and, possibly, also of IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 (which share JAK1 and JAK3 signaling molecules). Additionally, ruxolitinib has been shown *in vitro* to upregulate MHC-II expression and to block DC maturation, as well as inhibit neutrophilic migration, as discussed extensively elsewhere (12).

Other treatments have also been commonly used to treat SR-sGvHD: Extracorporeal photopheresis (ECP), an immune-modulatory treatment able to induce apoptosis of T cells, anti-inflammatory and Th2-promoting cytokines and, as well as increase the levels of circulating Tregs; anti-thymocyte globulin (ATG) which induces not only T cell depletion, but also apoptosis of B cells, as well as upregulation of Tregs and NK cells; inhibitors of calcineurin (the TCR signaling intermediate to the NFAT transcription factor) such as tacrolimus and cyclosporine A (CsA) that inhibit TCR signaling; several monoclonal antibodies (mAbs) against IL-2Ra (daclizumab and basiliximab), IL-6R (tocilizumab), TNF receptor, or TNF-α (infliximab, etanercept); inhibitors of the downstream signaling mTOR molecule (sirolimus, everolimus), or dihydrofolate reductase inhibitors (methotrexate) which block production of thymidylate and purines and suppress T cell activation and proliferation.

Treatments that have fewer data available and are therefore considered to be third-line treatment options include alemtuzumab (anti–CD52 receptor antibody) which induces T cell and B cell depletion; pentostatin (a potent inhibitor of adenosine deaminase, the purine salvage enzyme involved in the irreversible deamination of adenosine and deoxyadenosine) and inhibitors of lymphocyte proliferation such as mycophenolate mofetil (MMF) (5, 13). Most recently, abatacept, a fusion protein that selectively inhibits T cell co-stimulation by binding to CD80/CD86 on APCs and blocking CD28-mediated signaling has been proposed (14).

Other recent proposed biological drugs or treatments have been introduced, whose development is based on the known pathogenesis of aGvHD, but are still in the early clinical phases of development. These include fecal microbiota transplant to re-establish the microbiota balance through infusion of a fecal suspension from a healthy donor into a patient's gastro-intestinal tract, an anti CD3/CD7 immunotoxin to depletes T and NK cells, and finally vedolizumab, a mAb blocking the α4β7 integrin present on the surface of T lymphocytes and which inhibits their gastro-intestinal localization (5, 13). **Figure 1** summaries the major mechanisms of the drugs, shown in red, currently used for aGvHD treatment.

PROPOSED MECHANISM OF ACTION OF MSCs IN aGVHD

Following the first report of the possible efficacy of MSCs in a case of SR-aGvHD (15)(see below), many biological studies have been pursued in an effort to better understand and ideally potentiate the immunosuppressive/anti-inflammatory mechanism of MSCs (16). Consequently, basic research has produced an impressive amount of data on the different mechanisms by which MSCs may have immunosuppressive activity in GvHD. These include the secretion of different immunosuppressive molecules, such as prostaglandins E2 (PGE2), indoleamine 2,3-dioxygenase (IDO), heme oxygenase-1, TGF-β, IL-10, nitric oxide, galectins 1, 3, and 9, Leukemia Inhibitory Factor (LIF) and HLA-G5, the stimulation and induction of Treg differentiation, the inhibition of Th17 differentiation, the induction of IL-10 production by $CD5^+$ B cells (Bregs), the inhibition of B cells activation, proliferation and immunoglobulin secretion, as well as the inhibition of T and NK cell proliferation, the inhibition of IL-2 production by NK cells and the induction of T cells apoptosis. In addition, MSCs can dampen effector cell functions by cell-cell interactions *via* the PD-1/PDL-1 and HLA-G1 molecules. Furthermore, MSCs can secrete CCL2 and, through this chemokine, recruit monocytes and promote their differentiation to M2 type macrophages by upregulating expression of CD206, IL-10,and TGF-β and improve their phagocytic efficiency. MSCs can also inhibit monocyte differentiation into DCs and skew them into a more tolerogenic profile, reducing their expression of HLA-DR, CD1a, CD80, CD83, and IL-12 secretion. The monocytes/macrophages, after having phagocytosed MSCs, promote $Foxp3^+$ Treg formation. Moreover, it has to be stressed that, once infused *in vivo*, and in general after reaching or being influenced by the milieu in inflammatory active conditions, MSCs receive most probably the necessary "licensing" or activating signals to acquire a full immunosuppressive anti-inflammatory profile (17). In particular IDO, IFN-γ, TNF-α, IL-1α, and IL-17, as well as TLR3 activation, are thought to enhance MSC-mediated immunosuppressive activity *in vivo*, which of course would be a positive effect of inflammation. The licensing phenomenon is evidenced by MHC class I and class II expression, increased ICAM-1 and VCAM-1 adhesion molecule expression, as well as IDO, IL-6, IL-8, hepatocyte growth factor (HGF), PGE2, PDL-1 and COX2 expression. The full activation of MSCs, that takes place in presence of both IFN-γ and TNF-α, induces expression of CCR10, CXCR3, CXCL9, and CXCL10 (18–24). All these possible mechanisms have been nicely reviewed recently, and therefore, we refer the reader to these works for greater details as well as summary figures and tables (18, 21, 23, 25, 26).

It should be stressed that MSCs, even in an allogeneic setting, are not themselves APCs because they lack expression of the co-stimulatory molecules CD80 and CD86, and of MHC class II antigens and show low expression of MHC class I molecules. Furthermore they probably quite rapidly disappear *in vivo* (enacting therefore a hit and run mechanism, see below) (24, 27). Thus, a plethora of molecules can participate to MSC mediated immunosuppression *in vivo*.

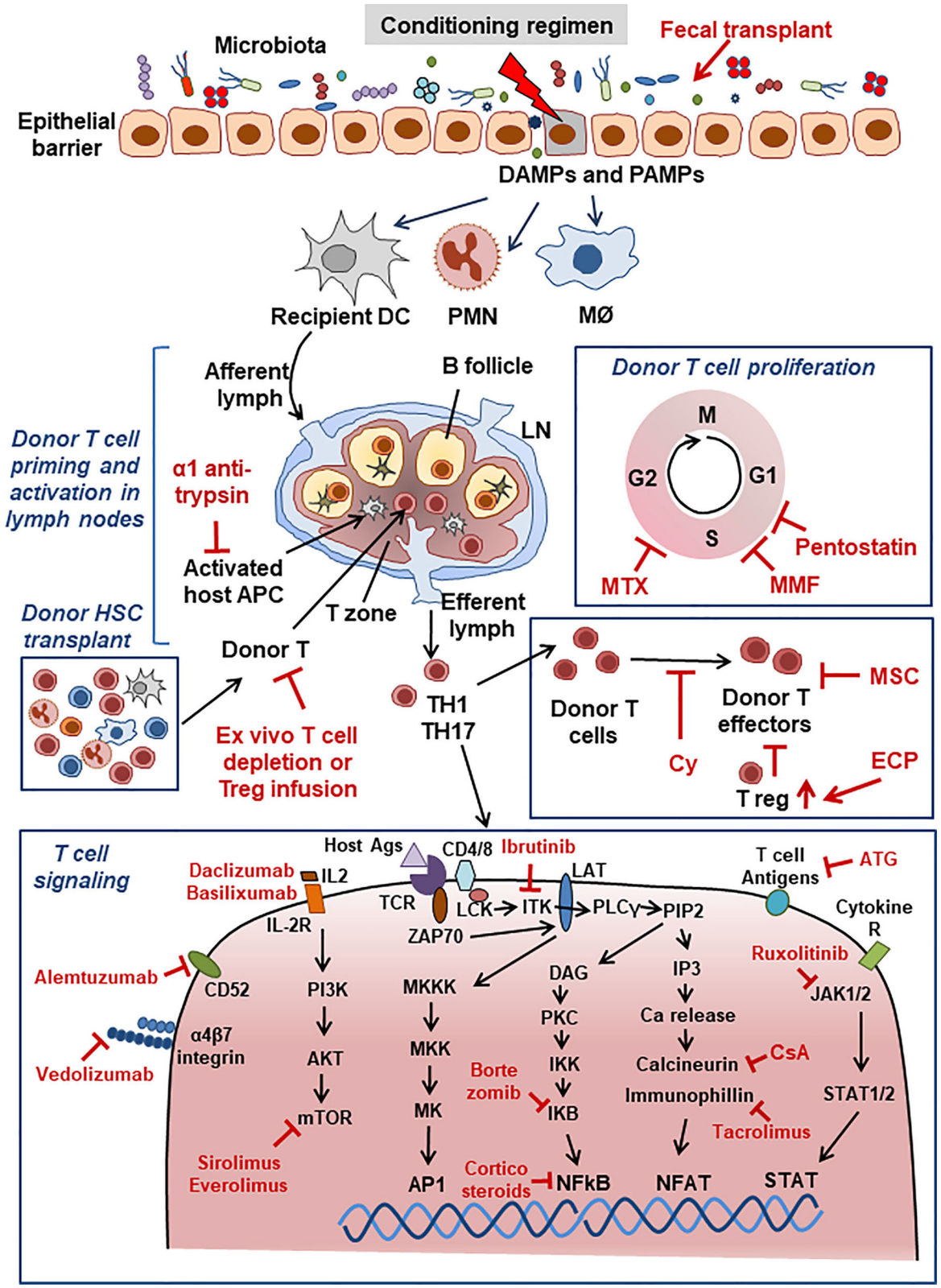

FIGURE 1 | Acute GvHD. Schematic view of major aGvHD mechanisms and points of interaction with drugs used for aGvHD treatment. Drugs are shown in red font. For abbreviations see **Table 1**.

PROPHYLAXIS OF aGVHD WITH MSCs

Prevention of GvHD (both acute and chronic) has been attempted by MSC infusion, generally given together with the HSC graft and in some cases with additional subsequent infusions up to 3 weeks following transplantation. These studies have been nicely reviewed recently by Morata-Tarifa et al., in a work which included the meta-analysis of 16 studies and a total of 654 patients (28). The data overall show a trend for a lower incidence of aGVHD, particularly grade IV and a reduced cGvHD, particularly extended cGvHD (see also below paragraph on cGvHD). No difference on overall survival between groups could however be identified in this prophylactic setting.

TREATMENT OF aGVHD WITH MSCs

MSCs have initially shown much promise in the setting of aGvHD treatment. Indeed, almost 20 years have elapsed since the first description of the treatment of a 9-year old boy, suffering from grade IV SR-aGvHD, using third party, bone marrow (BM) derived MSCs. The patient showed a complete response without any toxicity and a possible immunosuppressive role of MSCs was immediately hypothesized (15).

Following this report, a large number of phase I/II academic clinical trials have been conducted in severe SR (mainly acute) GvHD patients, treated with "similar" cells, derived from several different anatomical sources, expanded *in vitro* in various conditions and given with different schedules. A meta-analysis by Hashmi et al., including 13 non-randomized studies and comprising 336 patients, indicated a complete response rate (CR) of 28% with a 6 months survival rate of responders of 63%. Survival did not differ with respect to age, time of administration or dose of MSCs delivered (29). Similarly, a Cochrane-based extended meta-analysis of the outcome of treatment or prophylaxis with MSCs in acute or chronic GvHD, that included 12 randomized clinical trials and 879 patients, concluded that MSCs are not proven to be an effective therapy (30), despite the fact that a number of single reports suggest a positive effect of MSCs. Nonetheless, due to the considerable heterogeneity of the clinical results, and consistent, measurable, objective response in critical patients in most studies (22, 31), in the absence of clearly effective second- and third-line therapies, the use of cryopreserved unmatched allogeneic MSCs has become medical practice in many European countries. It was also originally recommended as a third line agent by the British Society of Blood and Marrow Transplantation (BSBMT) (22, 32), despite the fact that, more recently, the clinical commission report on GvHD treatment published by the UK National Health Service concluded that there was not enough evidence supporting the use of MSCs in GvHD patients (20). A more recent and complete review includes 14 clinical studies, reaching similar conclusions (20). Perhaps the most negative impact on the clinical arena were the results of the only placebo controlled phase III clinical trial, based on the infusion of BM derived MSCs (Remestemcel-L, produced by Mesoblast, although initially manufactured by Osiris Therapeutics under the name of Prochymal), which failed to meet its primary end-point (durable complete response lasting 28 days or more) either in 149 adults or 14 children (22, 33). However patients with liver involvement who received at least 1 cell infusion had a higher durable complete response and higher overall complete or partial response rate compared to the ones who received placebo administration (33). The patients were treated with 2×10^6 MSCs/kg, twice weekly for 4 consecutive weeks (33). Furthermore, a single arm, prospective study which enrolled 241 children suggests some benefit of MSCs in children. These were treated with a median of 11 MSC infusions (2×10^6/kg) following failure of conventional therapies; those with an early response to MSCs at day +28 appear to have also improved survival (34, 35). Nonetheless the Mesoblasts company's Biological Licence Application for the treatment of pediatric SR- aGvHD with MSCs was rejected on October 2020 by the FDA, who recommended to conduct at least one additional randomized controlled trial in adults and/or children.

As general comments about the clinical trials of MSCs for aGvHD, one can say that the pathogenesis of this disease involves many molecules, cells and pathways, which vary also according to the anatomical site involved as well as time during disease development, as described briefly above. Just to make matters even more complex, the same molecules can in some cases play opposite pro- and anti-inflammatory roles according to the disease status: one canonical example is IL-33 whose administration in animal models of GvHD may result in attenuation or exacerbation of the disease, according to the schedule at which it is administered (8). Furthermore, most clinical trials have been performed on groups of patients who had seen 3 and up to 6 different lines of therapy before receiving MSCs. Thus, the fact that MSCs can interact with multiple molecules, cells and pathways renders the identification of the most appropriate time and administration route of MSCs as yet very difficult. Some other specific factors that have delayed the optimization of MSC use in GvHD are discussed in more details in the following paragraphs.

FACTORS THAT HAVE DELAYED THE OPTIMIZATION OF MESENCHYMAL STROMAL CELLS USE IN GVHD

Heterogeneity of Cell Sources and Products

Cell therapy is naturally wrought in difficulties because of the potential variability of the products, linked to variable number of passages *in vitro*, heterogeneity of anatomical source (nowadays, MSC-like populations can be isolated from multiple tissues, including BM, adipose tissue, cord blood, umbilical cord wall and placenta, dental tissue, decidual endometrial blood as well as others), differences in composition due to their derivation from individual or pooled donors and the different culture conditions used (different media and additives, automated on non-automated methods). Furthermore, there is as yet no validated, standardized potency assay for the final drug product, as specifically underlined by the International Society of Cell Therapy MSC committee (36).

As an alternative to biological variability, a German group has expanded BM derived MSCs from 8 individual donors, pooled the cells at the moment of the first passage and then banked them at passage 2. Interestingly, the allo-suppressive potential of MSCs from individual donors was highly heterogeneous in mixed lymphocytes reactions *in vitro* (MLR), while the activity of the pooled MSC bank was reported to be significantly greater than the mean potency of the 8 individual donors. Indeed, the banked pooled MSCs demonstrated a reproducible and consistent allo-suppressive effect *in vitro* (37). This novel manufacturing protocol (referred to as "MSC Frankfurt am Main" or MSC FFM) was clinically tested in a first cohort of 51 children and 18 adults with refractory aGvHD (38) and, more recently, in a multicenter German trial, 92 patients have been treated, 88 with aGvHD grade III-IV. A median of three doses was administered without apparent toxicity, overall response rates were 82% and 81% at the first and last evaluation. At six months, the estimated overall survival was 64%, while the cumulative incidence of death from underlying disease was 3%, similarly favorable in children versus adults (39).

These data are encouraging that MSCs could be prepared in a more homogeneous and standardized way to offer perhaps more effective treatment. Interestingly the latter clinical use of MSCs was performed on the basis of the national hospital exemption authorization, which suggests also an innovative political strategy to cope with the national and international Good Manufacturing Practice (GMP) regulation which, in some cases, may delay testing of novel cell-based drugs in clinical trials (40–42).

Heterogeneity of the Inflammatory Environment of aGvHD In Vivo and Lack of Predictive and Validated Markers

To complicate the matter further, there is a wide heterogeneity of the inflammatory environment in the recipient at the moment of the infusion, a very imprecise knowledge of the real *in vivo* mechanism of action of the cells in the different phases of the disease and in different tissues involved and a lack of predictive biomarkers. These drawbacks have been the subject of in-depth critical revisions and discussions to which we refer the reader (18, 20, 22, 43, 44).

Several markers of aGvHD activity or tissue damage had been initially identified (IL-2Rα, TNFR1, IL-8, hepatocyte growth factor (45), but these as well additional molecules or effector cells (such as Th1, Th17, CD4, CD8 cells, and IL-6, HLA-G), measured in clinical studies to predict or follow GvHD, have given rather inconsistent results (20). More robust data have been obtained by monitoring the antimicrobial Paneth cell protein regenerating islet derived protein 3A or Reg3A, as well as the IL-33 receptor ST2, leading to the definition of an algorithm called MAP taking into account both markers. This method has been recently validated in an international clinical study and shown to predict GvHD gravity, mortality and response to treatment (46), so that these markers have been developed as a commercial kit (47). Interestingly, both molecules derive from the gastrointestinal tract and have complementary roles in the pathophysiology of aGvHD (9). Paneth cells are retained at the intestinal crypt base and contain antimicrobial peptides, including defensins, lysozyme, phospholipase a2. Reg3A concentrates in the mucus of the internal part of the gut mucosa and physically separates the microbiota from intestinal cells. Activated APCs, damaged stromal, endothelial and epithelial cells, as well as T cells trigger the release of alarmins such as IL-33 that bind to its receptor, ST2 (9). The possible role of Reg3A and ST2 as *in vivo* markers for aGvHD is therefore promising but will need to be confirmed in larger studies.

Difficulty in Tracing MSCs In Vivo and Unclear Pharmacodynamics

In addition to the problems mentioned above, it has proved difficult to detect infused MSCs *in vivo*. There is indeed a lack a solid evidence for their *in vivo* persistence (20). Since intravascular infusion is the most popular route for clinical MSC delivery, persistence of systemically infused MSCs has been mostly studied and these analyses have revealed that a large fraction of infused therapeutic cells are rapidly embolized and destroyed in the microvasculature after triggering an inflammatory reaction (23, 48, 49). Other reports suggest that infused MSCs trigger complement activation and that this results in their *in vivo* removal (48), and overall, very serious concerns have been recently raised on the hemocompatibility of the different MSC products to be injected. In synthesis, the most important potentially negative effects are linked with their highly procoagulant tissue factor (TF) activity, which is able to activate the instant blood mediated inflammatory reaction (IBMIR). There are several suggestions to perform preliminary test *in vitro* and *in vivo* on the products and it may also be useful to include therapeutics such as heparin to prevent the reaction (50). More in general the authors suggest to adopt a global safety strategy particularly for the MSCs derived from "alternative" sources, since the BM MSCs appear sufficiently safe, due to their extended clinical usage (50). As one recent example, careful *in vivo* toxicity study in both intra-arterially injected rats and intravenously treated mice with labeled human placenta derived decidual MSCs have been conducted and did not show any toxicity (51).

The observation that MSCs rapidly "disappear" *in vivo* has recently led to an alternative hypothesis as to their mechanism of action, which suggests that circulating MSCs may die by apoptosis, be engulfed by phagocytic cells and, in doing so, trigger IDO release and immunosuppression, as demonstrated in an experimental model (52). Indeed, further studies showed that patients displaying high *in vitro* cytotoxicity against MSCs, seemed to respond better to MSC therapy, while those with low or absent cytotoxic activity did not improve following MSC infusion, cytotoxicity thus possibly representing an innovative marker (52). Interestingly, this susceptibility to undergo apoptosis in a cytotoxic assay *in vitro* might also be used as a potency assay.

Regarding the rapid disappearance of the MSCs *in vivo*, alternative hypotheses have been proposed, in particular that only "fit" cells survive and reach the affected tissues. The observation that

the therapeutic activity of freshly collected MSCs was clearly superior to frozen and thawed cells in a mouse colitis model supports this explanation (53).

In the same vein, consideration should be given also to the route of administration with respect to the persistence of the "drug" *in vivo*, an element which has certainly been underestimated in most clinical trials conducted so far: in a recent elegant experimental work, mouse colitis was successfully treated by intraperitoneal or subcutaneous, but not intravenous administration (53) and extracellular deposits have been associated with increased persistence of MSCs in both experimental (54) and clinical settings (55).

On the other hand, the therapeutic benefit of MSCs has also been attributed in large part to the so called "hit and run" mechanism mediated by the production of extracellular vesicles (EV) and the secretion of cytokines, chemokines and growth factors that exert their activities during the initial days following injection. MSCs can also exert their immunomodulatory effects on cells *via* direct cell-cell contact, in a paracrine fashion and *via* the release of soluble factors (see above). EV contain a large array of cellular modulatory proteins, messenger RNAs and microRNAs (miRNA). MSC-derived EV can inhibit T, B and NK cells, possibly *via* the shuttling of specific miRNAs into the target cells. The capacity of B cells and monocytes to engulf EV seems particularly strong and, in addition, uptake of EVs by monocytes leads to their differentiation toward an immunosuppressive M2 signature, able to enhance the function of regulatory T cells (21). MSCs can also exert their healing effects by transferring mitochondria to target cells. This appears to be an important mechanism to revert metabolic damage and prevent apoptosis in target cells (24, 56, 57).

Clearly much work is still needed to understand the mechanism of action of MSCs *in vivo* in the context of aGvHD and measure their efficacy.

THE BIOLOGY AND IMMUNOLOGY OF CHRONIC GVHD

Chronic GvHD remains a major cause of non-relapse mortality in patients who survive longer than 2 years after allogeneic HSCT, and negatively influences both quality of life and long-term outcome of this procedure. Indeed, the incidence and severity of cGvHD have increased over the last 10 years, despite the advent of novel treatments and improved clinical practice (58).

cGvHD can involve not only the epithelial target tissues affected in classic aGvHD (gastrointestinal tract, liver, skin, and lungs) but also any other organ system, including oral, esophageal, muscoloskeletal, joint, fascial, ocular, and lympho-hematopoietic systems, hair and nails, and genital tissues (59). Although the highly inflammatory state of cGvHD can manifest itself as polyserositis and polymyositis, chronic disease more often is characterized by fibrosis with little inflammation and involves one or multiple organs in the integumentary, muscoloskeletal, cardiovascular, respiratory, gastrointestinal, reproductive, and both central and peripheral nervous systems (59).

Overall, cGvHD results from the excessive activation of immune effectors molecules and cells that cause inflammation in front of an insufficient presence of negative regulatory elements that help maintain tolerance (60). Schematically, the pathogenesis of cGvHD can be divided in 3 steps: 1) Early inflammation and tissue injury, both sustained by the innate immune system (not differently from aGvHD, see above). Endothelial cells contribute to the migration of donor's T cells into secondary lymphoid organs, such as the spleen and lymph nodes (LNs) and subsequently into GvHD target tissues. DAMPs and PAMPs lead to increased antigen presentation by inflammatory monocytes, plasmacytoid and myeloid dendritic cells and B cells. 2) Adaptive activation of immune system effector cells leads to germinal center (GC) formation in cooperation with donor T follicular helper (Tfh) cells through soluble factors such as IL-21, while IL-17A is directly involved in monocyte-macrophage differentiation, driving the latter towards a pro-fibrotic phenotype (61). An important step is thymic injury, where medullary (mainly responsible for negative selection) and cortical thymic epithelial cells (responsible for positive selection) are targeted by alloreactive T cells, often during the previous acute phase of the disease (62), leading to subsequent loss of central tolerance and the development of donor-derived T cells with specificity for host target antigens. In addition, there is a general loss of regulatory cell populations, including Tregs, Bregs, NKregs, invariant NK/T cells (iNK/T) and regulatory type 1 T cells (Tr1), with consequent loss of peripheral tolerance. Besides an immune response against the host MHC proteins, T cell and B cell activation and antibody generation against neo-antigens can be observed. As an example, while high avidity interaction of B cell receptors (BCR) with auto-antigens in the BM normally results in deletion of auto-reactive B cells, this does not occur in cGvHD patients who develop antibodies to minor histocompatibility antigens. cGvHD is closely associated with abnormally high BAFF levels, an activated B cell phenotype and a high BAFF/B cells ratio. Excessive B-cell activation of the BCR and increased levels of soluble BAFF (sBAFF), an activation and survival factor for B cells, are thought to be the cause an altered BAFF:B cell ratio. Furthermore, the pathogenic B cells are resistant to apoptosis, contributing to increased cell survival and expansion in response to sBAFF of inappropriately selected auto- or alloantigen reactive cells. In any case, the BCR is strongly hyperactivated and so are the associated Syk and Bruton tyrosine kinase (BTK) signaling molecules (63). The reduced number of $CD27^+$ memory and IgD negative post-GC B lymphocytes, together with increased infections, reduces the chances of a normal anti-microbial response. Why cGvHD patients produce allo-reactive B cells and antibodies, but do not show clinically relevant anti-microbial responses, still remains to be understood (60). 3) The propagation of tissue injury by dysregulated donor lymphocytes and aberrant tissue repair mechanisms set the stage for fibroblasts activation, collagen deposition, fibrosis and irreversible end-organ dysfunction, dominated by activated M2 macrophages that produce TGF-β and PDGF-α. Macrophages are major players in the control of inflammation, which has been shown to be an active, well defined process. A fundamental mechanism is the phagocytosis by macrophages of debris and of apoptotic

neutrophils (efferocytosis). M1 type macrophages, exacerbate tissue damage and initiate the inflammatory response. In contrast, M2 type macrophages release 'anti-inflammatory' cytokines (e.g. IL-10, IL-1 receptor antagonist, IL-1RA, and the IL-1 type II decoy receptor), express high levels of scavenging receptors and specific chemokines and contribute significantly to the resolution of inflammation (64). It is worth noting at this point that this dichotomic clear-cut (M1/M2) separation is today considered an over-schematized view of an actually continuous plastic differentiation between functional macrophages attitudes (65). Following skin damage, efferocytosis and TGF-β may skew macrophage function (66). Activated Th2 and Th17 T cells promote fibrosis by secreting IL-13 and IL-17. The healing of damaged tissue must be coordinated together with the end of the inflammatory process. The current view is that the reparative mechanisms initiate while the inflammation induced by the alloreactive stimulus is controlled and this is then followed by the restoration of tolerance (60). B cell activation contributes with auto- (when donor immune response occurs against donor cells) and allo- (when donor cells respond to recipient cells) antibody production, and this further activates macrophages to release TGF-β. Indeed, macrophages express high levels of Fcgamma receptors (FcγRs) and efficiently bind and become activated by antibody coated (opsonized) targets, which in turn can generate very high levels of TGF-β (59, 60, 67). **Figure 2** summarizes the major mechanisms of cGvHD.

THERAPY OF cGvHD

Prophylactic Therapies Available for cGvHD Other Than MSCs

In most protocols for cGvHD prevention, anti-thymocyte globulin (ATG) is given in various combinations with methotrexate (MTX), cyclosporine A (CsA), tacrolimus, mycophenolate mofetil (MMF) or sirolimus before the HSC transplant. More recently post-transplant cyclophosphamide in various combination with most immunosuppressive drugs, has had a revolutionary impact on the prophylaxis of cGvHD (68). These drugs presumably act by depleting mostly T cells (ATG) and inhibiting activation of lymphocytes T and B cells.

Standard Therapy for cGvHD

At present corticosteroids are the standard initial treatment of cGvHD, even though long-term steroid use results in infectious complications and other toxicities. Furthermore steroid resistance can occur. SR-cGvHD is defined as disease progression while on standard 1 mg/kg/day of prednisone for at least 2 weeks, stable disease at 4–8 weeks on 0.5 mg/kg/day or more of prednisone, or those unable to taper to less than 0.5 mg/kg/day.

In spite of the dramatic need for effective treatment and the enormous increase in our understanding of the pathogenetic mechanisms of cGvHD, consolidated second-line therapies are still lacking. As for the acute GvHD, also for the chronic form of GvHD the European Bone Marrow Transplantation GvHD management recommendation expert panel had reached the conclusion that "there are no data available allowing for comparison of the efficacy of different second-line treatment options for cGvHD and no standard second-line treatment exists" (see **Table 3**) (11). Nonetheless, a recent press release has announced that ruxolitinib has reached primary and key secondary endpoints in the phase III REACH3 trial comparing this Jak1/2 inhibitor to best available therapy, suggesting that this drug may become a standard second-line treatment also for cGvHD. Details of the results are therefore awaited. In the last few years, many exploratory clinical trials have been reported with the general perspective of reducing chronic inflammation and auto/allo B and T cell mediated immunity and results have been reported with a number of different drugs: rituximab which depletes B cells and therefore inhibits the allo-antibody response; ibrutinib, which irreversibly inhibits both Bruton tyrosine kinase (BTK) and IL-2 inducible tyrosine kinase (ITK), thus reducing B and T lymphocytes activation and additionally inhibits BAFF, IL-6, IL-4, and TNF-α production (69); fostamatinib, which specifically blocks the BCR associated SYK kinase; imatinib that inhibits TGFβ and PDGFRα signaling, and is therefore potentially active against fibrosis; ruxolitinib, a selective inhibitor of JAK1/2 (see above), low-dose subcutaneous IL-2, that induces an increase in Tregs; proteasome inhibitors (bortezomib), able to inhibit the degradation of IKB (NFKB inhibitor); extracorporeal photopheresis (ECP) with the aim of inducing the apoptosis of lymphocytes and facilitating the differentiation of DCs; others immunosuppressive agents previously reported for the treatment of the acute GvHD (calcineurin inhibitors, mycophenolate mofetil, mTOR inhibitors, pentostatin); finally, KD-025, an oral rho-associated coiled-coil kinase-2 (ROCK2) protein inhibitor is presently under investigation for the treatment of cGvHD (68). The most prevalent steps in the mechanism of action of cGvHD which are targeted by drugs are shown in **Figure 2**.

Given the variety of different organs affected during cGvHD, to a different extent in different patients, it is likely that the response to drugs may vary according to the disease site and that novel second-line therapies may not be as effective in all cases. Although new drugs have not provided a clear single option for all patients with cGvHD, promising complete response rates are starting to be observed. The general aim is of course to induce full tolerance while discontinuing immunosuppressive therapy, although it is presently still very difficult to identify those patients who will be responders and when immunosuppression can be tapered. In general, the standard immunosuppressive drugs are given until clinical amelioration and, later, slow tapering up to final discontinuation of drugs is the consolidated attitude. However, each attempt to taper drugs risks a subsequent return of GvHD and the need to restart immunosuppressive therapy at potentially higher dosages. Thus, currently, combination therapies incorporating novel drug targets and biomarkers are being investigated in clinical trials with the hope of diminishing toxicity while improving response rates (68).

MSCs AS PROPHYLAXIS FOR cGvHD

The report of Marata-Tarifa et al. mentioned above also includes a meta-analysis of 9 studies investigating the prophylactic use of

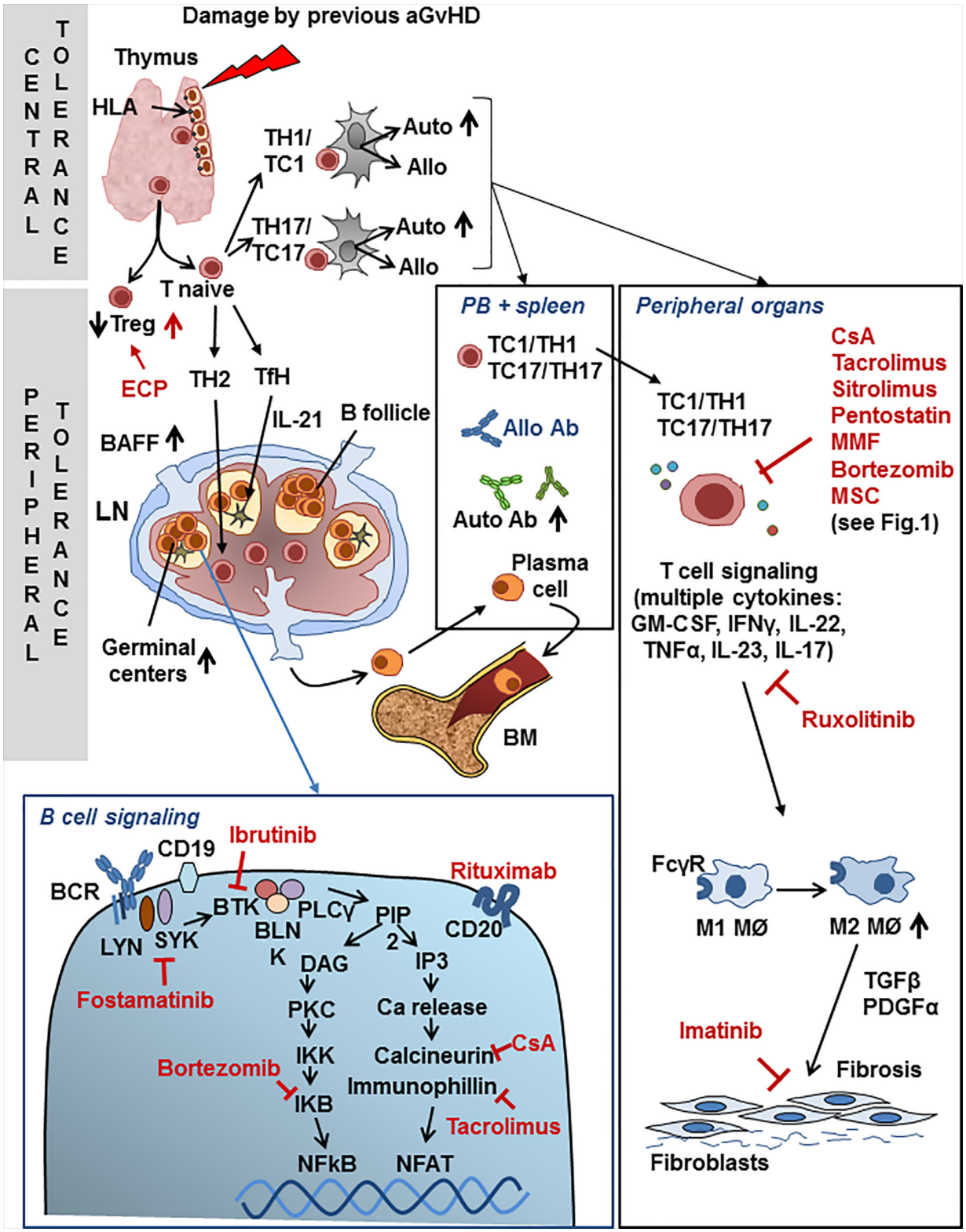

FIGURE 2 | Chronic GvHD. Schematic view of major mechanisms specific to cGvHD and points of interaction with drugs used for second-line cGvHD treatment. Drugs are shown in red font. For abbreviations see **Table 1**.

TABLE 2 | Major drugs used as second line treatment for aGvHD and their mechanisms.

Drug[a]	Major mechanisms identified
Alemtuzumab	Humanised anti CD52 monoclonal antibody, lymphocytolitic
Alpha-1 antitrypsin	Inhibition of dendritic cells activation and induction of Tregs
Basiliximab, daclizumab	Monoclonal antibodies against CD25, IL-2 receptor alpha chain, inhibit T cells proliferation
Extracorporeal photopheresis (ECP)	Apoptosis and phagocytosis by APC leading to inhibition of pro-inflammatory cytokines, increased production of anti-inflammatory cytokines, induction of Tregs
Fecal microbiota transplant	Reconstitution of proper microbiota
Cellular therapy with MSCs	Multiple, in general "anti-inflammatory"/ immunosuppressive
Cellular therapy with T regs	Increase of circulating levels of Tregs
Ruxolitinib[b]	Inhibition of JAK1 and JAK2, major intracellular kinases mediating signalling of a variety of cytokines
Mycophenolate mofetil (MMF)	Blocks de novo pathway of purine synthesis in T lymphocytes, antiproliferative
Methotrexate (MTX)	Inhibition of nucleotides synthesis, block T cells proliferation
Pentostatin	Adenosine deaminase inhibitor, inhibits purine metabolism and blocks proliferation of T lymphocytes
Rabbit anti-thymocytes antibody (ATG)	Antibody against various T antigens, cytolytic for T lymphocytes
Sirolimus	Mammalian Target of Rapamycin (mTOR) inhibitor, blocks T cells activation
Vedolizumab	Monoclonal antibody anti $\alpha4\beta7$ integrins, blocks gut homing of T lymphocytes

[a]*These drugs are used as second line treatments for SR aGvHD, as reviewed by Penack et al. (11).*
[b]*Ruxolitinib has been recently approved by FDA as second line therapy for SR aGVHD.*

TABLE 3 | Major drugs used as second line treatment of cGvHD and their mechanisms.

Drug[a]	Major mechanisms identified
Cyclosporin A, tacrolimus	Calcineurin inhibitors that block downstrem TCR signalling leading to NFAT regulated genes transcription; block T cells activation
Extracorporeal photopheresis (ECP)	Apoptosis and phagocytosis by APC leading to inhibition of pro-inflammatory cytokines, increased production of anti-inflammatory cytokines, induction of Tregs
Imatinib	Inhibits the abl kinase downstream of PDGFR and TGFβ receptors; inhibits fibroblasts proliferation and activation
Ibrutinib	BTK and ITK inhibitor: Inhibits B Cell Receptor (BCR) signalling and B cells activation and myeloid cell activation via inhibition of Bruton Tyrosine Kinase (BTK) expressed in B and myeloid cells
Sirolimus, everolimus	mTOR inhibitors that block T cells activation
Ruxolitinib	Inhibition of JAK1 and JAK2, major intracellular kinases mediating signalling of a variety of cytokines
Pentostatin	Adenosine deaminase inhibitor, inhibits purine metabolism and blocks proliferation of T lymphocytes
Rituximab	Monoclonal anti CD20 antibody, depletes B lymphocytes
Bortezomib	Proteasome inhibitor: Inhibits the proteolytic activity of proteasome in IkB degration, inhibits NFkB activation, inhibits T cells activation by cytokines
Fosfamatinib	Inhibits the BCR signalling via Spleen Tyrosine Kinase (SIK) inhibition in B lymphocytes and their activation
Mycophenolate mofetil (MMF)	Blocks de novo pathway of purine synthesis in T lymphocytes, antiproliferative

[a]*These drugs are used as second line treatments for SR cGvHD, as reviewed in …by Penack et al. (11).*

MSCs for chronic GvHD prevention (28). The studies included 148 MSC treated patients and 236 controls, both adults and children. MSCs from BM or umbilical cord were given in most cases together with HSCs, with a second infusions at day +21 in one case. The analysis shows that MSC infusion was associated with reduced cGvHD incidence (RR = 0.64; 95% CI, 0.47–0.88, $I^2 = 0\%$).

The largest clinical study included in the meta-analysis described above is that of Gao et al. (70). This study directly addressed the issue as to whether prophylactic administration of umbilical cord derived MSCs was safe and could prevent cGvHD incidence in a multicenter, double blind, randomized controlled clinical trial in patients undergoing HLA haplo-identical HSCT (70). The MSC dose was a fixed monthly 3×10^7 dose or saline as control, starting >4 months after transplantation in patients who had not developed aGvHD at day +100. 124 patients were enrolled (MSC N = 62, control n = 62). The average number of MSC infusions was 3.7 (range 2 to 4). cGvHD developed in 17 patients (24%): 14 mild/moderate, while 3 had a severe form. In the control group, cGvHD occurred in 30 patients (48,4%): 22 mild/moderate and 8 severe ($p<0.05$). No acute infusional toxicity nor adverse event were reported. 41 patients in the MSC group and 38 in the control group were still alive at the median follow up of 51 months (range, 24–70). Overall T cells numbers did not change, although Treg counts and the Th1/Th2 ratio increased after MSC infusion ($p<0.05$). Furthermore, the absolute numbers of memory B and NK cells in the MSC treated patient group were increased (70).

These data suggest that MSCs may have activity in cGvHD and larger controlled studies that include carefully studied biomarkers are warranted.

USE OF MSCs FOR cGVHD TREATMENT

The first clinical study of MSCs for the treatment of cGvHD reports the results of 19 patients treated with a median dose of 0.6×10^6/kg of third party BM-derived MSCs, for one (n = 8) up to five doses (n = 1) (71). CR (n = 4) or PR (n = 10) were reported for

a total 14 responders (73.7%). Immunosuppression was tapered after median 697 days in 5/14 survivors. Five patients (26.3%) died after the first MSC infusion. Reasons for death were invasive fungal infection (n = 2), primary malignant disease relapse (n = 2) and bronchiolitis obliterans (n = 1), this latter cause being related to cGVHD. No adverse events induced by MSCs were noted. The 2-year survival rate was 77.7%. MSCs seemed to be more effective for patients with cGVHD of the gastrointestinal tract or with liver and skin involvement. Interestingly, two patients with severe scleroderma had a PR or a minor PR and, clinical symptoms improved in patients with keratoconjunctivitis sicca. Clinical improvement was accompanied by an increased ratio of $CD5^+CD19^+/CD5^-CD19^+$ B and $CD8^+CD28^-/CD8^+CD28^+$ T lymphocytes suggesting effects on the immune system were taking place (71).

In a second study, four sclerodermic cGvHD patients were treated (72). The patients received four to eight infusions of 1 to 2×10^7 third-party donor BM-derived MSCs intra-bone. All four patients showed an improved clinical score and a reduction of symptoms (mainly sclerodermic), with a 14.1-month median follow-up and standard immunosuppressive drugs could be tapered to a significant extent. From the laboratory investigations, it was clear that the proportion of IL-10- and IL-4-producing cells gradually decreased, whereas the proportion of IL-2- and IFNγ-producing cells increased consistently in all patients (72).

In a third report, a total of 23 refractory cGvHD patients received three infusions of third party BM derived MSC at 10^6 cells/kg per infusion at 4 weeks intervals (73). 20/23 patients demonstrated an overall CR or PR at 12 months. Two PR patients died of fungal pneumonia, and three CR/PR patients died of leukemia relapse. Interestingly, best responses were observed in 16/23 with skin symptoms, 13/18 with oral mucosa and 13/15 with liver involvement. In most of the patients who achieved either CR or PR, the best therapeutic effects were observed 3 months after the first MSC infusion. In the responders, the absolute numbers of Bregs increased (74, 75). This was put in relation with *in vitro* data showing a higher survival rate and proliferation of $CD5^+$ B cells and an increased frequency of $CD5^+IL\text{-}10^+$ Bregs after co-culture with MSCs. The data presented finally suggested that MSCs can induce Breg *via* IDO and that this may be an immunosuppressive mechanism in cGvHD (73).

A more recent article reported the results obtained with 11 patients with severe, refractory, cGvHD treated with repeated infusions of allogeneic BM-derived MSCs over 6 to 12 months period, i.e. an extended schedule (76). At least 6 doses of 2×10^6 MSC/kg were administered with additional one to three doses to responders, while patients with progressive disease were taken off the study. Importantly, no patient could be defined as a non-responder until at least 6 months of treatment had been administered. With a median follow-up time of 76 months (range, 34–99) from inclusion, two patients have discontinued all systemic immunosuppression, and two have reduced steroids and calcineurin inhibitor. Organ responses were seen in joints (n = 8), skin (n = 4), eyes (n = 4), mouth (n = 3), gastrointestinal tract, and liver (n = 1 each). MSC treatment was well tolerated without immediate side effects. Overall, 6/11 patients showed long-term PR. 5 patients suffered grade 3 infections and 2 had dysplasia, as severe adverse events. The clinical effects were paralleled by reduced inflammatory cytokine levels and skin histology in the responders. Interestingly, the absolute number of naïve, but not memory T-cells, as well as the absolute numbers of naïve B cells ($CD19^+IgD^+CD38low$) and the $CD31^+$ CD4 subpopulation (early thymus exiters) at the time of treatment were higher in responders compared to non-responders. Finally, CXCL10, CXCL2 and CCL2 levels (mainly produced by inflammatory monocytes) decreased during treatment in long-term responders, while they were upregulated in non-responders, suggesting a continuation or worsening of the inflammation in the latter patients.

These results are important and suggest that the immune status of patients even before MSC infusion may influence treatment response, which may allow to predict which patients will benefit from MSC treatment. Furthermore the study identifies biomarkers that correlate with response during time (76). Clearly a larger study will need to confirm these results.

In good agreement with the pathogenetic mechanisms of cGvHD discussed above, it is interesting to observe that independent biochemical spectrometric studies have identified several molecules as likely candidates to become cGvHD markers, such as CXCL9, CXCL10, ST2, MMP-3, osteopontin, BAFF, the macrophage scavenging receptor CD163 and DKK3 (Dickkopf-related protein 3), as recently reviewed (77).

A conclusion that seems to emerge from the analysis of all clinical trials of MSCs in the treatment or prophylaxis of cGvHD described above is that MSCs may indeed have beneficial clinical activity in this setting. It is probably important to administer repeated doses of cells to obtain a significant effect. Clearly larger controlled trials, investigating specific biomarkers of response are therefore necessary, as well as a careful long-term clinical evaluation of the chronic lesions and of the possibility of tapering the standard immunosuppressive regimens. Finally, it seems a good suggestion to try and "equalise" a standard pool of MSCs obtained from several donors, which could also easily benefit from recently introduced "closed" standardized bioreactors in order to generate more homogeneous MSC preparations for therapeutic purposes (78).

ONE GENERAL COMMENT ON "TOLERANCE" IN THE CONTEXT OF GVHD

An extensive transcriptional profiling and statistical analysis of peripheral blood mononuclear cells from HSCT recipients has shown recently that, upon discontinuation of immunosuppressive therapy, two group of patients could be distinguished (from both acute and chronic GvHD): the ones who did not need any more drug therapy (tolerant) and the ones who still needed it (non-tolerant). The analysis of the identified genes confirmed the immunological nature GvHD and suggested a major role for NK cells, antigen presentation, lymphocyte proliferation and apoptosis (79).

Moreover, in a more recent updated analysis, the same group of researchers re-evaluated the consequence of immune suppression discontinuation on the HSC recipients. The results suggest that, during HSCT with standard immunosuppressive drugs and myeloablative conditioning, patients do not rapidly reach tolerance and tapering immune suppression therapy early does not prevent cancer relapse. Indeed, only 20% of patients were immune suppression-free survivors 5 years after HSCT. Interestingly, when all the variables associated with a successful discontinuation of therapy were analyzed, only the peripheral blood stem cells emerged to be significantly associated with an adverse event, in case of discontinuation of immunosuppression, suggesting, one more time, that the whole BM explants may offer an advantage over apheretic material (perhaps due to the well-known presence of MSCs in this tissue) (80).

These above interesting studies need obviously to be confirmed and extended.

THE ORIGIN AND DIVERSITY OF MESENCHYMAL STROMAL CELLS

The first demonstration of the presence in BM and other hematopoietic tissues of clonogenic progenitor cells capable of differentiating to fibroblasts as well as other mesodermal cells was published in the 1960's by Alexander Friedenstein (81). Haynesworth later set up the culture system to expand BM MSCs in the early 1990' [reviewed in (24)].

The notion of the stemness of MSCs was a concept initially proposed by Friedenstein (81, 82). Indeed, in the first 20 years following their initial characterization, there has been a diffuse emphasis on the stemness and pluripotency of MSCs, with a suggested unlimited differentiation capacity of these cells, indicating that they may be multipotent adult progenitor cells (83, 84). However already in 2005, the International Society of Cell Therapy (ISCT) published a position statement in Cytotherapy (85) clarifying the recommended designation of the cells in Multipotent Mesenchymal Stromal Cells (MSC) rather than Stem Cells, suggesting to abandon the stemness concept. In a subsequent position statement by ISCT, minimal criteria to define MSCs included the ability to differentiate to osteoblasts, adipocytes and chondroblasts, thus underlying their trilineage mesenchymal differentiation capacity, in addition to the property of adhesion to plastic surface and expression of CD73, CD90, CD105, but not CD34, CD45, CD14/CD11b, CD79a/CD19 and HLA-DR (immune lineage negative) (86). The subsequent experimental work, up to the present day, has indeed enormously revised this concept of the stemness of MSCs and, rather, their *in vitro* capacity to differentiate towards adipocytes, osteoblasts and cartilage, led to the redefinition of their property as stromal progenitor cells. Nowadays, the term "stromalness" is accepted as more appropriate than stemness for MSCs from different anatomical sources (44, 87). This definition does not rule out, obviously, that the ex-vivo expansion may hinder the presence of minimal subpopulations of true stem cells among the primary tissue MSCs, that may change their differentiation potential during ex vivo manipulations (see below).

MSCs are hypothesized to be present in tissues in the form of $CD146^+$ pericytes and adventitial cells in the perivascular niche, as well as interstitial fibroblast-like cells in most organs and tissues. It has been shown however that the transcriptome of these cells changes significantly during *in vitro* culture and expansion, so that it is unclear whether the biological properties of MSCs *in vitro* really reflect those of their tissue progenitors (24). This question is of interest but obviously quite difficult to unravel.

"MSCs" are characterized by rather non-specific markers which do not allow to distinguish MSCs from different sources and with different biological properties. Furthermore work performed in the last 10 years has clarified that $CD34^-CD45^-CD146^+$ "MSCs" isolated from different tissues have epigenetically different transcriptomes and differentiation programmes which are consistent with the tissue from which they have been isolated. Thus BM 146^+ cells are capable of giving rise to bone and BM stroma that support hematopoiesis such as adipocytes, but are not myogenic or chondrogenic *in vivo*. Muscle-derived $CD146^+$ cells are not skeletogenic and are myogenic, and cord blood-derived $CD146^+$ cells are not myogenic but are chondro-osteoprogenitors and able to form cartilage *in vivo*. Thus the selective purification of $CD34^-CD45^-CD146^+$ cells from several organs, leads to isolation of committed tissue-specific progenitors, not of multipotent or stem cells (88).

In the same vein, despite the standard expansion protocols generally applied for the production of clinical grade MSCs, differences have emerged upon careful analysis of genome wide methylation status, immunophenotype, transcription pattern and *in vivo* properties. These studies have shown for example that BM-derived MSCs spontaneously form a BM cavity in NOD SCID Gamma (NSG) mice *in vivo* through a vascularized cartilage intermediate that is progressively replaced by hematopoietic tissue or bone, at variance with MSCs derived from all other different sources, mainly adipose tissue, umbilical cord and skin (89). These observations may suggest a latent epigenetic program for endochondral differentiation present in BM-MSC and support the observation that BM-MSCs robustly build a functional human marrow niche whereas other MSCs may not (89).

Moreover, the important theme of MSC heterogeneity has been recently and extensively reviewed, with aspects such as donor variability, isolation procedures ex vivo, as well as differences in anatomical source being discussed. For example, a quite different proliferation potential has been observed *in vitro* in different MSCs with widely different timing for the appearance of senescence markers: senescence markers appeared already at passage 7 in BM derived MSCs, whereas adipose tissue derived, umbilical cord derived or endometrial derived MSCs showed much slower appearance of these markers (8, over 16 and 25–30 passages without sign of senescence, respectively) (44).

Similarly, heterogeneity is emerging in both xenogeneic and humanized mouse models of GvHD using adipose tissue, umbilical cord and BM derived MSCs, suggesting possible higher efficacy of umbilical cord and BM derived MSCs [carefully and extensively reviewed in (90)]. Nonetheless, overall, different MSCs administered in various dosages and schedules have shown only occasionally a statistically significant therapeutic effect. Whether this is due to inadequate models, MSCs sources or dosages is still

unclear (90). The question of the MSC sources and specific biological and therapeutic properties therefore still needs to be better understood in the context of GvHD.

MSCs IN TISSUE REPAIR AND FIBROSIS

It is worth pointing out at this stage that, whereas MSCs, as described above, may act as anti-inflammatory and immunosuppressive elements allowing the restoration of some tolerance, they may also play a role as a differentiation inducing and regenerative therapy, and this may play a role in the context of the chronic tissue damage seen in cGvHD.

This alternative "regenerative" role played by MSCs, largely explored in experimental and clinical settings of chronic lesions, might be reconducted to the secretion and paracrine effect of many molecules, such as vascular Endothelial Growth Factor, Fibroblast Growth Factor, Hepatocyte Growth Factor, Placental Growth Factor, Monocyte Chemoattractant Protein 1 (CCL2), Stromal differentiation factor-1, Ang-1, all critical for vascularization, as well as BCL-2, survivin, Insulin Growth Factor-I, Stanniocalcin-1 (STC-1), TGF-β, GM-CSF, all factors that inhibit cellular apoptosis and restore tissue homeostasis (23). Similar to the immune regulatory role of MSCs, their tissue repair capacity is thought by most authors to be mediated by the release either of EV or by intracellular components of the MSCs rapidly dying *in vivo* (48 h in most experimental animal studies, a hit and run mechanism as described previously).

Nonetheless, the most perplexing observation derives from the effects of MSCs on fibrosis mainly described in chronic inflammation (as for the cGvHD). MSCs have been shown in different contexts to ameliorate fibrosis by reducing the extent of monocyte/macrophage and B lymphocyte infiltration and inhibiting the expression of pro-inflammatory cytokines such as TNFα and IL-1β in liver and pulmonary fibrosis. As already mentioned above, MSCs appear able to reprogram pro-inflammatory macrophages (M1) towards an anti-inflammatory phenotype (M2), resulting in resolution of inflammation [as one example, see (91)]. In other experimental models, BM MSC have shown the ability to reduce liver fibrosis *via* induction of expression of MMPs by macrophages. Interestingly, also MSC-derived exosomes (see below) are able to reduce pulmonary fibrosis. To understand the role and mechanism of MSCs, it is worth noting that fibrosis appears to be the result of complex multiple interactions between molecules able to positively induce collagen deposition, as exemplified by TGFβ (see below), which appears also to be able to directly regulate the equilibrium between MMPs and tissue inhibitors of metalloproteinases (TIMPs). Also Wnt, one of the major signaling pathways involved in collagen deposition, is negatively regulated by Dickkopf protein 1 (Dkk-1), which is also under TGFβ control. In addition, as discussed above, MSCs are certainly able to induce Tregs, which are known to inhibit fibrocyte recruitment and fibrosis. Thus the *in vivo* anti-fibrotic activity of MSCs in liver, pulmonary and renal fibrosis is complex and, even if the local mechanism involved is far from being clarified, it has been very accurately detailed in a recent review article by Rockel et al. (92).

In contrast, MSCs may "contribute" directly to fibrosis. Indeed, the cell type most involved in development of fibrosis is the myofibroblast, which can secrete extracellular matrix proteins such as collagen and fibronectin. Many studies have demonstrated the plasticity of such cells as well as their organ specific developmental origin and localization. The pericytes, as well as fibroblasts and circulating BM derived mesodermic progenitors are believed to give rise to myofibroblasts and thus contribute to pathogenic fibrosis. Therefore, there is an emerging overlapping (from a morphological, phenotypic and *in vitro* differentiating potential point of view) between the cells which appear to be the MSCs precursors (pericytes) and the cells which appear to be the responsible of local fibrosis, even if a complete understanding and definition of such developmental programmes is far from being clarified. Nonetheless, it is tempting to speculate that what we define as *in vitro* expanded MSCs may have common properties and origin with the MSC-like cells which are responsible for tissue fibrosis and organ damage. The overall picture is also complicated by the observed plasticity *in vivo* in several experimental models which lead to the refusal of the concept of myofibroblasts as terminally differentiated cells, but rather as a transitory state in continuous evolution between deposition and regression of fibrosis (93).

Thus, the role of MSCs in the development and resolution of fibrotic conditions in cGvHD needs to be more fully understood to better explore how to manipulate these cells for their best therapeutic effects.

MSCs BEYOND GVHD: FROM THE "UNIVERSAL DRUG FOR ANY DISEASE" TO MORE SOLID CLINICAL PERSPECTIVES

MSCs have been proposed and tested in many different clinical conditions beyond GvHD. Indeed the original misinterpretation on the stemness nature of MSCs and hypothesized multi-lineage differentiation, has unfortunately been abused by some private "direct to consumers" clinics, which have marketed MSCs to treat patients affected by a wide range of diseases and pathological conditions, without a strong rationale or scientific link between these cells and the disease etio-pathogenesis, with little subsequent demonstrated benefit and in some cases even resulting in adverse reactions. This critical point has been already raised by detailed position papers (49, 94–96).

Interestingly, in early 2019, a search of the "clinicaltrial.gov" website, using as keywords "MSC", "mesenchymal cells" "stromal cells", evidenced the registration of over 900 clinical studies globally (44), including more than 10.000 patients treated and ten phase III studies (97). In addition to Prochymal remestemcell, as discussed before, the use of MSCs was approved in Japan following the act on the safety of regenerative medicine (98). In 2018 the European Medicines Agency has approved Alofisel to treat Crohn's disease (99). A search performed in april 2020 identified 12 different proprietary allogeneic and 4 autologous MSC products, utilized in 1094 ongoing clinical trials, 64 being phase II/III and 6 phase III/IV studies (including 47 studies for GvHD) (18). Approval was granted for allogeneic MSC therapies

in Europe, Japan and India, such as Alofisel for the treatment of perianal fistulas in Crohn's patients, based on the results of the Adipose Derived Mesenchymal Stem Cells for Induction of Remission in Perianal Fistulizing Crohn's Disease (ADMIRE-CD) phase III study. Mesoblast's TEMCELL HS was approved in Japan for the treatment of aGvHD in BM transplant recipients. Stempeucel, marketed by Stempeutics, has received limited approval in India for the treatment of critical limb ischemia (associated with Buerger's disease) (100). In another review, Godoy and co-authors report 16 MSC-based commercial products, 6 for bone regeneration, 2 for perianal fistulas, 2 for regeneration of subcutaneous tissue, 1 for wound repair, 1 for cartilage repair, 1 for traumatic osteoarthritis, 1 for GvHD, 1 for acute myocardial infarction, 1 for acute radiation injury (18).

It is reassuring, therefore, that these approvals so far concern pathological conditions which can be reconciled with the mesodermic "stromalness" previously described (conjugated with anti-inflammatory and immune-suppressive activity). Indeed these activities are widely justifiable in diseases such as GvHDs and Crohn's, due to the autoimmune and inflammatory pathogenesis of most connective tissue diseases. In contrast, the very unrealistic and unlikely activities in the direction of the pluripotent stemness are disappearing.

Nonetheless, the increasing understanding of the true heterogeneity of the MSC preparations obtained from different sources and by different methods, may in the future lead to a more precise identification of ideal tissue targeting for MSC products. Whereas the immunosuppressive and anti-inflammatory capacity of different MSC preparations may differ, as described above, this argument has not yet been addressed with respect to their use in different clinical conditions. For example, some clinical uses of MSCs (closing fistulae or wound repair), may require a "fibroblastic" ability, whereas others (treating the sclerotic lesions in cGvHD) will not. Rather, in the latter case, the immunosuppressive and anti-inflammatory activities shown by MSCs may rather induce the de-differentiation of myofibroblasts.

Lastly, but not less important, is the consideration that, in trying to cope with the clinical demand, several "GMP compliant" expansion methods ex vivo are available and have been applied, which may, by themselves, represent a confounding factor, as for the different anatomical source, individual donor variability, isolation procedures, and expansion conditions, as well as final formulation, scaling up, dosages, release tests and routes of administration. Many of these issues have been recently reviewed (44). As one example, crucial aspects of the supposed *in vivo* activity of MSCs, such as the immunosuppression of T and B cells, have been found to differ according to the different GMP compliant expansion protocols used (101, 102). Ideally, disease specific MSCs will soon be identified, in conjunction with optimized culture conditions and manufacturing.

CONCLUSIONS

Despite several decades of *in vitro* and *in vivo* studies on MSCs, their ready availability from different tissues and their multiple functions have led to the conduction of many clinical trials and the approval of several commercial products for different clinical conditions, including GvHD. MSCs have shown some activity in aGvHD and perhaps more convincingly in cGvHD. Nonetheless, a number of hurdles still need to be overcome to make these drugs more effective *in vivo* for GvHD as well as other diseases: we need to better understand the heterogeneity of MSCs due to donor, cell source, subsets, culture conditions, using more extensive and refined methods, which should include more standardized or disease- and function-specific potency assays; we need to identify reliable biomarkers *in vivo*, to predict which patients will be responders, and to more precisely follow the early and late clinical response *in vivo*; we need to investigate and refine best dosing and schedules of administration according to disease type and stage, for examples investigating repeated dosages for GvHD in an early setting.

We believe that MSCs have not yet said their last word and that well conducted studies will bring a more consolidated clinical use of these cells in the future.

AUTHOR CONTRIBUTIONS

Both authors have written, discussed, and structured the manuscript. All authors contributed to the article and approved the submitted version.

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Detection and Monitoring of Regulatory Immune Cells Following Their Adoptive Transfer in Organ Transplantation

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Application of cell-based immunotherapy in organ transplantation to minimize the burden of immunosuppressive medication and promote allograft tolerance has expanded significantly over the past decade. Adoptively transferred regulatory immune cells prolong allograft survival and transplant tolerance in pre-clinical models. Many cell products are currently under investigation in early phase human clinical trials designed to assess feasibility and safety. Despite rapid advances in manufacturing practices, defining the appropriate protocol that will optimize *in vivo* conditions for tolerance induction remains a major challenge and depends heavily on understanding the fate, biodistribution, functional stability and longevity of the cell product after administration. This review focuses on *in vivo* detection and monitoring of various regulatory immune cell types administered for allograft tolerance induction in both pre-clinical animal models and early human clinical trials. We discuss the current status of various non-invasive methods for tracking regulatory cell products in the context of organ transplantation and implications for enhanced understanding of the therapeutic potential of cell-based therapy in the broad context of control of immune-mediated inflammatory disorders.

Keywords: cell therapy, adoptive transfer, cell tracking, regulatory T cell, mesenchymal stromal cell, regulatory myeloid cell, transplantation

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INTRODUCTION

Cell-based therapy using naturally occurring or genetically modified immune cells, having now been successfully translated to the clinic for cancer treatment, is undergoing clinical development to promote tolerance and prolong graft survival after solid organ transplantation. Cell products under active investigation for clinical use in kidney or liver transplantation include donor-antigen alloreactive regulatory T cells (darTreg) and polyclonally expanded Tregs (pTreg), regulatory macrophages (Mreg), regulatory dendritic cells (DCreg), and mesenchymal stromal cells (MSCs). Findings from the recent ONE Study, the largest multi-center consortium to date assessing adoptive cell therapy in kidney transplant patients, have confirmed the safety of infusing various regulatory immune cells, paving the way for further development (1). The main challenge in clinical testing of

regulatory cell therapy, however, is that the *in vivo* fate and localization of the cell product remains largely unknown which leads to major gaps in understanding of tolerance induction mechanisms and hinders cell therapy protocol design. Non-invasive, accurate, and durable techniques to monitor exogenous cell products after infusion in both pre-clinical and clinical human studies are critical in addressing 1) variability in clinical outcomes, 2) potential cell toxicity and adverse side effects of infusion, 3) anatomic localization and 4) duration and magnitude of desired tolerogenic activity (2).

Historically, cellular staining and subsequent flow cytometry has been a reliable approach to detecting adoptively transferred cells (3–5) however, more advanced approaches to longitudinal *in vivo* cell monitoring using whole-body imaging with novel reporter systems, initially developed for cancer immunotherapy, are increasingly being incorporated into both pre-clinical and clinical transplant studies. This review will discuss current techniques used to track and monitor the major regulatory immune cells under clinical investigation for tolerance induction (**Table 1**) and how understanding the in vivo fate of these cell products has helped advance cell-based therapy in organ transplantation.

TRACKING/MONITORING OF POLYCLONAL OR DONOR AG ALLOREACTIVE TREGS IN EXPERIMENTAL ORGAN TRANSPLANTATION

Naturally occurring Tregs are a rare, specialized subset of thymic-derived $CD4^+CD25^+$ T cells characterized by high expression of the transcription factor forkhead box P3 (Foxp3). In addition to these thymic Tregs (tTregs), naïve $Foxp3^-CD4^+$ T cells can differentiate in the periphery to become $Foxp3^+$ cells, that are known as induced Tregs (iTregs) or peripheral Tregs. Distinctions between tTregs and iTregs have been reviewed recently (27). T cell receptors (TCRs) that recognize antigens to which an organism is chronically exposed promote the generation of iTregs (28, 29).

Tregs have been implicated extensively in tolerance induction and maintenance pathways. Their potential to regulate allograft rejection after transplantation is the most extensively evaluated of the regulatory cell types under current investigation. Given their paucity in the peripheral circulation in the healthy steady

TABLE 1 | Methods used to track and monitor regulatory immune cells adoptively transferred for transplant indications.

Species	Cell type	Transplanted allograft	Detection method	Duration of tracking post-infusion (days)	Reference
Rodent					
	Tregs/autologous	Heart	Treg-specific mAb staining	98	(6)
	Tregs/donorAg-reactive	Skin	CSFE dye/GFP	60	(7)
	Tregs/autologous	Pancreatic islet	CSFE/PKH-26 dye	10	(8)
	Tregs/autologous and non-autologous	VCA	Luciferase gene-reporter system	294	(9)
	Tregs/autologous	Heart/Lung	IOPC-NH2/MRI	3	(10)
	Tregs/autologous	Skin	99mTcO4-/SPECT	1	(11)
	Tregs/CAR	Skin	Luciferase gene-reporter system	21	(12)
	Tregs/CAR	Pancreatic islet, skin	Luciferase gene-reporter system	21	(13)
	Mregs/donor-derived	Heart	Donor-discriminatory Mreg mAb staining	28	(14)
	DCregs/autologous	Heart	PKH-26 dye	5	(15)
	DCregs/donorAg-pulsed	Heart	PKH-67 dye	1	(16)
	DCregs/donor-derived	Heart	Donor-discriminatory DCreg mAb staining	7	(17)
	MSCs/autologous	Kidney	PKH-26 dye	1–2	(18)
	MSCs/donor-derived	Heart	PKH-26 dye	21	(19)
NHP					
	Tregs/autologous and non-autologous	–	CSFE/VPD450 dye	21	(20)
	Tregs/autologous	–	CSFE dye	40	(21)
	Tregs/autologous	–	CSFE/VPD450 dye	87	(22)
	Tregs/autologous	–	CSFE dye	100	(23)
Human					
	Tregs/autologous	Kidney	Deuterium labeling	180	(24)
	Mregs/donor-derived	Kidney	^{111}In labeling/SPECT	1	(25)
	DCregs/donor-derived	Liver	Donor-specific MHC mAb staining	7	(26)

CAR, chimeric antigen receptor; NHP, non-human primates; Tregs, regulatory T cells; Mregs, regulatory macrophages; DCregs, regulatory dendritic cells; GFP, green fluorescence protein; VCA, vascularized composite allotransplantation; mAb, monoclonal antibody; donorAg, donor-antigen; CSFE, carboxyfluorescein succinimidyl ester; VPD450, violet proliferation dye 450; IOPC-NH2, superparamagnetic nano-sized iron-oxide particle; MRI, magnetic resonance imaging; 99mTcO4-, technetium-99m pertechnetate; SPECT, single-photon emission computed tomography; ^{111}In, ^{111}Indium tropolonate; MHC, major histocompatibility complex.

state, one salient issue in using Tregs to promote tolerance induction is whether these cells can persist or self-expand in host peripheral blood and tissue to exert a sustained therapeutic effect after administration. Localization and physical distribution of Tregs within allograft tissue, in particular, have been associated with enhanced immunomodulatory function *in vivo*, implicating the importance of cell homing in adoptive Treg cell therapy for transplant tolerance (30–32). As such, methodologies to track the fate of infused Tregs are critical and have been incorporated into both pre-clinical and early clinical studies (**Table 2**).

Early studies investigating *ex vivo*-expanded polyclonal Tregs adoptively transferred to skin- or pancreatic islet-engrafted mice relied on direct labeling of the *ex vivo*-expanded $CD4^+CD25^+Foxp3^+$ cells with intracellular carboxyfluorescein succinimidyl ester (CFSE) dye or on Treg generated from green fluorescent protein (GFP) transgenic donors to study tissue homing and survival of these cells after intravenous administration (6, 7). Flow cytometric analysis detected labeled autologous (6) or darTregs (7) in peripheral blood, spleen, draining lymph nodes (LN) and allograft tissue, up to 60 to 98 days post-infusion demonstrating the persistence and trafficking of adoptively transferred autologous Tregs to secondary lymphoid organs. These studies also determined the cell surface molecules integral to Treg migration. E and P selectin ligands were found to be important in Treg homing to the graft, while chemokine receptors CCR7, CCR2, and CCR5 were required for their migration to secondary lymphoid tissue (8).

TABLE 2 | Observations of adoptively transferred Treg survival and migration in various species.

Species	Cell origin	Transplant allograft	Sites of cell trafficking	Duration of *in vivo* detection post-infusion	Comments	Reference
Rodent						
	Autologous	Heart	Peripheral blood, spleen, mesenteric LN, allograft	98 days	Tregs detected in blood at day 7 and 98, all others at day 98	(6)
	Autologous, donorAg-pulsed	Skin	Spleen, draining and mesenteric LN, allograft	21 days (spleen) 60 days (LNs, allograft)		(7)
	Autologous	Pancreatic islet	Spleen, draining and non-draining LN, allograft	4 days	Tregs migrate first to allograft then to LNs	(8)
	Autologous and non-autologous	VCA	Axillary and inguinal LNs, allograft	4–294 days (non-autologous) 4–14 days (autologous)	Tregs migrate first to LNs (day 4) then to allograft (day 6); Tregs failed to persist after 2 weeks in syngeneic recipients	(9)
	CAR and non-autologous	Skin	Allograft, draining LN	2–21 days (CAR) 2–7 days (non-autologous)	Polyclonal Tregs homed to both HLA- A2-expressing allograft and non-A2 skin while CAR Tregs homed to A2-expressing skin allograft only	(12)
	CAR and non-autologous	Pancreatic islet, skin	Islet and skin allograft, draining LN, spleen	1–21 days (CAR)	FITC-H-$2D^d$ -mAbCAR Tregs show enhanced localization to the islet allograft.	(13)
	Autologous	Heart/Lung	Allograft	24–48 h	Labeled Tregs detected in both heart and lung allograft on MRI	(10)
	Autologous	Skin	Spleen, liver, intestines, heart, tail, thymus, muscle	24 h	Only study to demonstrate uptake of labeled Tregs in non-lymphoid tissues	(11)
NHP						
	Autologous and non-autologous	–	Peripheral blood	21 days (autologous) 6 days (non-autologous)	In non-transplanted model, auto Treg survival higher than MHC-mismatched Treg	(20)
	Autologous	–	Peripheral blood, bone marrow (BM), LNs	16 days (peripheral blood) 37 days (peripheral blood, + rapamycin) 13 days (BM) 6 days (LN)	Rapamycin therapy enhanced *in vivo* persistence of infused Tregs in blood	(21)
	Autologous	–	Peripheral blood, spleen, inguinal LN, mesenteric LN	71 days (peripheral blood) 50 days (lymphoid tissue)		(22)
Human						
	Autologous	Kidney	Peripheral blood	90 days	Infused Tregs peaked at 2–8% of total Tregs in peripheral blood dropping below detection by 3 months	(24)

BM, bone marrow; CAR, chimeric antigen receptor; FITC, fluorescein isothiocyanate; LN, lymph node; mAb, monoclonal antibody; MRI, magnetic resonance imaging; NHP, non-human primates; Tregs, regulatory T cells; VCA, vascularized composite allotransplantation; MHC, major histocompatibility complex.

More recent studies in transplanted rodents have shifted towards non-invasive whole-body *in vivo* cell tracking and imaging of adoptively transferred Tregs. Tregs isolated and expanded *ex vivo* from luciferase transgenic rats were adoptively transferred into major histocompatibility complex (MHC)-mismatched vascularized composite allotransplant rat recipients and visually tracked using bioluminescence imaging (BLI) longitudinally. In contrast to the limited detection of labeled Tregs in cross-sectional samples of earlier studies, real-time *in vivo* imaging allowed Cheng et. al to identify migratory patterns of infused Tregs first to draining LNs and then to grafted tissue over a prolonged period of 42 weeks (9). A novel method using superparamagnetic nano-sized iron-oxide particle, IOPC-NH2, to label transferred T cells and magnetic resonance imaging (MRI) was developed by Liu et. al and successfully demonstrated localized infiltration of IOPC-NH2-labeled autologous T cells into allograft tissue within 24 h in a rat heart-lung transplant model (10). Radiolabeling of *ex vivo*-expanded Tregs with technetium-99m pertechnetate ($99mTcO4^-$) was performed both directly and indirectly via retroviral transduction with a construct expressing the hNIS glycoprotein ion channel gene (11). These studies localized adoptively transferred labeled Tregs in spleen, liver, lungs, and the allograft after administration and skin transplantation in mice with the approach allowing longitudinal detection of transferred Tregs *in vivo* over time.

Tregs have been well-characterized in nonhuman primates (NHP) (20, 21, 33–35).The *in vivo* persistence and homing of adoptively transferred pTregs to secondary lymphoid organs demonstrated in rodent models have been corroborated by several NHP studies evaluating the survival, migration, and function of exogenous Tregs after administration. *In vivo* detection of *ex vivo*-expanded autologous or allogeneic Tregs infused systemically into non-transplanted cynomolgus or rhesus macaques was accomplished through direct CSFE or violet proliferation dye 450 (VPD450)-labeling and subsequent flow cytometric analysis of the labeled cells in peripheral blood, mesenteric and inguinal LNs, and spleen at various timepoints post-infusion (20, 21). Pharmacokinetic analysis of CSFE-labeled autologous Tregs detected an initial rapid phase of elimination from the peripheral blood between day 0 and day 3 post-infusion after which these transferred cells persisted at low levels in the blood up to 3 weeks (21). Persistence of these cells in secondary lymphoid organs was not as durable. Labeled autologous Tregs were detected in inguinal and mesenteric LNs harvested at days 1 to 2 post-infusion, but lost to detection by day 6 (21). Administration of concurrent immunosuppression (IS) therapy substantially increased survival of transferred autologous Tregs in peripheral blood and LNs. Labeled autologous Tregs persisted longer in peripheral blood and LNs in monkeys given rapamycin alone or with concurrent IL-2 and were detected in these compartments in greater numbers when compared to non-immunosuppressed conditions 50 to 84 days post-infusion (22, 23). These studies highlight the wide variability in survival of infused Tregs under numerous different conditions, including the presence and type of IS, as well as cell production techniques, particularly cryopreservation.

Studies in splenectomized, kidney-transplanted NHP treated early post-transplant with cyclophosphamide and then infused with *ex vivo*-expanded autologous Tregs support their efficacy in prolonging allograft survival and function. In addition, multiple Treg infusions in NHP pretreated with anti-thymocyte globulin (ATG) and post-operative rapamycin prolonged renal allograft survival (36). In contrast, Ezzelarab et. al failed to demonstrate enhanced heart allograft survival after adoptive transfer of autologous pTreg to ATG-treated heart allograft recipients, possibly reflecting, in part, reduced survival capacity of the pTreg product *in vivo* (37). Overall, *in vivo* detection of the transferred regulatory cells in the majority of these studies was limited, as they focused primarily on allograft survival outcomes.

TRACKING/MONITORING OF CAR TREGS IN EXPERIMENTAL ORGAN TRANSPLANTATION

While the majority of pre-clinical studies investigating the efficacy of Treg cell therapy have focused on polyclonal autologous and non-autologous Tregs, several groups have evaluated the potential of using chimeric antigen receptor (CAR) modified Tregs as a more potent and targeted cellular method of tolerance induction after transplantation. Investigators have demonstrated that adoptive transfer of genetically engineered donor HLA-specific CAR Tregs successfully prevents the rejection of transplanted allogeneic cells and graft tissue in humanized mouse models (12, 13, 38, 39). *In vivo* BLI utilizing the luciferase-GFP reporter system showed rapid and specific trafficking of adoptively transferred HLA-A2-specific CAR Tregs (12) or mAb-directed CAR Tregs targeted to $H\text{-}2D^d$ (13) to transplanted skin or pancreatic islet allografts respectively, persisting up to 21 days after transfer. Additionally, both studies demonstrated that, compared to their polyclonal counterparts, CAR Tregs achieved a more targeted localization and longer persistence in allograft tissue.

TRACKING/MONITORING OF TREGS IN HUMAN ORGAN TRANSPLANTATION

In humans, early phase clinical testing of adoptively transferred autologous Tregs in transplant patients is well underway. Deuterium-labeled autologous pTregs were infused and tracked in the peripheral blood of 3 kidney transplant recipients on maintenance IS regimen of tacrolimus, mycophenolate mofetil ± prednisone with subclinical inflammation on 6-month surveillance biopsy (24). $CD4^+CD127^{lo}CD25^+$ Tregs were purified via FACS from peripheral blood and single cell suspensions from kidney biopsies. DNA was then extracted from all purified cells and subjected to gas chromatography and mass spectrometry (GC-MS) analysis to measure deuterium enrichment in circulating Tregs. Infused Tregs peaked within 7 days of infusion and were detected by

deuterium signals at 30 days. Deuterium-labeled cells fell to the limit of detection within 3 months of infusion (24). In this study, infused Tregs demonstrated patterns of persistence and stability comparable to those observed in prior corresponding immunosuppressed NHP models and non-immunosuppressed type 1 diabetes mellitus patients receiving autologous pTreg therapy (40). Anatomic biodistribution of clinical grade Tregs after therapeutic infusion was ascertained in a non-transplant autoimmune hepatitis (AIH) clinical pilot study by radiolabeling of good manufacturing practice (GMP)-grade Tregs with ^{111}Indium tropolonate (^{111}In) (41). Serial gamma camera and SPECT-CT imaging taken at serial timepoints after infusion tracked the presence of transferred indium-labeled Tregs. 22% to 44% of infused Tregs migrated to the liver, spleen and bone marrow of 4 AIH patients for up to 72 h without any off-target organ localization (41). This provides an additional effective cell tracking method that can be implemented in current and future transplant Treg therapy human clinical studies to assess spatial distribution of infused cell therapy non-invasively in real-time.

TRACKING/MONITORING OF REGULATORY MYELOID CELLS IN EXPERIMENTAL ORGAN TRANSPLANTATION

The myeloid cell lineage includes multiple regulatory immune cell subsets under active investigation to induce and maintain transplant tolerance in solid organ transplantation, including DCregs, Mregs, and myeloid-derived suppressor cells (MDSCs) (42) (**Table 3**).

MDSCs comprise a heterogeneous population of immature myeloid progenitor cells that have been associated with modulation of T cell differentiation. There is evidence from pre-clinical rodent models that MDSCs may play a role in promotion of transplant tolerance by inducing Treg and inhibiting alloreactive T cell proliferation in an inducible nitric oxide synthase (iNOS)-dependent manner (43, 44) however, adoptive transfer of ex vivo-generated MSDCs has not been found in pre-clinical animal studies to improve allograft survival (45) and, as such, has not reached clinical testing in humans to date (46). Tracking of infused MDSCs has thus far been restricted to mouse cancer models with one study using a ^{64}Cu-labeled CD11b-specific mAb and PET scanning (47).

Mregs, characterized by a CD14$^-$CD63$^+$HLA-DR$^+$ phenotype and IL-10 production, have been demonstrated to suppress T cell proliferation *in vitro* (48). In a heterotopic heart transplant mouse model, administration of donor-specific Mregs significantly prolonged allograft survival in an iNOS-dependent manner (14). Mregs were tracked *in vivo* using donor-discriminatory Mreg staining and flow cytometry analysis of cells from recipient blood, spleen, liver, LN, BM, and lung suspensions at serial timepoints post-infusion. 24 h after administration, Mregs were readily detected in the blood, spleen, liver, and lung but not in LN or BM. Persistence of infused Mregs decreased in all tissue compartments thereafter up to 2 weeks, after which Mregs were no longer detectable (14). Notably, cross-dressing of recipient antigen-presenting cells (APCs) with donor-specific Ag was not observed in this study.

DCregs are another myeloid-derived immune cell subset whose tolerogenic properties have been well-characterized (49) and have thus, garnered significant attention for clinical testing and use in transplant tolerance induction therapy (50). Extensive pre-clinical testing in organ- and skin transplanted mouse models, has demonstrated that the adoptive transfer of *ex vivo*-generated autologous or donor-derived DCreg prolongs allograft survival and promotes donor Ag- specific tolerance. These effects have been achieved either in the absence of, or in combination with, short-term IS (51–57). Two reports have suggested that

TABLE 3 | Observations of adoptively transferred regulatory myeloid cell survival and migration in various species.

Species	Cell type	Cell origin	Transplanted allograft	Sites of cell traf-ficking	Duration of *in vivo* detection post-infusion	Comments	Reference
Rodent							
	Mregs	Donor-derived	Heart	Peripheral blood, spleen, LN, BM, liver, lung	14 days	24-h post-infusion, infused Mregs detected most in lung/liver, but dissipate thereafter	(14)
	DCregs	Autologous	Heart	Spleen	5 days		(15)
	DCregs	donorAg-pulsed	Heart	Spleen	24 h		(16)
	DCregs	Donor-derived	Heart	Spleen	24 h		(17)
Human							
	Mregs	Donor-derived	Kidney	Lung, liver, spleen, BM	30 h	Majority of labeled infused Mregs detected in lungs, then dissipate to liver and spleen after 2.5-h post-infusion	(25)
	DCregs	Donor-derived	Liver	Donor-specific MHC mAb staining	1 h (intact) 7 days (donorAg)	Intact infused DCreg were not detected after 1-h post-infusion, however donor-specific Ag detected on recipient DC up to 7 days	(26)

BM, bone marrow; DCregs, regulatory dendritic cells; Mregs, regulatory macrophages; mAb, monoclonal antibody.

donor-derived DCreg can prime the alloimmune response (58, 59). In a heterotopic cardiac transplantation rat model, infused autologous DCregs were labeled with PKH-26 red fluorescent cell linker which allowed their detection in spleens of recipient rats using immunofluorescence imaging of histological sections 5 days after administration (15). The use of additional fluorochromes allowed elucidation of interferon-gamma production induction as a potential mechanism of immunoregulation. The lipophilic membrane dye PKH was also used to label rapamycin-treated autologous DCregs pulsed with alloAg that were also administered to heart transplanted mice (16). DCreg homing to spleen was unaffected by rapamycin treatment, but conferred the capacity to suppress alloAg-specific T cell proliferation. Donor discriminatory MHC staining and flow cytometry analysis have also been utilized to detect *in vivo* survival of infused donor-derived DCregs in heart-transplanted mice, which has been shown to be short-lived likely due to killing/removal by host natural killer (NK) cells (60). Thus, the therapeutic effect of pre-transplant infusion of donor-derived DCreg does not appear to depend on the in vivo persistence of intact donor DCreg which offers a potential advantage over other cell therapy approaches for which immunosuppressive ability that may depend on in vivo persistence of the transferred regulatory cells.

TRACKING/MONITORING OF REGULATORY MYELOID CELLS IN HUMAN ORGAN TRANSPLANTATION

In a human study published in 2011, two kidney transplant recipients were infused with donor-derived Mreg pre-operatively and shown to successfully wean to low-dose tacrolimus monotherapy within 24 weeks of transplantation, with no evidence of adverse effect or rejection (25). A small proportion (12%) of adoptively transferred Mregs were radiolabeled using ^{111}In prior to infusion allowing for *in vivo* Mreg tracking in real-time using SPECT-CT scanning. Scintigrams reconstructed from SPECT imaging demonstrated initial trapping of labeled Mregs in the pulmonary vasculature, but after 2.5 h re-distributed to the peripheral blood, liver, and spleen. 24 h after infusion, Mregs were no longer detectable in the lungs or peripheral blood and were seen to accumulate in lymphoid and non-lymphoid organs (25). Pre-transplant administration of Mreg therapy in two enrolled kidney transplant recipients was most recently assessed for safety and feasibility as part of the multi-center ONE study, however efficacy and *in vivo* cell tracking/distribution were not evaluated (1, 61).

Donor discriminatory HLA staining is being used to track donor-derived DCregs infused 7 days before transplant into prospective living donor liver transplant recipients. Detection of the donor DCreg and their products is enhanced by image-based flow cytometry methods that can directly visualize the expression of MHC Ags and other gene products of donor or recipient origin by APCs in the circulation and host lymph nodes (26).

TRACKING/MONITORING OF MSCS IN EXPERIMENTAL ORGAN TRANSPLANTATION

MSCs are naturally occurring, bone marrow-derived precursor cells, unique in their activation and migration to inflammatory sites, including allograft tissue, where they can exert their immunoregulatory effects, including upregulation of Treg differentiation in the inflammatory microenvironment (48). Administration of *ex vivo*-expanded MSCs has now consistently proven to be effective in prolonging allograft survival in murine models of solid organ transplantation (62). For *in vivo* tracking, cell labeling with PKH -26 red fluorescence cell linker has been used in murine models infused with autologous or donor-derived MSCs 7 days before kidney or semi-allogeneic heart transplantation (18, 19). In kidney allografted mice, adoptively transferred autologous MSCs infused 1 day prior to kidney transplantation preferentially migrated to the spleen, correlated with better graft survival, whereas post-transplant administration of MSCs was associated with infiltration of the allograft and subsequent C3 complement deposition without any therapeutic effect on allograft function (18). In cardiac allografted mice, PKH-26+ donor-derived MSCs infused prior to transplantation localized to liver, lung, primary and secondary lymphoid organs after infusion with none detected in peripheral blood. Survival in lymphoid tissue and lung was short-lived as PKH-26+ MSCs were not detected in these compartments at day 7 and 21 timepoints, while transferred MSCs were still detected in liver at day 7 post-infusion.

TRACKING/MONITORING OF MSCS IN HUMAN ORGAN TRANSPLANTATION

Multiple human studies investigating the safety, feasibility, and efficacy of adoptively transferred MSCs in solid organ transplantation are currently ongoing (63–65). One large randomized, controlled trial using MSC-based induction therapy in living donor kidney transplantation has already demonstrated reduced incidences of acute rejection, lower rates of infection, and improved 1-year graft function (63). Cell tracking/localization experiments in published human studies are lacking, however the importance of tissue localization following MSC administration is bound to prompt current or future human studies to incorporate non-invasive in vivo detection methods of this infused regulatory cell product.

CONCLUSIONS

Cell-based therapies are increasingly being considered and investigated for minimization of IS and induction/maintenance of tolerance in solid organ transplantation. As such, gaps in understanding of the *in vivo* fate of adoptively transferred

regulatory immune cells after administration need to be filled in order to advance translation of these treatments to the clinic. Current direct cell labeling and flow cytometric analyses of target cells using intracellular dyes or surface marker tags have been efficacious in determining persistence of transferred cells in pre-clinical animal models; however, they lack anatomic information and are cumbersome to apply routinely to human studies due to the need for frequent blood draws and/or tissue biopsies. Cell radiolabeling in conjugation with imaging modalities such as SPECT or MRI has proven to be a more effective strategy of longitudinal *in vivo* cell monitoring in humans given its non-invasive approach, but commonly used radionuclides are often severely limited by their short half-lives. Advanced multi-modal approaches utilizing a dual reporter gene/radiolabeling system and whole-body imaging would provide the highest resolution and sensitivity of monitoring infused cell therapy in the most comprehensive and non-invasive way.

As current early phase human studies investigating various regulatory immune cell products for transplant tolerance advance to higher stages of clinical testing, incorporating some method of *in vivo* monitoring of the infused regulatory cell products without detriment to their function/survival will become imperative to ensure patient safety and maximize therapeutic potential.

AUTHOR CONTRIBUTIONS

Both authors contributed to the content design, literature searches, writing of the manuscript, and manuscript review. All authors contributed to the article and approved the submitted version.

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REVIEW
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Current Preventions and Treatments of aGVHD: From Pharmacological Prophylaxis to Innovative Therapies

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Graft versus host disease (GVHD) is one of the main causes of mortality and the reason for up to 50% of morbidity after hematopoietic stem cell transplantations (HSCT) which is the treatment of choice for many blood malignancies. Thanks to years of research and exploration, we have acquired a profound understanding of the pathophysiology and immunopathology of these disorders. This led to the proposition and development of many therapeutic approaches during the last decades, some of them with very promising results. In this review, we have focused on the recent GVHD treatments from classical chemical and pharmacological prophylaxis to more innovative treatments including gene therapy and cell therapy, most commonly based on the application of a variety of immunomodulatory cells. Furthermore, we have discussed the advantages and potentials of cell-free therapy as a newly emerging approach to treat GVHD. Among them, we have particularly focused on the implication of the TNFα-TNFR2 axis as a new immune checkpoint signaling pathway controlling different aspects of many immunoregulatory cells.

Keywords: hematopoietic stem cell transplantation, graft versus host disease, T cells, immunoregulation, tolerance induction, cell therapy, TNFα-TNFR2 signaling pathway

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INTRODUCTION

Bone marrow transplantation (BMT), also called hematopoietic stem cell transplantation (HSCT), is a process of infusing stem cells taken from healthy donors into recipient patients. Though initially developed to treat damage caused by exposure to high doses of radiation, today allogeneic HSCT is the treatment of choice for many blood malignancies such as acute leukemias, myelodysplastic syndrome and lymphomas (1), and inherited or acquired non-malignant blood disorders, such as sickle-cell anemia and aplastic anemia (2, 3).

Abbreviations: ATG, anti-thymocyte globulin; BM, bone marrow; CD, cluster of differentiation; EVs, extracellular vesicles; GVHD, graft versus host disease; GVL, graft versus leukemia; GVT, graft versus tumor; HSCs, hematopoietic stem cells; HSCT, hematopoietic stem cell transplantation; IL, interleukin; ILC, innate lymphoid cells; MSCs, mesenchymal stromal cells; NK, natural killer cells; PB, peripheral blood; TBI, total body irradiation; Tc, cytotoxic T cells; TCR, T cell receptor; Teffs, effector T cells; Th, T helper cells; TNFR2, tumor necrosis factor receptor 2; TNFα, tumor nerosis factor alpha; Treg, regulatory T cells; UCB, umbilical cord blood.

In allogeneic HSCT, patients first receive a conditioning regimen consisting of combination chemotherapy sometimes associated with radiotherapy and T-cell-depleting antibodies. Patient conditioning is followed by the infusion of donor HSCs which could be harvested from the bone marrow (BM) or, more commonly nowadays, from the peripheral blood (PB) of donors that have been treated with granulocyte colony-stimulating factor (G-CSF) to induce the release of immature hematopoietic progenitors into the circulation. BM cells and G-CSF-mobilized peripheral blood stem cells (PBSCs) are both enriched in hematopoietic progenitors; however, they also contain mature $CD4^+$ and $CD8^+$ T cells. In general, donor T cells present in the graft are essential for three main purposes: 1) They are involved in hematopoietic engraftment (4). 2) Reconstitution of T cells immunity (particularly in adults with reduced thymic function, i.e. the majority of transplanted patients, as recipients' age has significantly increased over the last 2 decades) (5). 3) Mediating a potent beneficial antitumor effect, known as graft versus leukemia/tumor effect (GVL/GVT) (6).

Unlike solid organ transplantation, the main reason to apply HSCT is not only to replace a non-functioning tissue, but to benefit from a strong GVL/GVT effect. About 60 years ago, Barnes and Loutit suggested that BM transplantation was associated with an anti-tumor effect that could not be explained by pre-transplantation chemotherapy or irradiation (7). Furthermore, Butturini showed the loss of anti-tumor effect after T cell depletion (8). The first precise work focused on GVL effect was conducted by Horowitz et al., on a sample of 2,254 patients who received BM graft. Horowitz demonstrated that the relapse risk of leukemia was correlated with the occurrence of GVHD, mostly in its chronic presentation; i.e. those patients developing chronic GVHD had a lower risk of relapse as compared with patients developing only acute GVHD or no GVHD at all. On the other hand, the highest risk of leukemia relapse was observed among recipients of T cell-depleted grafts or in case of a syngeneic donor (6). In parallel, the concept that allogeneic cells have an anti-leukemia effect independent of GVHD is supported by studies on mice, where T cells with GVL but not GVHD activity were identified (9). This supports the independency of GVHD from the GVL effect at least in mouse models. Today we clearly know that these effects result from the recognition of residual malignant host tumor cells and other non-malignant residual cells by alloreactive donor T cells within the graft. In addition, NK cells have been also shown to have anti-tumoral activities (10). Other studies gave rise to the hypothesis that NK cells attack targets that do not express "self" MHC class I molecules (11). Interestingly, due to the presence of killer cell immunoglobulin-like receptors (KIRs), MHC class I receptors, NK cells can distinguish between normal and tumoral cells and kill those that do not have MHC class I molecules specific for their KIRs (12).

Despite the beneficial effects, several serious complications might occur after HSCT. One of the principle causes of post-HSCT mortality is GVHD, which is also a major cause of morbidity in up to 50% of transplanted recipients (13).

Around 50 years ago, GVHD was initially reported by Barnes, Loutit, and Micklem as a "secondary disease of radiation chimera" and was classically defined by Billingham as a syndrome in which donor immunocompetent cells recognize and attack host tissues in immuno-compromised allogeneic recipients (14, 15). Billingham formulated three conditions for the development of GVHD:

1. The graft must contain immunologically competent cells. Mature T cells are the principle immunocompetent cells of the graft that are responsible for development of GVHD. Moreover, the severity of GVHD is directly correlated with the number of transfused T cells (16).
2. The recipient must express tissue antigens that are not present in the transplant donor. The incompatibility between donor's and recipient's tissues, in particular MHCs (Major Histocompatibility Complex), known in human as HLA (Human Leukocyte Antigen), is directly correlated with the incidence of GVHD (17). Today, thanks to a better understanding of the exact immunological bases of GVHD, we are sure that not only differences of MHCs, but also the diversity of minor histocompatibility antigens could cause this disease. In full HLA-matched allogeneic HSCT, minor H antigens disparities between donor and recipient are associated with severe GVHD (18, 19).
3. The patient must be incapable of rejecting the graft. Since the presence of alloreactive recipient T cells would cause the rejection of the allograft, recipients must primarily undergo immunosuppressive treatments.

In 2006, these old criteria have been revised with the addition of a fourth and essential condition: donor lymphocytes must be able to migrate and home to host target tissue of GVHD. T cell have the necessary combination of homing and chemokine receptors to interact with the endothelium at the target tissues (20).

As mentioned earlier, it is now clear that the main immunologically competent cells in the triggering of acute GVHD are donor T cells of the blood or bone marrow transplants (21). Generally, patients whose immune systems are suppressed and receive white blood cells from another individual are at high risk of developing the disease. However, GVHD can seldom develop in various clinical settings other than HSCT, such as solid organ transplantation when T cells within the donor's tissues are not eliminated, or after transfusion of blood products (post-transfusion GVHD) (22–24).

In humans, GVHD is either acute (aGVHD), which classically occurs within 100 days of transplant, but can also develop later following reduced-intensity conditioning (RIC) regimens ("late-onset acute GVHD") or chronic (cGVHD), which typically develops 100 days after transplantation (25–27). The mechanisms involved in these two manifestations are different; aGVHD demonstrates an exacerbated inflammatory mechanism, whereas cGVHD displays autoimmune features.

The development of GVHD and its severity in transplanted recipients depend on several factors like the donor/recipient HLA-matching, recipient's age, genetic polymorphisms, toxicity of the conditioning regimen, stem cell source (bone marrow versus peripheral blood), donor/recipient sex pairs (higher risk for female donor into male recipient) and prophylaxis approach of

GVHD (28). Classically, corticosteroids at the dose of 2 mg/kg/day are the first line treatment of established grade II or higher aGVHD, but patients with steroid-refractory aGVHD have a dismal outcome with long term mortality rates that historically reached 90% (29). In this review, we discuss in detail the current strategies of prophylaxis and treatments of GVHD. We have categorized these treatments into classical ones based mostly on pharmacological prophylaxis and innovative therapies such as gene, cell and immune therapy of aGVHD.

Classical Pharmacological Prophylaxis

Despite our profound understanding of aGVHD at the molecular level, the limited successes of established immune therapies for prevention and treatment of GVHD remain unsatisfactory. This might be in turn due to the observed controversial effects of the majority of molecules involved in the pathophysiology of this disease, thus complicating the establishment of the best mechanism of prevention. The ideal clinical achievement in HSCT would be to extenuate harmful effects of donor T cells while preserving and accentuating GVL/GVT effect, a scenario that has not been completely yielded yet. Since the main cause of GVHD is the presence of donor T cells in the graft, most prophylaxes are focused on either inhibition or depletion of these T lymphocytes or induction of tolerance.

Inhibition of Alloreactive T Cells

In 1980s the introduction of two new immunosuppressive agents, Cyclosporine A and Tacrolimus, which prevent T cell activation *via* inhibiting calcineurin, significantly improved allograft survival rate. To work, they fix themselves on calcineurin-calmodulin-$Ca2^{+}$ complex and inhibit the phosphatase activity of calcineurin, which in turn stops the translocation of nuclear factor of activated T cell (NFAT) and NF-κB into nucleus (**Figure 1**) (30–32), therefore, hamper the transcription/expression of IL-2 and IL-2 receptor (IL-2R or CD25).

The standard prophylaxis of GVHD is the combination of a calcineurin inhibitor with methotrexate, a drug that interferes with alloreactive T cells division (33). In the setting of unrelated donor transplantation, the addition of anti-thymocyte globulin (ATG) can reduce the incidence of both acute and chronic GVHD, without any significant increase in relapse risk (34). In the late 90's, the advent of RIC regimens came with new "methotrexate-free" GVH prophylaxis protocols (35), such as the combination of cyclosporine and ATG (36), that can also be associated with mycophenolate mofetil, mainly in case of unrelated donor transplantation (37).

Sirolimus (rapamycin) is a molecule that forms a complex with mammalian target of rapamycin (mTOR), and therefore debars the PI3K-AKT-mTOR pathway and also that of NF-κB with the concomitant reduction of DNA transcription/translation, cell cycle progression and ultimately T cell suppression (**Figure 1**) (38). Rapamycin is highly used in solid organ transplantation (39, 40) and in autoimmune diseases like type 1 diabetes, which demonstrates that rapamycin not only depletes effector T cells but also enhances the expansion of regulatory T cells (Tregs) that can further suppress effector

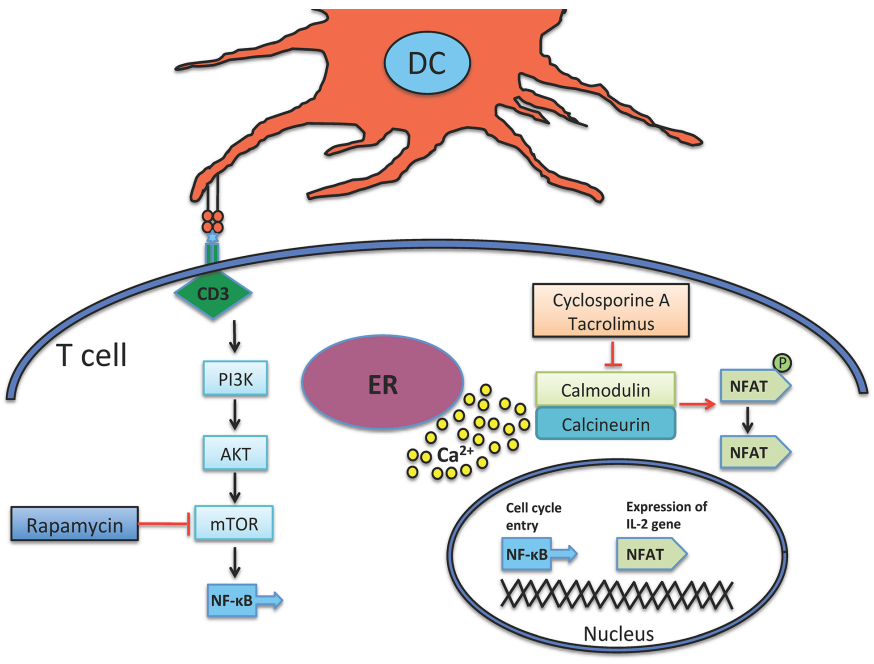

FIGURE 1 | Mechanism of action of immunosuppressant agents. Both Cyclosporine and Tacrolimus inhibit calcineurin, a calcium-dependent phosphatase that dephosphorylates and further activates NFAT, which in turn provokes IL-2 production. Calcineurin is activated by liberated calcium from ER. mTOR is another target down-stream of CD3 signaling, it is activated by the PI3K enzyme. mTOR induces cellular division and is inhibited by Rapamycin. ER, endoplasmic reticulum.

activity of T cells (41, 42). In case of GVHD, some clinical trials have shown its protective effect (43–45).

Despite partial achievements, none of the above-mentioned therapeutics could satisfactorily prevent GVHD, knowing that still 50% of transplanted patients show the disorder. Additionally, because all these agents are conferring a general immunodeficiency, they unfortunately interfere with the desired GVL effect (46).

In case of aGVHD occurrence, standard first-line treatment relies on high doses (2 mg/kg/day) of corticosteroids (47). Unfortunately, all attempts to improve on the curative treatment of established aGVHD have turned into repeating failures, either with strategies aiming at increasing the doses of steroids (48), or combining them with other drugs (49, 50). In case of steroid-refractory aGVHD, many second-line treatments have been tested, and until recently, none of them had demonstrated superiority over others, and thus no standard treatment was recognized in this setting (47). However, a recent phase III study has established ruxolitinib, an oral selective inhibitor of JAK1 and JAK2, as the most potent molecule in steroid-refractory aGVHD, with an acceptable safety profile, making it a new standard of care (51). The rationale for targeting JAK1/2 is the major role of its signaling in inflammation, tissue damage, T-cell activation, lineage commitment and survival, but also activation of neutrophils and differentiation and maturation of dendritic cells, all of which are involved in the pathogenesis of aGVHD (52–55).

Depletion of Alloreactive T Cells

The idea of depleting T cells from the infused cell product is not new and dates back to 1980s and 1990s; for such, three main strategies were considered effective: 1) *Ex-vivo* negative selection of T cells. 2) *Ex-vivo* positive selection of $CD34^+$ stem cells. 3) In-vivo depletion of T cells by antibodies.

Heeding these strategies, total T cells removal from the graft resulted in reduced incidence and severity of aGVHD (56–58). Nevertheless, the presence of T cells in graft was demonstrated as very important, so their depletion caused poor hematopoietic engraftment, increased incidence of disease relapse and opportunistic infections (56, 59, 60). Later on, the invention of magnetic beads led to more accurate targeting and also more efficient depletion of T cells. Interestingly, three separate clinical trials, targeting $CD3^+$T cells removal, $CD3^+$T cells plus $CD19^+$ B cells depletion, or αβ T cells plus $CD19^+$ B cells elimination, ended in lower incidence of aGVHD and better engraftment rate (61–63).

Positive selection of $CD34^+$ stem cells by magnetic beads is potentially an effective method to deplete alloreactive donor T cells prior to transplant which resulted in remarkable reduction of aGVHD and cGVHD (64–66). The major limitations of this method are increased risk of infections, which resulted in 40% mortality, and a high incidence of cancer recurrence.

ATG is a polyclonal antibody preparation that triggers simultaneous in-vivo depletion of donor and host T cells *via* induction of apoptosis, which enables a better control of transplant rejection or GVHD occurrence (**Figure 2**) (67, 68). Although ATG seems more convenient for the purpose, its high doses were associated with increased infections (69). In addition, ATG affects B cells, NK cells and APCs, thus works as a non-specific targeting agent (70). In a recent consensus, ATG/ATLG (anti-T lymphocyte globulin) was strongly recommended as part of myeloablative conditioning regimen prior to matched or mismatched unrelated allogeneic HSCT to prevent both aGVHD and cGVHD. In reduced intensity or non-myeloablative

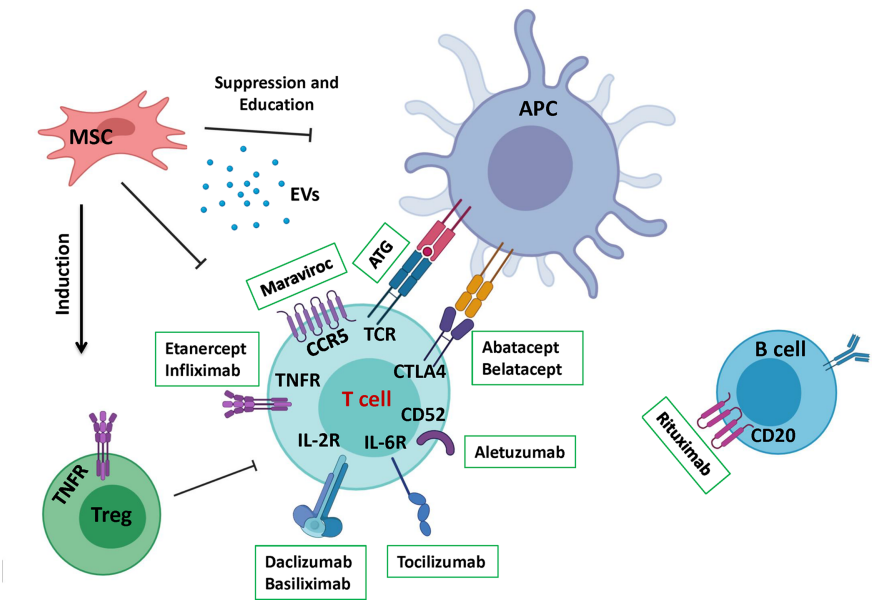

FIGURE 2 | A summary of the different therapeutics applied in aGVHD treatment. This schematic depicts a summary of recent therapeutics that has been developed to control GVHD. Therapeutic approaches are divided to pharmacological drugs mostly aiming to target a signaling pathway or cellular therapy that renders vaster regulatory effects.

conditioning regimens, ATG/ATLG was estimated appropriate to reduce the incidence of GVHD, but an increased risk of relapse was suggested to take into account (71).

Introduction of monoclonal antibodies made T cells depletion even more specific. T10B9 is a monoclonal antibody (mAb) which targets T cell receptor (TCR) αβ heterodimer region of $CD3^+$T cells (72). A combination of this mAb and Cyclosporine A was compared with methotrexate and cyclosporine A treatment in a phase 2/3 clinical trial, and the results showed reduction in grades 3 to 4 aGVHD but a higher risk of chronic myelogenous leukemia relapse (73). Another example of this kind is Alemtuzumab (Campath), which targets CD52 antigen (**Figure 2**) expressed on the surface of T and B cells but not on $CD34^+$ stem cells (74). Its first application was reported to reduce multiple sclerosis (MS) severity and relapse (75). A recent study was performed on 201 adult patients receiving a RIC allograft. With a median follow-up of 24 months, the cumulative incidences of aGVHD and late acute GVHD grades II-IV (grades III-IV) were 34% (13%) and 20% (8%) respectively. Furthermore, the cumulative incidences of cGVHD and overlap syndrome were 4% and 7% respectively (76). Although Alemtuzumab administration before HSCT, from related or unrelated donors, resulted in a lower incidence of GVHD, it could remain in the blood at lympholytic level for 1 to 2 months after transplantation. Consequently, the immune system reconstitution was considerably delayed, leading to a high incidence of viral infection and relapse (77).

More recently, the use of post-transplant cyclophosphamide (PT-Cy) has brought T-cell replete haplo-identical transplantation up to date, with remarkable results regarding GVHD incidence in this high-risk setting, thanks to the selective depletion of alloreactive T cells, while sparing regulatory T cells (78, 79). PT-Cy has also shown efficacy in transplantation with HLA-matched related and unrelated donors (80), and phase III clinical trials comparing PT-Cy and standard GVHD prophylaxis are currently ongoing (NCT03818334, NCT02345850).

Although the inhibition and depletion of alloreactive T cells are classically more studied to prevent GVHD, several other research works have been investigating on alternative strategies to block T cell migration towards GVHD target organs. This is in accordance with the more recently defined fourth criteria of GVHD development (20). A variety of molecules have been testing for this effect, notably maraviroc that blocks CCR5 (**Figure 2**) (81, 82), fingolimod (FTY720) that mostly interferes with T cells' infiltration into skin (83–85), and natalizumab that has been shown to mediates homing of lymphocytes to the gastrointestinal tract (86), with promising results.

Innovative Therapies

Current progress in biomedical research has opened the door for new innovative therapy approaches including gene and cell therapies. Gene transfer technologies, including the suicide gene approach, are promising tools to manipulate donor T cell immunity, to boost the GVL effect, to foster functional immune reconstitution, and to prevent or control GVHD. Cell therapy of aGVHD is based on distinctly ex-vivo or in-vivo expansion of Tregs, which are the natural immunosuppressant cells of the body. Moreover, the application of mesenchymal stromal cells (MSCs), regulatory macrophages, innate lymphoid cells (ILCs), NKT cells and endothelial progenitor cells (EPCs), based on their immunoregulatory and/or regenerative properties have also been, or are currently being investigated, showing very promising results.

Gene Therapy of aGVHD

Gene therapy of aGVHD consists in transferring a suicide gene into donor T lymphocytes, which can be selectively controlled after transplant. Herpes simplex virus thymidine kinase (HSV-TK) has already been introduced as a cell-cycle dependent suicide gene (87, 88). In the presence of ganciclovir (GCV), an anti-herpes drug, infected cells catalyze the generation of triphosphate ganciclovir that further inhibits DNA chain elongation, which is toxic to proliferating cells (89, 90). In-vitro and in-vivo preclinical studies in mice (91, 92) and afterward phase I/II clinical trials have demonstrated that the retroviral-mediated transfer of HSV-TK suicide gene into donor T cells prior to graft infusion allows efficient control of donor T cell alloreactivity (93, 94). In the latter clinical trial, the investigators also showed that these infected T cells improve immune reconstitution and could provide GVL effect. However, there are some possible drawbacks for applying this strategy. In immuno-compromised patients, TK may lead to undesired elimination of transduced cell populations as a result of the immunogenicity of this viral protein. In addition GCV is a drug used to treat cytomegalovirus (CMV) infection which commonly affects immuno-compromised patients. Administration of GCV in CMV infected patients could result in undesired TK-cell killing (95). Also, as suggested in the study by Maury et al., elimination of TK^+ cells after ganciclovir administration may not prevent GVHD caused by a putative in-vivo expansion of the small proportion of TK^- alloreactive T cells in this lymphopenic setting (94).

Another suicide gene that has also been tried in a phase I clinical trial, is inducible human caspase 9 (iC9), a hybrid protein consisting of a human FK506-binding protein (FKBP12) linked to a modified human caspase 9 lacking the caspase recruitment domain (CARD). This transgene can be activated by a single administration of a small-molecule drug (AP1903). Thanks to the accelerated immune reconstitution, patients have immediate and sustained protection from major pathogens, including cytomegalovirus, adenovirus, BK virus, and Epstein-Barr virus in the absence of acute or chronic GVHD (96).

In an attempt to reprogram progenitor cells in order to evaluate their engraftment, differentiation, and safety, NSG mice $CD34^+$ cells were ex-vivo transduced with a proprietary lentiviral vector encoding a human gene or a mock (GFP) vector. The result revealed that the mice treated with transduced $CD34^+$ cells had lower aGVHD outcome such as lymphohistiocytic inflammatory cell infiltrates and microgranulomas in the liver and lungs in comparison to control mice injected with naive $CD34^+$ cells (97).

CELL THERAPY OF aGVHD

Mesenchymal Stromal Cells

Mesenchymal stromal cells (MSCs) are non-hematopoietic self-renewal cells that have the ability of multipotent differentiation

mainly into mesodermal lineages like chondrocytes, osteocytes and adipocytes (98–100). These cells that are known for their adherence capacity to plastic, neither express the hematopoietic and monocyte markers such as CD34, CD45 and CD14, nor endothelial markers like CD31 and CD144. Additionally, they do not express MHC II molecules like HLA-DR, and co-stimulation molecules like CD80 and CD86. However, they do express markers such as CD90, CD73, CD105, CD146, and CD29, plus a poor expression of MHC I molecules. MSCs can be isolated from different adult, prenatal and neonatal tissue sources including but not limited to BM, adipose tissue (AT), dental tissues, endometrium, amniotic fluid, umbilical cord and many others (101–103). It has been revealed that MSCs from diverse tissues have different regenerative and immunoregulatory features (101, 104). Moreover, source tissue diversities were correlated to variable expression quantities of highly procoagulant tissue factor (TF) CD142 on their cell surface (102), which remarkably affects their safety profile for intravenous (IV) infusion due to triggering of the "instant-blood-mediated inflammatory reaction" (IBMIR) (105). This is indeed a crucial aspect for the cells' safety and efficacy profile (106) as also interestingly discussed by Moll G et al, for recent COVID-19 MSC based therapies (107).

MSCs can support hematopoietic cells and possess non-specific immunosuppressive and immunomodulatory functions against both innate and adaptive immune responses (108, 109). They can directly inhibit the proliferation of alloreactive T cells or convert them to Foxp3 expressing regulatory T cells through a cell-cell contact dependent and independent manner (**Figure 2**) (110–114). Additionally, MSCs can program macrophage plasticity by polarizing them towards less pro-inflammatory M1 and more anti-inflammatory M2 subpopulations (**Figure 2**) (115). Contrary to their in-vitro suppressive capacity when used in a 1:1 MSCs/T cells ratio, they had no clinical usefulness in terms of graft survival or severity of aGVHD in mice (116). However, few years ago Baron et al., revealed that, a third party, ex-vivo expanded, MSCs co-injection in a high risk, mismatched, unrelated-donor HSCT could reduce the severity of GVHD (117). On the other hand, co-injection of MSCs and HSCs in an HLA-identical sibling HSCT although resulted in a decrease of aGVHD severity, the incidence of relapse was remarkably higher (118). Recently a case report for a 15 years old boy, showed a dramatic decrease of aGVHD after treating with 2×10 (6) MSCs/kg 8 times in 4 weeks followed by MSCs administration once/week in the next 4 weeks (119).

Most cells release membrane-derived extracellular vesicles (EVs) carrying biomolecular payloads that offer significant potential in both detecting and treating diseases. EVs have a lipid bilayer and are ranging from 50 nm to ~2μm secreted from nearly all mammalian cell types (e.g., endothelial cells, neuronal cells, muscle cells, stem cells) that can be found in various body fluids such as breast milk, semen, saliva, urine, and serum (120). Based on their biogenesis pathways, EVs are categorized into three main classes: exosomes, microvesicles, and apoptotic bodies (121).

MSC-EVs could alter $CD4^+$ T cells through an APC-related pathway, increasing the population of $CD4^+CD25^+Foxp3^+$ Treg, consequently, increasing the immunosuppressive effects of MSC-EVs (**Figure 2**) (122). Furthermore, recent studies support the crucial role of MSC-EVs in regulating the M1/M2 macrophage subpopulation balance. For instance, MSC-EVs could interfere with the activation of M1 macrophages while favoring their M2 counterparts. This is accompanied by reduced secretion of TNFα, IFNγ, VEGF, and IL-12 and increased IL-10 production (123–125). EVs were shown to have the similar tissue repair capabilities as MSCs making them a promising non-cellular approach for GVHD treatment (126). It has been demonstrated that MSC-EVs could enhance the survival rate and reduce the grade of aGVHD in mouse models. This was followed by a modification in the naive and effector T cell ratio (127). Other studies reported reduced clinical symptoms including diarrhea and hormone consumption after MSC-EVs therapy. They showed that MSC-EV treatment reduced the PBMC secretion of IL-1β, TNFα, and IFNγ (128).

The encouraging point in using MSCs is that they are very well tolerated in-vivo, however, the efficiency of MSCs treatment is variable in different studies. This could be due to the fact that MSCs are very heterogeneous cells. Recently, we have demonstrated that compared to MSCs harvested from WT mice, their counterparts from TNFR2 KO mice are significantly disabled to suppress Teffs and convert them to $Foxp3^+$Tregs (111). Sorting TNFR2 enriched MSCs or up-regulating this marker with a proper agonist could potentially lead to a more homogeneous cell product with increased immnuregulatory features. Taken together, the optimized source, dose, frequency and treatment intervals of MSCs administration require better understanding of the mechanisms of MSCs treatment.

As previously mentioned MSCs can exert their therapeutic effect either directly or indirectly through educating/reprogramming other cells such as macrophages and T cells. In the next sections, we discuss the role of regulatory macrophages and regulatory T cells in GVHD treatment.

Regulatory Macrophages

Recipient macrophages are known to resist the conditioning regimen and to remain in patients for many weeks after HSCT (129). This might provide the opportunity to modulate donor T cell immunity. This hypothesis proved valid in a mouse model of GVHD indicating that macrophages resisted in lymphoid tissues after lethal irradiation and elimination by anti-colony stimulating factor 1 receptor (anti-CSF-1R), which is expressed on all monocytes and tissue macrophages and plays a key role in their homeostasis (130), led to exacerbated GVHD (131). They further showed that pre-transplant CSF-1 therapy could expand recipient regulatory macrophages resulting in amelioration of aGVHD through an IL-10 dependent mechanism. The infiltration of macrophages can add to GVHD occurrence, however, macrophages have different subpopulations which act differently in GVHD (132). Macrophages recruitment is one of the main steps in aGVHD initiation, and a higher ratio of M1/M2 macrophages correlates to a higher incidence of grades 2 to 4 acute GVHD (133, 134). Pro-inflammatory M1 macrophages have been shown to contribute and infiltrate more in aGVHD,

whereas anti-inflammatory M2 macrophages are reported to be more predominant in cGVHD and refractory aGVHD. Due to the secretion of anti-inflammatory cytokines, such as IL-10 and TGFβ, M2 macrophages could suppress different immune cells, particularly T cells. Therefore, they could be potentially a good cell therapy product to target GVHD. Bouchlaka et al, showed that MSC educated M2 macrophages have enhanced CD206, CD163, IL-6, TGF-β, arginase-1 expression and reduced IL-12 and TNFα production and can attenuate GVHD. This was mostly due to controlled T cell proliferation and enhanced fibroblast proliferation (135). Very interestingly, it has been demonstrated that the polarization of M2 macrophages by MSCs is also TNF-TNFR2 dependent (136). This could demonstrate once more the importance of TNFR2 targeting to take the better advantage of M2 macrophages or change the balance of M1 and M2 macrophages in GVHD treatment.

Regulatory T Cells

Natural regulatory T cells (nTregs) are defined as natural immunosuppressive cells that are able to inhibit alloreactive lymphocytes and control innate and adaptive immune responses (**Figure 2**) (137–140). Any impairment in Tregs functionality or imbalance in their recovery after HSCT is associated with a loss of tolerance and development of autoimmunity and also GVHD (141, 142). Compared to previous cell therapy approaches of aGVHD, Tregs are the most studied and applied cellular based therapy that has shown very promising results. Studies in mouse models have proved that depletion of Tregs before transplantation significantly accelerates the occurrence of aGVHD and inversely, others reported that adoptive transfer of freshly-purified donor Tregs or donor derived ex-vivo expanded Tregs were remarkably efficient to control aGVHD (143–145). The attractive point of Treg cell therapy is that GVL effect is acceptably preserved which is probably due to retention of donor T cells or differences in homing pattern of effector versus regulatory T cells (146). In addition to Treg suppressive activity they have other beneficial effects like facilitating the engraftment of hematopoietic cells and participating in immune reconstitution (60, 147). However, the low percentage of Tregs (5–10% of peripheral $CD4^+$ T cells) represents a major obstacle for their vast clinical application. This barrier has been overcome by means of ex-vivo expansion of Tregs with anti-CD3 and anti-CD28 in the presence of IL-2, to yield non-specific polyclonal Tregs. Although, the application of polyclonal Tregs has shown promising outcomes in different complications such as GVHD (148), solid organ transplantations like kidney transplantation (149), non-immune diseases such as cardiovascular diseases, obesity, type 2 diabetes (T2D), and degenerative diseases (150), it was less convincing in other disorders such as type 1 diabetes (T1D) and multiple sclerosis (MS) mainly due to the heterogeneity of expanded Treg cell population (150, 151).

The other proposed solution was ex-vivo expansion of Tregs through TCR-mediated activation by alloantigen of recipient (recipient specific Treg or rs-Treg) in the presence of IL-2. This process permits to obtain a satisfying number of rs-Tregs that are capable of specifically suppressing donor T cells and consequently providing more promising results regarding aGVHD control compared with polyclonal Tregs (152, 153). These rs-Tregs could hamper the activation and differentiation of donor T cells in-vivo leading to a total and sustained protection of transplanted mice while preserving immune reconstitution and GVL effect (147, 154). Unfortunately, due to the difficulty to sort purified Tregs under clinical grade practice conditions, rs-Tregs involve a risk of contamination of cell product with highly alloreactive and thus pathogenic recipient specific effector T cells (rs-Teffs), which precludes their therapeutic application. To overcome this issue, Martin GH et al. suggested an alternative strategy utilizing Tregs which are specific for a single exogenous antigen (HY antigen specific Tregs or HY-Treg) that is neither expressed in donor nor in recipient (HY antigen is only expressed in males). In this case, the contaminating Teffs are maintained non-pathogenic as the exogenous antigen is transiently presented by few host APCs and is not expressed by host target organs of aGVHD. In a semi-allogeneic mouse model of HSCT, when both donors and recipients were female, the co-transfer of Teffs and HY-Tregs alone could not protect against aGVHD, however, modifying the gender of recipients to male mice that express HY antigen, was enough to completely protect against aGVHD. Alternatively, to re-activate HY-Tregs in-vivo in the presence of their cognate Ag, three intravenous injections of HY-peptide at D0, D3 and D6, resulted in entire protection against aGVHD (155). The hallmark of this strategy is that it potentially provides an OFF-ON system to benefit from the alloreactive effect of donor T cells on demand i.e. to destroy malignant cells when Tregs are off (non-activated), and to turn them on (activated with their cognate Ag) as soon as observing the primarily signs of aGVHD.

Further studies to identify the mechanism of action of Tregs in such an inflammatory environment revealed that in murine model of aGVHD, Treg immunosuppressive effect was dependent on the secretion of TNFα by Teffs and the expression of TNFR2 by Tregs. In this context, the blockade of TNFα-TNFR2 signaling pathway either by administration of an anti-TNFR2 mAb or harvesting Tregs from TNFR2-KO mice to block the possibility of signal transduction through the TNFR2, or using T cells harvested from TNFα KO mice led to the interruption in Treg suppressive function resulting in high grades of aGVHD (156, 157). The advantage of this finding is that it provides an OFF button for Tregs. Thus, after their proper immunosuppressive function (ON status) we have the possibility to turn them off until the next need.

Such promising results acquired with animal models over the last decade encouraged its application in human. Two phase 1 clinical trials using adoptive transfer or ex-vivo expanded Tregs before (day 4) or just after (day+1 +/− day+15) transplant resulted in notable reduction in the severity of aGVHD (158, 159). In further clinical update, Brunstein et al., have reported that the incidence of grades 2 to 4 aGVHD at 100 days was 9%, and cGVHD at 1 year was 0% without any difference in infection density (148). Moreover, a significant faster recovery of total $CD4^+$T cells and a subset of naive $CD4^+$T cells were observed. The rationale for using cord blood derived Tregs in the study by Brunstein et al. was in part based on their similar capacity to

express the essential Tregs markers (160), in addition to their resistance against the classical immunosuppressant drugs that usually interfere with Treg viability or function, and therefore abrogate their therapeutic effect (161).

Another strategy to increase Treg percentage in patients is through in-vivo expansion of these cells by the administration of low doses of IL-2. Previous clinical studies had already revealed that IL-2 therapy induces the selective expansion of Tregs following HSCT and in patients with solid tumors (162–164). This strategy was tried in a phase 2 clinical trial which achieved an expansion of Tregs from a mean of 4.8% pre IL-2 to 11.1% after therapy, with the greatest change occurring in recipients of matched related donor transplants. Interestingly, no IL-2–treated patient developed grades 2 to 4 aGVHD. Additionally, IL-2–treated recipients maintained T cells reactive to viral and leukemia antigens and on the whole, the rate of infection was significantly lower compared with non-treated patients (165).

The low dose IL-2 administration was also studied in cGVHD with remarkable in-vivo Treg expansion and promising clinical results, particularly in pediatric patients (166–168).

Innate Lymphoid Cells

Innate lymphoid cells (ILCs) are different from their B and T cell counterparts as they do not express rearranged Ag specific receptors (169). ILCs are a heterogeneous family of cells that are classified on the basis of their transcriptional factors and their functionality. Like T lymphocytes, ILCs are also grouped into cytotoxic and helper subsets. New classifications consider NK cells as cytotoxic ILCs that express T-bet and eomesodermin (Eomes) and are able to secrete IFNγ and TNFα, thus yielding cytotoxic effects (169, 170). Helper ILCs are further subdivided into three distinct populations: ILC1, ILC2 and ILC3. Briefly, ILC1 population needs T-bet for their development and they are able to secrete IFNγ. However, the difference between this population and NK cells is that they neither express Eomes nor exert cytotoxic activities (170). ILC2 express GATA3 and produce Th2 cytokines (171). Finally, ILC3 cells are themselves heterogeneous populations that are further subdivided into more subsets. They are known to express RORγt and to mainly produce IL-17 and IL-22 cytokines (172). In general, ILCs contribute to host defenses against a broad variety of pathogens (173, 174). In the context of GVHD, due to the damage caused by the conditioning regimen and the further tissue damage resulted from donor T cells attack, the role of ILCs is supposed to be essential. Hanash et al., identified intestinal ILC3 subset as the main IL-22–producing cells after TBI, highlighting their crucial role in the protection against epithelial cells damage and in preserving intestinal stem cells (175). The same results were reported by another team showing that IL-22 treatment in mice after HSCT could increase intestinal stem cell recovery, increase epithelial cell regeneration, and eventually reduce intestinal GVHD (176). The role of ILCs in tissue repair is not limited to intestinal cells since another study has described the promising role of ILC3 in thymic epithelial recovery, through IL-22 production, causing a more efficient T cell reconstitution (177). Similar results were obtained in lung epithelial tissue repair (178). The latter is in accordance with another study demonstrating a critical role of lung ILCs in restoring airway epithelial integrity and tissue homeostasis after infection with influenza virus (179). The possible protective effect of ILCs in aGVHD was firstly discussed by Hanash et al., showing that host-derived IL-22 could substantially limit aGVHD development (175). Moreover, Munneke et al., have suggested that once ILCs (regardless of origin, donor or recipient) are activated they could reduce aGVHD development and tissue damage (180). Nevertheless, the exact role of IL-22 in inflammatory conditions such as GVHD is not completely clear and might be controversial. For instance, Couturier et al, reported that the IL-22 deficiency in donor T cells could attenuate murine aGVHD mortality while preserving the GVL effect (181). Altogether, the positive role of ILCs in tissue repair, stabilization of stem cells and maintenance of tissue hemostasis is currently the subject of discussions and ILCs are potentially an interesting candidate to be tested in back to back therapies i.e. with classical pharmacological treatments or more interestingly with novel therapies such as gene therapies and regulatory T cells (182). In other words, testing the immune suppression caused by any of these approaches versus tissue repair and hemostasis that could be induced by ILCs.

NKT Lymphocytes

NKT cells simultaneously express TCR and markers of NK cells. Within this population, invariant NKT (iNKT) are characterized by an invariant alpha chain of TCR that has a capacity to recognize glycolipids, like the *glycolipid* alpha-*galactosylceramide* (alpha-GalCer) antigen presented by CD1d molecules (183, 184). This glycolipid induces a fast and massive activation of NKT cells which are involved in the regulation of allogeneic responses *via* production of IL-4 and IFNγ. They can also regulate other cells of the immune system towards a tolerogenic or a cytotoxic response, particularly against tumors (185–187). In a mouse model, it was shown that $CD4^-CD8^-$ iNKT lymphocytes of bone marrow origin, could control aGVHD without attenuation of GVL effect (188). Authors also suggested that this protection effect is through production of IL-4 by NKT cells that can consecutively induce Treg proliferation. In another study, administering a low dose of $CD4^+$NKT at the same time of the BM graft significantly reduced the incidence of aGVHD. Once again, this was associated with IL-4 secretion by NKT cells and subsequently altering the secretion of pro-inflammatory cytokines such as IFNγ and TNFα by donor T cells, without hampering their proliferation (189). In patients who received Total Lymphoid Irradiation (TLI) conditioning regimen, a very good reconstitution of iNKT was observed and this was linked to a remarkable decrease in the incidence of higher grades of aGVHD (190). Moreover, Rubio et al., have provided a proof of concept that early post-allogeneic HSCT iNKT cell recovery can predict the occurrence of aGVHD and an improved overall survival (191). This was confirmed in another study showing that proportion of $CD4^-$ iNKT cells of the graft could be predictive of aGVHD in recipients (192).

Endothelial Progenitor Cells

Endothelial progenitor cells (EPCs) are the BM-derived hematopoietic cells that are responsible for neo-vascularization and repairing tissue damages at the endothelial sites (193). These

cells that express classical endothelial markers such CD31, CD144, VEGFR2 and CD133 demonstrate some unique features that make them especially interesting for treatment of degenerative, cardiovascular and hematopoietic disorder. For instance, Loisel et al. have shown a successful administration of autologous EPCs for the treatment of right ventricle (RV) failure in a piglet model of chronic thromboembolic pulmonary hypertension (CTEPH) (194). Similar to MSCs, EPCs have shown some levels of immunosuppressive and immunomodulatory properties (195). Our team has recently demonstrated that human EPC derived from CB are tolerated in xenogeneic mouse models of ischemia and contributed to vascular formation (196). We further revealed that EPCs' immunosuppressive effect was entirely TNFR2 dependent since administration of an anti-TNFR2 mAb abolished their regulatory functions (197). Accordingly, we showed that priming EPCs with TNFα enhances their immunosuppressive effect through a TNFR2 dependent interaction (198). These interesting features encouraged scientists to evaluate their therapeutic effect in GVHD models. EPCs injection was reported to be have some protective roles in accelerating hematopoietic and immune reconstitution, restoring vascular niche in BM and ameliorating GVHD grade through improving the integrity of BM sinusoidal endothelial cells (199–202). Further investigations revealed that the administration of anti-vascular endothelial cadherin antibody **(AAVE)** remarkably interrupted those mentioned effects (200). Based on our recent experiences, we think it would be very interesting to specifically target TNFR2 molecule in EPCs *via* its proper agonist, in order to selectively upregulate this marker and benefit from increased EPC immunosuppressive and pro-angiogenic effects. Controlling these two crucial aspects leads to higher HSCs engraftment, better immune reconstitution and, if necessary, improved GVHD prevention.

CONCLUSIONS

In spite of great advancements in treating GVHD, it still remains a major complication of HSCT. Here, we described a series of novel therapeutic approaches that target different cells that contribute to GVHD occurrence. Additionally, we have discussed the application and the potential therapeutic benefits of a variety of cells with immunoregulatory functions with the special attention in Tregs that have been proved to be a very promising approach to control GVHD. Cell free therapies including the administration of EVs, in-vivo amplification of regulatory cells and targeting immune checkpoint signaling pathways such as the TNF-TNFR2 axis are among some new emerging approaches to selectively control the reaction and intensity of the immune response which potentially could lead to better control of GVHD.

AUTHOR CONTRIBUTIONS

SN, ML, and SS wrote the manuscript. SN, ML, and GU reviewed and revised the manuscript. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Tregs and Mixed Chimerism as Approaches for Tolerance Induction in Islet Transplantation

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Pancreatic islet transplantation is a promising method for the treatment of type 1 and type 3 diabetes whereby replacement of islets may be curative. However, long-term treatment with immunosuppressive drugs (ISDs) remains essential for islet graft survival. Current ISD regimens carry significant side-effects for transplant recipients, and are also toxic to the transplanted islets. Pre-clinical efforts to induce immune tolerance to islet allografts identify ways in which the recipient immune system may be reeducated to induce a sustained transplant tolerance and even overcome autoimmune islet destruction. The goal of these efforts is to induce tolerance to transplanted islets with minimal to no long-term immunosuppression. Two most promising cell-based therapeutic strategies for inducing immune tolerance include T regulatory cells (T_{regs}) and donor and recipient hematopoietic mixed chimerism. Here, we review preclinical studies which utilize T_{regs} for tolerance induction in islet transplantation. We also review myeloablative and non-myeloablative hematopoietic stem cell transplantation (HSCT) strategies in preclinical and clinical studies to induce sustained mixed chimerism and allograft tolerance, in particular in islet transplantation. Since T_{regs} play a critical role in the establishment of mixed chimerism, it follows that the combination of T_{reg} and HSCT may be synergistic. Since the success of the Edmonton protocol, the feasibility of clinical islet transplantation has been established and nascent clinical trials testing immune tolerance strategies using T_{regs} and/or hematopoietic mixed chimerism are underway or being formulated.

Keywords: Tregs, islet transplantation, hematopoietic stem cells, mixed chimerism, transplant tolerance

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INTRODUCTION

Type 1 diabetes (T1D) arises from an autoimmune attack of the insulin-producing, islet beta cells of the pancreas. Patients with T1D exhibit abnormalities in immune regulation that contribute to its etiology. Organ/tissue transplantation is complicated by adaptive $CD4^+$ and $CD8^+$ T cell responses that can contribute to allograft rejection (1–4). Owing to the combined specters of auto- and allo-immune responses, islet transplantation is one of the most challenging settings to prevent immune rejection.

Pharmacologic immunosuppressive drugs (ISDs) in islet transplantation traditionally target effector T cell proliferation and function to prevent graft rejection (5). However, most of these ISDs

require life-long administration and have increased risk of multiple adverse reactions, including susceptibility to infection and incidence of secondary cancers (6, 7). In addition, survival of the transplanted islets is shortened due to direct toxic effects of the ISDs on islet β cells (8). One of the major goals in islet transplantation is the induction of immunosuppressive drug-free tolerance to the islet graft (9–11).

By virtue of their role in controlling alloreactive T cell responses to organ and tissue grafts, regulatory T cells (T_{regs}) are considered as promising alternatives to pharmacologic agents to promote engraftment and survival of the transplanted organs/tissues (12–14). Peripheral tolerance established by T_{regs} is crucial to prevent immune-mediated rejection of the transplanted graft (15, 16). Several preclinical studies have demonstrated induction of immune tolerance in different transplantation models such as heart, kidney, skin, liver, and islets (17–20). Multiple clinical trials are in progress evaluating the efficacy of recipient T_{regs} in organ transplantation tolerance (clinicaltrials.gov). One promising strategy in preclinical studies is the adoptive transfer of *in vitro* culture expanded T_{regs} to prevent the rejection of donor islet grafts (21, 22) and at least one clinical trial testing this approach is underway (NCT03444064). This phase I clinical trial aims to assess the safety and feasibility of autologous polyclonal T_{regs} in islet transplant patients. However, pre-clinical studies and clinical studies with recipient-derived T_{regs} in solid organ transplantation have shown that peripheral T cell tolerance is not necessarily durable and methods to enhance T_{reg} function is an active area of research.

Another cell-based strategy for inducing islet allograft tolerance originates from studies which showed that the establishment of hematopoietic mixed chimerism between the donor and recipient results in donor allograft tolerance (**Figure 1**) (23, 24). Subsequent preclinical islet transplantation

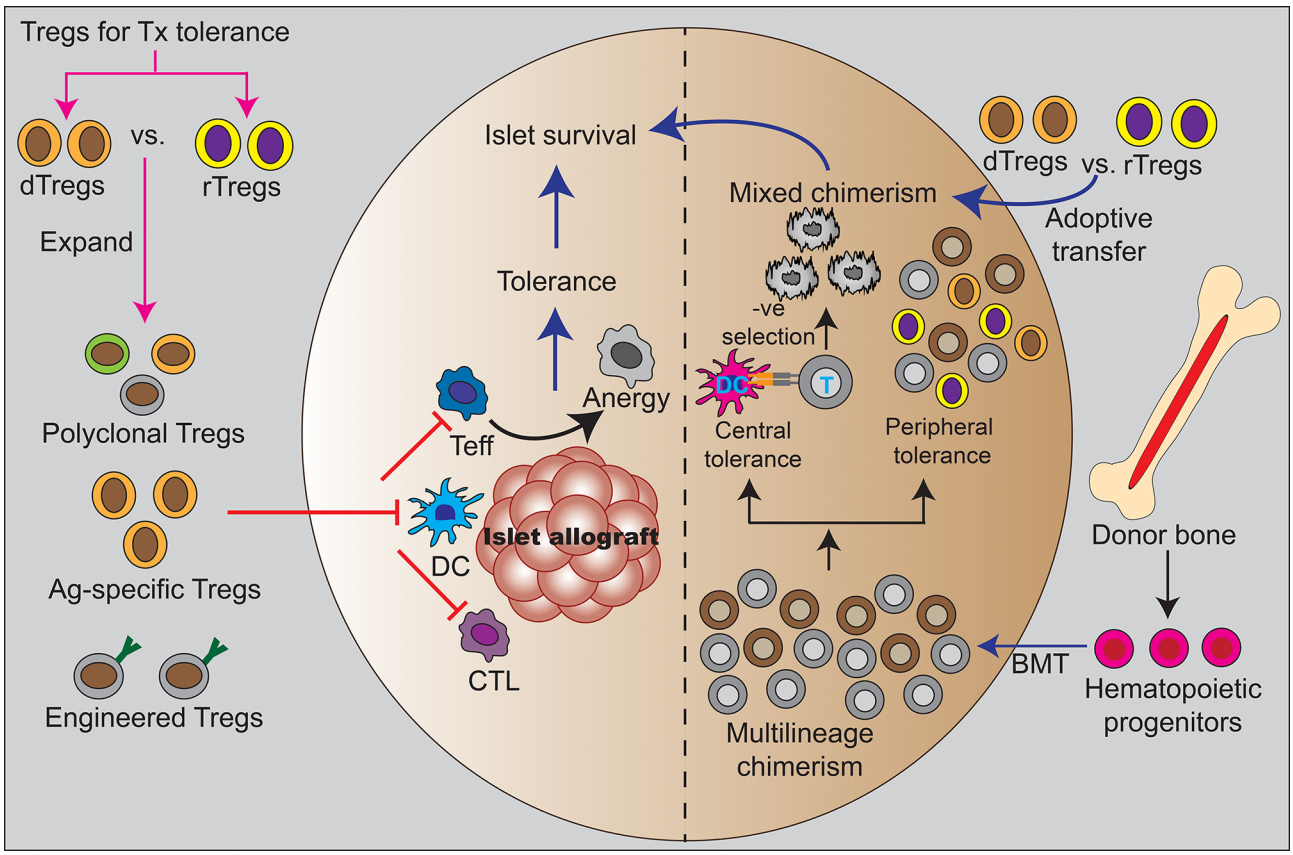

FIGURE 1 | T_{reg} and hematopoietic mixed chimerism as clinical strategies for tolerance induction. The left half of the figure shows direct effect of T_{regs} in inducing peripheral tolerance by regulating different immune cells such as dendritic cells and T cells to suppress alloreactivity. The adoptive transfer of different types of T_{regs} that been used in preclinical studies to support mechanisms of peripheral islet tolerance including polyclonal T_{regs}, antigen-specific T_{regs}, and engineered T_{regs}. These studies suggest T_{regs} might be used to reduce or eliminate systemic immunosuppression. The right half shows establishment of mixed hematopoietic chimerism through combined donor islet and hematopoietic stem cell transplantation. This is a state of coexistence of donor and recipient hematopoietic cell precursors with evidence to indicate that both mechanisms of central deletion of alloreactive responses and peripheral tolerance pathways regulate allograft tolerance. The administration of exogenous T_{regs} have been used to promote mixed hematopoietic chimerism and tolerance in preclinical studies. T_{reg} are necessary for sustained chimerism and tolerance in these models and human clinical studies have shown T_{reg} exert allo-antigen specific regulation in the setting of mixed chimerism. Ag, antigen; BMT, bone marrow transplantation; CTL, cytotoxic T lymphocytes; DC, dendritic cell; dTregs, donor-derived regulatory T cells; rTregs, recipient-derived regulatory T cells; T, T cell; Teff, effector T cell.

models which rely on mixed chimerism for tolerance induction have developed clinically translatable approaches (25–28). Encouragingly, clinical trials of combined kidney and hematopoietic cell transplantation (HCT) from living donors have demonstrated that immune tolerance to solid organs is possible by establishing hematopoietic mixed chimerism. Over 80% of HLA-matched patients enrolled in these trials are completely off ISDs (29, 30).

In the case of islet transplantation for T1D, experiments in preclinical murine models first reported almost 20 years ago have reproducibly shown that the establishment of hematopoietic mixed chimerism not only provides durable allograft tolerance but also prevents autoimmune islet destruction (31). A major problem in the translation of combined islet and HCT has been the traditionally toxic conditioning required for HCT, but the bone marrow transplantation field is rapidly evolving and significantly less toxic approaches have been developed or are in early phase clinical trials (32–34). Thus, combined islet and HCT is a promising area of translational investigation.

Since T_{regs} play a critical role in the establishment of tolerance in the setting of hematopoietic mixed chimerism (**Figure 1**), it is important to better understand T_{regs} in this setting. It is also possible that a combined immune therapy of T_{regs} and HCT may be synergistic (35).

TREGS IN AUTOIMMUNE DIABETES

T_{regs} are a small subsets of $CD4^+$ T cells, characterized by the surface expression of CD4 and CD25, and the expression of the transcription factor forkhead box protein 3 (Foxp3) which is critical for their function (36). T_{regs} are well-known for their suppressive function and are responsible for safeguarding against various autoimmune diseases, including T1D (37, 38). This review focuses on major exogenously-administered T_{regs} that have been used in bone marrow and islet transplantation settings with a special emphasis on the classical $CD4^+CD25^+Foxp3^+$ T_{regs}.

Non-obese diabetic (NOD) mice spontaneously develop autoimmune diabetes and share many features of human T1D (39, 40). Preclinical studies in NOD mice have shown that T_{reg} can prevent autoimmune diabetes (41–43). The NOD mice which have defective CD28/B7 costimulation pathway and are prone to exacerbated T1D pathology showed delayed diabetes progression when injected with $CD25^+$ T_{regs} (44). Moreover, adoptive transfer of islet-specific T_{regs} reversed T_{reg} defect in CD28 deficient NOD mice and successfully prevented the disease progression (45). These series of findings suggest ex vivo-expanded T_{regs} as a way to satisfy T_{reg} deficiency in the treatment of T1D.

T1D is characterized by presence of defective T_{regs} function and activation, particularly in the IL-2 pathway which can also affect T_{reg} function (44–47). The role of IL-2 signaling in T_{reg} development, metabolism, and function has been discussed in a recent review (48). Defective IL-2 signaling is associated with impaired T_{reg} metabolism and diminished suppressive function (49). Ex vivo expansion of T_{regs} derived from T1D patients may be a principle way to correct for any inborn deficiency and these T_{regs} have been tested for their safety in phase 1 clinical trials with no evidence of therapy-related adverse events reported (50). Another clinical study has shown similar safety in pediatric T1D patients, and suggests disease modulation with observed reduced daily insulin requirement in treated patients (51, 52).

TREGS FOR PROMOTING ISLET ENGRAFTMENT

T_{reg} therapy can be applied in two settings in islet transplantation: promoting islet survival in initial engraftment and inducing peripheral tolerance to eliminate immunosuppression.

The most common implantation site for clinical islet transplantation is within the liver *via* hepatic portal vein infusion (53). It is estimated that >50% of the initial islet mass that is infused is lost within the first few days due to local inflammatory changes and coagulation at the islet implantation site; the phenomena is termed as instant blood-mediated inflammatory reaction (IBMIR) (54, 55). The addition of T_{regs} at the time of islet infusion has been explored as a method for reducing initial islet graft loss and improving islet engraftment (56–58).

In addition to potentially changing the inflammation at the islet implantation site, experiments in which T_{regs} are either co-cultured, co-aggregated, or co-infused with islets have shown that T_{regs} appear to affect the islets themselves (59–62). In a preclinical study, co-culture of T_{regs} with the pancreatic islets altered production of inflammatory chemokines such as CCL2, CCL5, CXCL9, and CXCL10, produced by the islets themselves, benefitting islet graft survival after implantation under the kidney subcapsule (60). TGF-β secreted from the T_{regs} have been shown to improve islet viability and function in islet-T_{reg} coculture experiment (63). T_{regs} might therefore improve islet viability and potentially reduce their immunogenicity.

A number of studies have provided preclinical evidence that T_{regs} incorporated into the islet graft itself or co-administered with the islet allograft can inhibit adaptive immune responses (59, 61, 64). In one example, Takemoto et al. constructed co-aggregates of BALB/c islets and C57BL/6 T_{regs} and transplanted into the liver of C57BL/6 mice where a long-term survival of the allogenic islets was observed for over 100 days without any immunosuppression (65).

TREGS FOR MODULATING ADAPTIVE RESPONSES IN ISLET TRANSPLANTATION

Adoptive transfer of recipient-derived T_{regs} in preclinical models has shown to be effective in preventing islet allograft rejection

through the establishment of transplant tolerance (**Figure 1**). Polyclonal T_{regs} have been used either to protect islets from direct contact-mediated immune attack or to modulate systemic immune response (59–61, 65, 66). Zhang et al. adaptably transferred donor antigen-specific T_{regs} in mice and found a profound synergistic effect with rapamycin in the islet allograft transplant setting (67). In another study by Lee et al, adoptive transfer of donor-reactive T_{regs} in T cell depleted mice resulted in indefinite survival of islet allografts. Moreover, *in vitro* expanded T_{regs} have been shown to delay porcine islet xenograft rejection in humanized mice by inhibiting graft-infiltrating effector T cells (64). In addition, multiple studies have demonstrated that local co-injection of islets and T_{regs} promotes islet engraftment (59, 60, 65).

CHOICE OF TREGS IN ISLET TRANSPLANTATION

An essential question remains unanswered in studies that examine the use of T_{reg} therapy: which is better, donor or recipient T_{reg}? This has perhaps been shaped by the perception that the only available source of clinical-grade T_{regs} is from the recipient, but T_{regs} can potentially be obtained from cadaveric spleen and bone marrow for clinical use.

It would be reasonable to hypothesize that both donor and recipient T_{regs} may reduce inflammation during islet transplantation. Perhaps donor T_{regs} would be more effective because of alloreactive responses to recipient MHC Class II expressed by local APCs or other cells. Likewise, recipient T_{regs} co-cultured with islets themselves might be more effective in changing islet profiles, as recipient T_{regs} may be more able to exert effector function through alloreactive TCR responses.

In case of T_{reg} modulatory effects on adaptive immune responses, recipient T_{regs} might be favored as their initial alloreactive responses to islet tissue could locally shape the recipient adaptive immune response to allow alloreactive T_{regs} to persist and expand. Alternatively, donor T_{regs} might be more able to modulate adaptive responses by early and critical interactions with infiltrating recipient immune cells. In T1D patients, it is possible that recipient T_{regs} may also have deficiencies that could be avoided with the use of donor T_{reg} therapy, however other methods such as ex vivo expansion or genetic modification of T1D T_{regs} are being explored (68–70). Alternatively, both donor or third-party T_{regs} could be utilized.

IMPROVING TREG FUNCTION

Outside of HCT, clinical trials with T_{regs} have generally shown an excellent safety profile but generally unclear efficacy. This may be because many studies do not use lymphodepletion, which may help with T_{reg} engraftment (71, 72). Furthermore, the persistence of T_{regs} may be affected by the concomitant use of immunosuppressive drugs (73), with some evidence pointing to low dose IL-2 and rapamycin as a more effective strategy than other immunosuppressive regimins (74). Likewise, the use of low dose IL-2 and protein engineered IL-2 derivatives is being explored (75). Other promising methods of inducing T_{regs} *in vivo* such as the administration of tolerogenic $CD11c^+$ DCs or pharmacologic stimulation of T_{reg} are well described elsewhere (76–78).

Gene modification techniques have been proposed as alternative strategies to produce more active and efficacious T_{regs} in a large scale, involving two approaches: engineering T_{regs} with T cell receptor (TCR) (79–81) or chimeric antigen receptor (CAR) (82–84). Islet antigen-specific T_{regs}, generated using lentiviral-mediated TCR gene transfer, were capable of inhibiting effector T cells through antigen-specific suppression (81). This demonstrates the potential applicability of islet antigen-specific T_{regs} in the prevention of diabetes progression as well as in islet transplant settings. Islet antigen-specific T_{regs} generated using lentiviral transduction showed strong suppressive activity in an antigen-specific manner, providing a proof-of-concept for the potential use of TCR gene transfer technology-enhanced T_{reg} activity in islet transplantation (81). Thus, gene transfer technology is likely to be adapted to enhance the therapeutic efficacy of T_{regs} while avoiding the pan-immunosuppression effect of polyclonal T_{regs}.

CAR T_{regs} are genetically engineered cells which express single chain variable fragment that recognizes specific antigen on target cells in an TCR-independent fashion. Recently, CAR T_{regs} have received growing attention in different transplantation models (84). Insulin-specific CAR T_{regs} generated using retroviral transduction were shown by Tenspolde et al. to be functionally stable and suppressive *in vivo* (85). The adoptive transfer of ex vivo expanded recipient T_{regs} transiently expressing CAR to target the MHC-I of donor islets in murine models showed improved initial allograft engraftment and survival, with donor-specific tolerance mechanisms observed (86). These studies suggest CAR T_{regs} could exert site-specific and localized immunosuppression.

COMBINED ISLET AND BONE MARROW TRANSPLANTATION

The use of bone marrow to induce donor-specific tolerance has been tested in different solid organ transplantation models in preclinical and clinical studies in living donor transplantation (87–89). In a seminal study nearly 20 years ago, Sykes showed in murine models that immunological tolerance to allogeneic islets could be achieved in NOD mice with established disease through the bone marrow mixed chimerism across MHC barriers generated using a non-lethal dose of irradiation and a combination of anti-CD4, anti-CD8, anti-Thy1.2, and anti-CD40L mAbs (31). Since then, a number of studies have explored different conditioning regimens including those with

different radiation doses or chemotherapy (fludarabine, cyclophosphamide, or busulfan) without irradation (26, 90, 91). A common thread to these studies, lymphodepletion was generally required for alloreactive graft tolerance and sustained chimerism (92).

One remarkable finding of a number of studies that explored NOD recipients is that the mixed chimerism induced from donors was sufficient to overcome autoimmune islet attack. Zeng et al. explored how the degree of MHC mismatch might affect autoimmunity. They showed that increased MHC mismatch from NOD recipients more effectively protects against autoimmune islet rejection (93). It is likely that human cadaveric donors of human islets will be HLA-mismatched, a major question in the clinical translation is which HLA alleles might be overlapping or not.

Oura et al. evaluated islet allograft survival in non-human primates using MHC-mismatched cynomolgus monkeys and found that islet allograft rejection is prevented as long as mixed hematopoietic chimerism is obtained. This is different from tolerance to kidneys transplanted into the same monkeys that were obtained even with a transient mixed chimerism (25, 94). This suggests that islet allografts may be more immunogenic or complicated than solid organ allografts in terms of tolerance induction in humans.

COMPLICATIONS OF HEMATOPOIETIC CELL TRANSPLANTATION LIMITING THE APPLICATION OF MIXED CHIMERISM

One of the major issues with bone marrow transplantation is the intensity of conditioning which has evolved over the past decades with some approaches such as the use of total lymphoid irradiation and antithymocyte globulin (TLI/ATG) have a good safety profile in combined organ and HCT (95). Current developments in safer conditioning in sickle cell are also being explored (96, 97). One of the most promising approach for newer and far less toxic HCT is the use of monoclonal antibodies against hematopoietic stem cell niche constituents instead of radiation or chemotherapy that is now being explored in patients with immune deficiency (98–100). Another complication is graft versus host disease (GVHD) (101) which in part is in large part mediated by donor T cells (102, 103). Early trial results of an ongoing phase 2 clinical trial of T_{reg} therapy given at the time of HCT reinforce their capacity to prevent GVHD (104, 105). Aside from GVHD, dysregulated immunity can be a complication of GVHD which can include viral reactivation of cytomegalovirus (CMV) or Epstein bar virus (EBV) as well as susceptibility to pathogens or opportunistic infections (106, 107). Studies in the HCT setting have not shown an increased risk of viral reactivation with T_{reg} therapy. T_{reg} may help to regulate viral latency (108). In combined kidney and HCT studies in the HLA-matched and haploidentical setting, CMV reactivation might occur more frequently than in kidney transplant alone and appears controlled with antiviral medications (95), however the risks of immune dysregulation in the fully HLA-matched deceased donor setting remains unknown.

CRITICAL ROLE OF TREGS IN MIXED CHIMERISM AND GRAFT TOLERANCE

Following bone marrow transplantation, T_{regs} have been used to to prevent GVHD and to prolong allograft survival through the induction of mixed chimerism in combined marrow and organ transplantation (109). In this regard, studies report development of transplant tolerance by T_{regs} in the setting of mixed chimerism (35, 110), the dependency of tolerance on the presence of recipient T_{regs} (111), as well as the need for donor T_{regs} to prevent GVHD (112). In our murine studies, recipient T_{regs} have been shown to promote hematopoietic engraftment after HCT (86). Multiple other studies have shown that the addition of T_{regs} to conditioning increases donor hematopoietic engraftment (105, 113–115). It is interesting to observe a long-term graft tolerance even with the incorporation of T_{regs} which disappear shortly after infusion (73, 116). These findings suggest that the long-term graft survival might be due to the ability of the transferred T_{regs} to induce infectious tolerance. Recent studies show that both the donor and recipient T_{regs} contribute to suppress the alloreactive responses after HCT (105, 117, 118). The integration of T_{reg} therapy into combined organ or islet transplantation is therefore a potentially non-toxic method for improving tolerance induction and establishing mixed hematopoietic chimerism. We are currently testing this in an ongoing trial in combined kidney and HCT from living donors (NCT03943238).

Finally, in the context of islet transplantation with concomitant HCT to induced mixed chimerism, donor T_{regs} are likely more effective in preventing graft-versus-host disease (GVHD) based on preclinical models in which donor T_{regs} were found to prevent GVHD when given at the time of HCT (112).

CLINICAL TRIALS WITH TREGS

Clinical islet transplantation for T1D patients with severe hypoglycemia unawareness is an approved therapy in the majority of advanced nations (119). This population has severe morbidity and mortality; therefore, clinical trials are needed. Clinical trials integrating T_{reg} therapy and/or hematopoietic mixed chimerism into islet transplantation have been limited. An ongoing clinical trial (NCT03444064) is testing the integration of autologous polyclonal T_{regs} in T1D patients who are receiving the conventional Edmonton islet transplantation protocol. Another clinical trial (NCT03162237) of islet xenotransplantation is currently underway and involves transplantation of 10,000 islet equivalent (IEQ) of porcine islets and infusion of 2 million/kg autologous T_{regs} in the recipients receiving induction immunotherapy with belatacept

and maintenance immunotherapy with tacrolimus and mycophenolate mofetil. In the only report of combined islet and hematopoietic transplantation, a small six patient phase 1 trial integrating an infusion of cadaveric hematopoietic stem cells intravenously post-transplant did not successfully lead to donor chimerism or graft tolerance, but showed that the infusion of bone marrow cells from a cadaveric source is safe and potentially feasible (120).

CONCLUSION AND FUTURE PERSPECTIVES

Since the success of the Edmonton protocol in showing the benefit of islet transplantation to patients with hypoglycemia unawareness, the major challenge of achieving and maintaining tolerance remains. The integration of cell therapy approaches such as T_{reg} therapy, mixed hematopoietic chimerism, or a combination of both remain promising.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Potential Application of T-Follicular Regulatory Cell Therapy in Transplantation

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Regulatory T cells (Tregs) constitute a small proportion of circulating $CD4^+$ T cells that function to maintain homeostasis and prevent autoimmunity. In light of their powerful immunosuppressive and tolerance-promoting properties, Tregs have become an interesting potential candidate for therapeutic use in conditions such as solid organ transplant or to treat autoimmune and inflammatory conditions. Clinical studies have demonstrated the safety of polyclonally expanded Tregs in graft-versus-host disease, type 1 diabetes, and more recently in renal and liver transplantation. However, Tregs are heterogenous. Recent insights indicate that only a small proportion of Tregs, called T follicular regulatory cells (Tfr) regulate interactions between B cells and T follicular helper (Tfh) cells within the germinal center. Tfr have been mainly described in mouse models due to the challenges of sampling secondary lymphoid organs in humans. However, emerging human studies, characterize Tfr as being $CD4^+CD25^+FOXP3^+CXCR5^+$ cells with different levels of PD-1 and ICOS expression depending on their localization, in the blood or the germinal center. The exact role they play in transplantation remains to be elucidated. However, given the potential ability of these cells to modulate antibody responses to allo-antigens, there is great interest in exploring translational applications in situations where B cell responses need to be regulated. Here, we review the current knowledge of Tfr and the role they play focusing on human diseases and transplantation. We also discuss the potential future applications of Tfr therapy in transplantation and examine the evidence for a role of Tfr in antibody production, acute and chronic rejection and tertiary lymphoid organs. Furthermore, the potential impact of immunosuppression on Tfr will be explored. Based on preclinical research, we will analyse the rationale of Tfr therapy in solid organ transplantation and summarize the different challenges to be overcome before Tfr therapy can be implemented into clinical practice.

Keywords: regulatory T cell, T-follicular regulatory cell, transplantation, cell therapy, immunosuppression

INTRODUCTION

Over the last three decades, despite an improvement in short-term outcomes after solid organ transplantation, long-term outcomes have not drastically improved (1). There has been a massive leap in understanding the mechanisms that cause chronic allograft dysfunction (CAD) leading to graft loss, particularly in kidney transplantation. Immune mediated injury is the predominant cause of CAD, many cases of which are associated with the presence of donor specific antibodies (DSA), directed predominantly against donor human leukocyte antigens (HLA). These DSA are produced by B cells in response to alloantigen stimulus through a process that is T-cell regulated (2). The majority of traditional therapeutic strategies attempted have focussed on either targeting B cells or whole T cell population or on removing the DSA themselves, without more precise targeting. Targeting the immune allo-responses to regulate DSA responses might be a way to improve patient outcomes.

$CD4^+$ T cells play an important role in both activating other cells (including B cells) and in regulating the immune response (regulatory T cells-Tregs) (3). The old concept of T cell ability to help B cells has been further clarified recently with the discovery of a new small subpopulation of $CD4^+$ T cells, called T follicular helper cells (Tfh) (4–6). Tfh are responsible for the interactions with B cells in the germinal centers (GC) within the secondary lymphoid organs (SLO) (spleen or lymph nodes) (7). They support B cells in the process of antibody production (4). In addition, Tfh help in promoting the differentiation of B cells into memory B cells and long-lived plasma cells (8). Human and murine Tfh display similarities in phenotype (expressing CD4, CXCR5, PD1, and Bcl6; lacking expression of CCR7 and IL-7Rα and secreting IL-21) (9–11) and function, responsible of interaction and activation of GC B cells leading to antibody production.

Tfh display a Treg counterpart population called T follicular regulatory cells (Tfr), which have the role of regulating specific interactions between B cells and Tfh (12–14). Tfr have been extensively studied in mouse models, with fewer studies in humans, due to the challenges of sampling human SLO. In human, they have been characterized as being Tfh-like [expressing CD4, the C-X-C chemokine receptor type 5 (CXCR5) and Bcl-6 in association with CD25 and FOXP3] and express varying levels of PD-1 and ICOS depending on their localization. They can be found in SLO like tonsil, lymph nodes, spleen, and ectopic lymphoid structures and even in blood (bTfr) (15). The bTfr remain to be further characterized in humans and their function remains poorly understood. In general, while the role of Tfr in modulating autoimmune responses seems to be crucial, the exact role of these cells in the transplantation settings remains to be elucidated. Here, we review the current evidence around the origin of Tfr, the different subtypes and associated functions. We will discuss the role of Tfr in human diseases with a focus on transplantation and explore the potential of cell therapy using Tfr.

DEFINITION AND ORIGIN OF TFR

Definition of Tfr and Tfh With Focus on the Differences Between Mouse and Human Tf Cells

The discovery that only a small fraction of $CD4^+$ T cells was involved in antibody production (Tfh) and modulation (Tfr) within the GC is recent. Interestingly, although the role of Tregs in regulation of the GC response was first described more than fifteen years ago in human (16, 17), the formal discovery of the Tfr subtype occurred later in murine models (12–14). Tfr are proposed to form when $FOXP3^+$ precursors acquire a Tfh-like phenotype that includes expression of Bcl-6, CXCR5, PD-1, and ICOS. Although Tfr share some Tfh features, they do not express/produce the same cytokines which characterize Tfh, such as IL-21, IL-4. In addition to FOXP3, Tfr cells express the typical markers expressed by Tregs such as GITR, Blimp-1, and CTLA-4 (12–14).

Precise definition of Tfr as a sole entity is complicated by the dynamic expression of some chemokine receptors, particularly as the cells navigate between compartments within the SLO, and within the body (see section on *Maturation of Tfr*), as they can be found in blood and SLO like tonsil, lymph nodes, spleen and ectopic lymphoid structures. However, distinguishing between Tfh and/or Tfr with only one specific marker is almost impossible. Most of the work on these two subtypes of T cells has been done in mouse models with this work inevitably influencing study of human counterparts. CXCR5 is probably the most accepted and used marker for Tfh and Tfr (12–14). CXCR5 is a G protein receptor for the chemokine CXCL13. It enables T cells to migrate to the B cell zones of the lymph nodes. This is supported by the evidence that CXCR5 knock out mice present a complex pattern of lymph node developmental defects and a completely disorganized splenic microarchitecture, lacking segregated T- and B-cell areas (18). However, it has been demonstrated recently that some Tfr can access the GC independently of CXCR5 (19), and that after interaction with B cells they proliferate less than their $CXCR5^+$ counterparts. Interestingly CXCR5 expression seems to be regulated by nuclear factor of activated T cells 2 (NFAT2) in Tfr (20) but by achaete-scute homologue-2 (ASCL2) in Tfh (21). The involvement of NFAT2 regulation in Tfr was confirmed in Nfat2fl/fl x Cd4cre mice by the demonstration that NFAT2 knock out mice displayed reduced numbers of Tfr (20).

Until recently, studies describing $CD4^+CXCR5^+$ cells did not differentiate between Tfh and Tfr (4, 7, 11). It is not clear what proportion of the $CD4^+$ $CXCR5^+$ population are Tfr cells. A recent report suggested that $CD4^+CXCR5^+FOXP3^+$ cells accounted for only 12.8% of circulating $CD4^+$ $CXCR5^+$cells (22), implying that the majority of circulating $CD4^+CXCR5^+$cells were Tfh, although without confirmatory evidence of a more detailed phenotypic characterization (15).

Current evidence suggest that Tfr derive from Tregs, at least in mouse (see below), and represent 18.57±6.55% of the total $CD25^+FOXP3^+$ T cells (23). Therefore, FOXP3 is expressed in

Tfr at different levels through their differentiation process (see **Figure 1**). Another way to identify Tfr would be the use of the combination of markers $CD4^+CD25^{high}$ $CXCR5^+CD127^{low}$, as circulating Tfr are $CD25^{high}CD127^{low}$ as they originate from Tregs, while blood Tfh express heterogenous amount of CD25 (negative to low) and a low level of CD127 (24).

The phenotype and function of Tfr and Tfh both depend on the expression of Bcl-6 and STAT3 (5, 12, 14, 25, 26). However, only Tfr express Bcl-6 alongside the Bcl-6 antagonist Blimp-1, although this has only been demonstrated in mouse and not yet in human Tfr (23). While Bcl-6 is important for the Tfh-like properties of Tfr, Blimp-1 is associated with the Treg-like phenotype and function of Tfr (12, 27–29). Blimp1 is a transcriptional repressor protein that suppresses Bcl6 expression. Tfr numbers are regulated through a balance between Bcl-6 and Blimp-1 (12). IL-2 is a key factor regulating Tfr differentiation, promoting Blimp1 expression while repressing Bcl6 in Tregs to preclude Tfr cell development (30).

In humans, they have been characterized as being $CD4^+$ $CD25^+FOXP3^+CXCR5^+$ cells with different levels of PD-1 and ICOS expression depending on their localization (31, 32) (and on the transcription factor Bcl-6 for differentiation and localization into the B cell follicle (4–6, 14, 33). ICOS expression in human does not discriminate Tfr from other Tregs (34).

As none of the individual markers described above seems to be specific for Tfh/Tfr and blood, a combination of surface markers is necessary to be able to characterize these two subsets properly, from their origin to the fully matured T cells (see **Table 1** and **Figure 1**). The function of Tfr in a normal immunological response is described below.

Germinal Center Reaction

GC are defined structures that develop within the SLO during ongoing immune responses; they have been extensively studied and described in mouse models. Through a process called GC reaction, a naïve mature B cell first undergoes clonal expansion and somatic hypermutation within the dark zone of the GC before moving to the light zone (38). There, B cells demonstrating a relevant affinity toward the antigen of interest form cognate interactions with Tfh cells primed by the same antigen. These Tfh help promote B cell responses by providing cytokines (such as IL-21, IL-4) and co-stimulation (through the inducible costimulatory molecules ICOS and CD40L) (7, 35, 39–41). Continued cognate interaction between Tfh and B cells drives immunoglobulin class switching, somatic hypermutation, and B cell differentiation (42) leading to the production of long-lived plasma cells and memory B cells.

Mechanisms of Regulation by Tfr and Antibody Production

In adoptive transfer experiments of Tfr and Tfh in mice lacking these population of cells, the group of Sage et al. have demonstrated that Tfr have a direct impact on B cell effector function by decreasing antibody secretion, and inhibiting somatic hypermutation and class-switch recombination (23, 31, 36). However, Tfr can also act indirectly to inhibit antibody responses by specifically suppressing production of IL-4 and IL-21 by Tfh, leaving other functions intact (36).

Different approaches have been used to address the specific role of Tfr in regulation of GC responses. The first series of reports used Bcl-6 as a surrogate marker of Tfr in genetic and/or bone marrow chimera models. In these models, Bcl-6 was deleted in $FOXP3^+$ cells. The results were contradictory, with some studies indicating that Bcl-6 was essential for Tfr function (12, 43). Fu et al., for example, using Bcl-6fl/flFOXP3Cre (KO) mice, which have reduced numbers of $CXCR5^+PD1^+CD4^+FOXP3^+$ Tfr cells, demonstrated enhanced protection against influenza virus associated with an increase in humoral autoimmunity (43). Others, however have demonstrated that the lack of Bcl-6^+FOXP3^+ cells did not impact on the development of the Tfh-cell population and numbers of GC B cells, but did alter the levels and avidity of the antigen-specific IgG response (44).

As described earlier, Bcl-6 is not an absolute marker of Tfr and could be expressed by other Tregs. Therefore, Clement et al. (45), designed an inducible Tfr cell-deletion model aiming to study the role of Tfr in an intact host. They generated a strain of mice called T_{FR}-DTR (for Diphtheria Toxin receptor) where DTR expression is under the control of a recombinant *Cxcr5* gene in cells expressing FOXP3. Thus, only Tfr cells expressing both FOXP3 and CXCR5 expressed DTR on their surface so were susceptible to deletion by DT. After immunization with (4-hydroxy-3-niotrophneyl)acetyl-ovalbumin (NP-OVA) Tfr in these mice regulated only early GC responses to antigen-specific antibody and B cell memory. Lack of Tfr was associated with a surge of self-reactive IgG and IgE, demonstrating a key role of Tfr in preventing these potentially deleterious responses post-vaccination. Both blood Tfh (bTfh) (46) in HIV+ patients, and bTfr seem to have a memory function and able to be redirected toward antigen re-exposition in other diseases (8, 36).

Although the mechanisms of regulation by Tfr *in vivo* are still under investigation and what is known has been reviewed recently (23, 47, 48), a summary of some of the most important functions of Tfr is set out below. CTLA-4 expression by Tfr has been shown to be key for their function, as conditional deletion of CTLA-4 on Tfr inhibits their function, GC B cells are not inhibited and this leads to increased amounts of antibody produced (47). Furthermore, Tfr cells inhibit antigen-specific IgG levels when adoptively transferred into CTLA-4 inducible knockout (KO) mice (47) or mice immunised with NP-OVA. MOG-CFA and NP-HEL (31, 36). This will be discussed more when we describe the effect of immunosuppressive drugs on Tfr later on in this review. The modulation of the metabolism of GC B cells and Tfh could be another regulatory mechanism used by Tfr, leading to inhibition of production of IL-21 and IL-4 by Tfh and inhibition of class-switch recombination and antibody production by B cells (23). Tfr have been shown to produce TGF-β and IL-10 which is turn could lead to inhibition of B cells responses (47). It could be possible that Tfr produce granzyme B leading to B-cell and/or Tfh cytolysis (23, 47). Eventually, a direct mechanical disruption of Tfh and GC B cell has been hypothesised, but this has not been

TABLE 1 | Different expression of markers in different types of Tfr and Tfh compared to Tregs and T naive.

	T Naive	bTfh (blood)	cTfh (central GC)	Treg	eTfr	bTfr	iTfr	mTfr	Ref
CD4	++	++	++	++	++	++	++	++	(8, 11–15, 24, 31, 35)
CXCR5	-	+++	+++	-	++	++	++	+++	(8, 11–15, 24, 31, 35, 36)
CD25	-	-	-	+++	+++	+++	+	–	(8, 11–15, 24, 31, 35)
FOXP3	-	-	-	++	++	++	++	++	(8, 11–15, 24, 31, 35)
ICOS	+	-	++	+	?	-	++	+++	(8, 11, 12, 15, 24, 31, 35, 36)
PD-1	-	-	++	±	–	-	+	++	(8, 11–15, 24, 31, 35)
Bcl6	-	-	++	-	+	-	+	++	(8, 11–13, 15, 24, 35)
Blimp1	-	-	-	+	+	?	+	+	(8, 11, 12, 15, 24, 31)
CTLA-4	-	+	?	+++	?	+++	+++	+++	(12, 15, 37)

Expression of different surface markers (CD4, CXCR5, CD25, ICOS,PD-1) and intracellular markers (FOXP3, Bcl-6, Blimp-1, CTLA-4) in different subtypes of T cells: Naïve T cell, blood T follicular helper cells (bTfh), central Tfh (cTfh), regulatory T cells (Tregs), early T follicular regulatory cell (eTfr), blood Tfr (bTfr), intermediate Tfr (iTfr), and mature Tfr (mTfr). The markers described have been identified in both mouse and human (dark gray), in mouse only (light gray), or hypothesized in the publication from Fonseca et al., in Immunol rev 2019 (white). Of note, "bTfr" refers to blood Tfr, and "cTfr" to circulating Tfr as indicated in some publications (7, 15). mTfr refers to mature Tfr and these subtype of Tfr has been described within the germinal centre.
"-" refers to no expression, "+" refers to low expression, "++" refers to intermediate expression, and "+++" refers to high expression.

proven yet (47). Finally, Tfr regulates the interaction between Tfh and B cell during the GC reaction and limits the size of the GC reaction. They inhibit the production of high-affinity antibodies specific for self-antigens (48) and limits both self-reactive and non-specific responses (see **Figure 1**).

A distinct population of helper cells involved in B cell responses has been recently described by Rao et al. (49), as PD-1^{hi} $CXCR5^{-}$ $Bcl6^{lo}$and called T peripheral helper (Tph) cells. The original description of these cells was in a model of Rheumatoid Arthritis, but they have also been recently described as important in type 1 diabetes (50) and in the pathogenesis of lupus (51). It is currently not known whether Tfr can regulate this population, nor whether Tph are relevant to transplantation.

Origin of Follicular Regulatory T Cells

Tfr have been found in spleen, lymph nodes and lymphoid tissues as well as in the lymphatic and blood circulations. Tfr cells were initially thought to arise from natural (thymus-derived) Tregs (12), that become induced upon TGFβ signaling in the periphery (14). Linterman et al. (12) found Tfr resembled Treg more closely than Tfh due to the elevated expression of many Treg associated genes; *FOXP3, Ctla4, Gitr, Klrg1*, and *Prdm1* as detailed above. However, Tfr also expressed high amounts of the *CXCR5, Pdcd1, Bcl6, CXCL13* (9) and *ICOS*, the typical Tfh genes. Tfr did not express receptors for the helper cytokines IL-21 or IL-4 or the costimulatory ligand CD40L. Furthermore Linterman et al., reported that 97% of Tfr cells express Helios, a transcription factor expressed by thymus-derived Treg cells. Thus, the origin of Tfr cannot be determined by genetic analysis alone.

To shed further light on the origin of Tfr, Linterman et al., transferred naïve cells ($CD4^{+}CD44^{lo}CD25^{-}$) from mice expressing the 3A9 TCR transgene recognising a hen egg lysozyme peptide (HEL) into congenic mice. After being challenged with HEL, no donor originating Tfr could be identified with all Tfr deriving from recipient cells. Furthermore 6 days after selective ablation of all $FOXP3^{+}$ Tregs using a diptheria toxin receptor inserted in the FOXP3 locus, Tfr were absent in diptheria treated mice, indicating that Tfr cannot form if $FOXP3^{+}$ cells are absent, suggesting that Tfr development requires the presence of $FOXP3^{+}$ Tregs.

Chung et al. (14), similarly sought to trace the origin of $CXCR5^{+}$ Treg in mice. They found that $CXCR5^{+}FOXP3^{+}$ Tregs were essentially absent in the thymus compared to the spleen. To determine whether $Bcl6^{+}CXCR5^{+}$ Treg cells were generated from naïve $CD4^{+}$ or natural Treg precursors in the periphery, they mixed $CD45.1^{+}$ naïve $CD4^{+}$ T cells ($CD25^{-}GITRCD44^{lo}CD62L^{hi}$) and $CD45.2^{+}CXCR5^{-}$ Treg from FOXP3gfp mice, The T cells were injected into Tcrb-/- mice, which were deficient in alpha beta T-cell receptor and consequently had ~ 6% $CD4^{+}CD8^{+}$ of *wt* (52). This was followed by immunization with keyhole limpet hemocyanin (KLH) in complete Freund's adjuvant (CFA). 98.2% of $Bcl6^{+}FOXP3^{+}$ cells in the recipient mice were $CD45.2^{+}$, indicating that the origin of $Bcl6^{+}CXCR5^{+}$ Treg is from $CXCR5^{-}$ Treg. Furthermore, they found that the majority of $CXCR5^{+}$ $FOXP3^{+}$ cells expressed Helios. While Chung et al. and Linterman et al., showed Tfr differentiate primarily from $FOXP3^{+}$ Treg precursors, the former concluded that $Bcl6^{+}$ $CXCR5^{+}$ Treg cells are absent in the thymus but induced in the periphery from $CXCR5^{-}FOXP3^{+}$ natural Tregs.

It may be that the differentiation of Tfr requires numerous stimulations. Thus, the thymus provides the microenvironment for Treg precursors to acquire CD31 (53) and Helios but then the subsequent differentiation of Tfr occurs by further stimulation in peripheral lymphoid tissue (15).

As a counter to Tfr deriving solely from $FOXP3^{+}$ natural Tregs, Aloulou et al. (54), proposed they may also derive from FOXP3 negative precursors such as naive $CD4^{+}$ T cells. They demonstrated that naive $CD4^{+}$ T cells can become Tfr cells in murine models using an adjuvant that promotes peripheral Treg cell formation. This may occur in the context of a stimulus that promotes the conversion of $CD4^{+}$ $FOXP3^{-}$ cells into $FOXP3^{+}$ Treg cells, specifically, one that enhances PD-L1 expression on antigen presenting cells. Whether these 'induced' Tfr cells emerge from Tfh cells that acquire FOXP3 expression, or from peripheral Treg cells that acquire the follicular fate through CXCR5 expression awaits further investigation. However, Tfh cells cannot be induced to switch on FOXP3 *in*

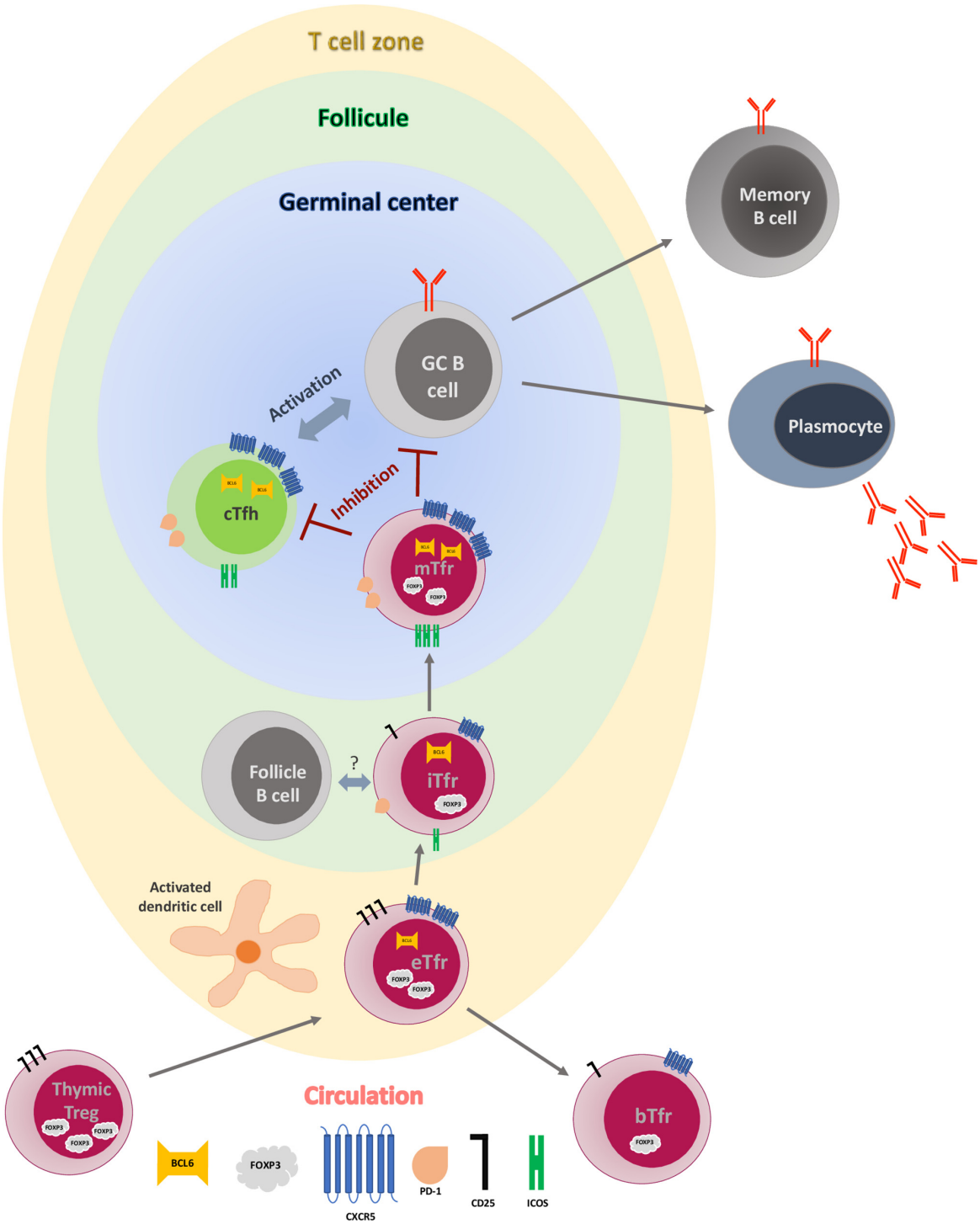

FIGURE 1 | Early germinal center reaction and T follicular regulatory cell maturation process. Early Tfr (eTfr) derive from natural regulatory T cells (Tregs) after expression of CXCR5 and Bcl-6 and down regulation of FOXP3. After interaction with activated dendritic cells (DC) in the T cell zone, some eTfr lose expression of Bcl-6 and enter the circulation (blood Tfr-bTfr), while some migrate to the follicle (intermediate Tfr-iTfr), where they interact with the follicular B cells and start expressing PD-1 and ICOS. Eventually, they move to the germinal center (GC), becoming mature Tfr (mTfr), where they inhibit both the central T follicular helper cell (cTfh) and the GC B cells, leading to regulation of antibody production and B cell differentiation.

vitro (13), once again suggesting that it is more likely that it is peripheral Treg cells that give rise to Tfr cells.

Again, the concept that Tfr can arise from FOXP3$^-$ T cells has been countered by Maceiras et al. (55). They used congenic mice to investigate the precursors of Tfr cells following immunization in two distinct genetic backgrounds without the confounding issue of lymphopenia. They found that the adoptive transfer of thymic-derived FOXP3$^+$ Treg in mice can differentiate into Tfr but FOXP3$^-$ T cells only differentiate into Tfh. Additionally, they demonstrated that Tfh and Tfr pools are generated from distinct TCR repertoires, with Tfh cells expressing antigen-responsive TCRs to promote antibody responses, and Tfr cells expressing potentially autoreactive TCRs to suppress autoimmunity, strengthening the idea that Tfr and Tfh are derived from distinct populations. The proposed origin of Tfr in murine models is summarized in **Table 2**.

As discussed above, early Tfr (eTfr), derive most probably from natural Tregs in the periphery, and, after expression of Bcl-6 and CXCR5, are attracted to the T cell zone of SLO, where they interact with activated dendritic cells (**Figure 1**). This priming step is required by the Tfr as the number of these cells were reduced in a model of immunised mice with 4-hydroxy-3-nitrophenylacetyl hapten–conjugated OVA (NP-OVA), where dendritic cells have been ablated (36). Within the T-zone eTfr can have two different fates. Following the interaction with follicular B cells, they can lose their Blimp-1 expression, upregulate Bcl-6, ICOS, and PD-1 and transfer to the T-B border in a CXCR5 dependent manner, becoming "intermediate Tfr" (iTfr) (15). Again, this step is crucial for full differentiation of Tfr, as Tfr were almost absent in draining lymph nodes of immunized mice that lack B cells (36). The molecular mechanisms associated with the interactions between follicular B cells and iTfr remains to be determined, however, there is some evidence that this step could be antigen-independent (36, 55–57). Conversely, eTfr can retain an immature phenotype, fail to express Bcl6, and access the circulation, becoming CD25$^+$ CXCR5$^+$FOXP3$^+$Bcl6$^-$ blood Tfr (bTfr) (34, 36). Therefore, the presence of bTfr could be the footprint of a truncated GC formation regulation and the consequence of this could be an increase proliferation of Tfh and antibody production. In some auto-immune diseases the presence of bTfr in the blood correlated with the severity of disease (53, 58, 59). Although bTfr have been described in human (15), they have not been fully characterized and their function remain poorly understood. In particular, their precise role in transplantation remains to be elucidated.

The iTfr migrate then to the GC where they can become fully mature Tfr (mTfr) with very efficient suppressive capacities (30). It is at this stage that they lose the CD25 expression and upregulate Bcl-6, ICOS and PD-1 (60). In human, these cells are able, not only to inhibit Tfh activation (therefore decreasing their production of IL-4, IL-21) and suppress Tfh cell-GC B cell interactions leading to antibody production (61) but also decrease the activation of B cells through PD-1/PD-L1 interactions and the inhibitory function of CTLA4 molecules. Moreover, it has been demonstrated that they inhibit the capacity of class switching from IgM to IgG in mouse (23) and decrease IgA production by B cells in human (62), therefore inhibiting the selection of non-antigen-specific B cells (including those with self-reacting BCR) and limiting the number of B cells (indirect regulation). The regulation of B cells by Tfr is mechanistically complex and context-dependant as demonstrated very recently by Lopez-Ocasio Maria et al. (63). They showed with experiments *in vitro* that when the BCR was engaged, B cells were more resistant to suppression by Tfr, and this was dependent on a CD40-CD40L-associated mechanism.

The localization of these potential Tfr subtypes are not exclusive, and it has been demonstrated histologically that both iTfr and mTfr can be present in the Follicle and in the GC (60). It remains unknown if mTfr and iTfr could recirculate in the blood. However, it seems to be possible for bTfr to migrate back to Follicle and GCs (36). The Tfr regulation within GC seems to be happening in early GC formation stages (45), as Tfr seem to be less frequent in fully developed GCs (64) and might be inhibited by cytokines produced in GCs (65).

In summary, even if further work is needed in human, Tfr seem to derive from thymic Trefs. bTfr seems to be CD4$^+$CD25$^+$FOXP3$^+$CXCR5$^+$PD-1^{low}ICOS^{low}, whereas mTfr could be described as CD4$^+$CD25$^+$FOXP3$^+$CXCR5$^+$ PD-1$^+$ICOS$^+$Bcl-6$^+$. The exact role of these different subpopulations still needs to be investigated, particularly in the context of transplantation.

TABLE 2 | Proposed cells of origin of Tfr in mouse models.

Reference	Conditions	Originating Cell	Definition of Tfr
Linterman et al., *Nat Med. (2011)* (12)	Selective ablation of FOXP3+ Tregs using diphtheria toxin meant no Tfr developed	FOXP3+ Tregs	CD4+CXCR5^{high} PD-1^{high}FOXP3+
Chung et al., *Nat Med. (2011)* (14)	Induced in the periphery from CXCR5-FOXP3+ natural Tregs	CXCR5- Treg	*CD4+Bcl6+CXCR5+ FOXP3+*
Aloulou et al., *Nat Commun. (2016)* (54)	Incomplete Freund's Adjuvant promotes conversion of CD4 +FOXP3- cells into FOXP3+ Tregs	FOXP3- naive CD4+ T cells	*CD4+ CXCR5+ PD-1+ FOXP3+*
Maceiras et al., *Nat Commun. (2017)* (55)	Adoptive transfer of thymic-derived FOXP3+ but not FOXP3- cells into congenic mice showed development of Tfr	Thymic-derived FOXP3+ Treg	*CD4+CXCR5+ PD-1+FOXP3+*

ROLE OF TFR IN HUMAN DISEASES

Tfr have been described in responses to influenzae vaccination and chronic infections associated with hepatitis C virus (HCV), human immunodeficiency virus (HIV) and hepatitis B virus (HBV) (46, 66–68) and they are particularly relevant in the settings of autoimmune (AI) diseases. Tregs are at the core of the physiopathology of autoimmune diseases as their role is to regulate the responses to self-antigen as demonstrated by an association between autoimmune conditions and defects in Treg function (37). Tfr have been described and characterized in several autoimmune diseases (53, 58, 59, 69). They represent a critical peripheral tolerance mechanism, to prevent GC derived auto-immunity. Patients suffering from auto-immune diseases may have an unbalance of Tfr between blood and LN in favour of blood naïve Tfr, leading to a non-specialized response in the LN (59). In this review, we will focus on the role of Tfr and their impact on the immune responses in the setting of bone marrow and solid-organ transplantation.

Relevance of Tfr in Bone Marrow Transplantation

Graft *versus* host disease (GvHD) is a significant complication of allogeneic hematopoietic stem cell transplantation, whereby transplanted donor cells recognise recipient antigens as foreign. This may be acute (aGvHD) or chronic (cGvHD). In cGvHD, alloreactive Tfh cells and germinal center (GC) B cells have a crucial role in GC reactions to produce pathogenic antibodies, as evidenced by the reduction in severity of cGvHD in mouse models when these antibodies are inhibited (70). Although Tfr can inhibit GC reactions by acting as negative regulators of B cell function (71), Treg numbers are reduced in patient samples of cGvHD (72) likely contributing to cGvHD pathogenesis.

McDonald-Hyman et al. (73) found mice with cGvHD had significantly fewer Tfr in line with patient data, suggesting that a loss of regulation by Tfr associates with cGvHD. However daily therapeutic interleukin-2 complexed with the JES6-1 clone of anti-IL-2 antibody (IL-2/mAb) increased Tfr numbers, due to the fact they preferentially bind to $CD25^{hi}$ cells, while Tfh numbers were consequently reduced. Markers of cGvHD were also reduced as assessed by tissue pathology scores and pulmonary function tests. This effect was not seen in aGvHD since treatment with IL-2/mAb complexes led to an expansion of total Tregs and $CD8^+$ Tconv likely counterbalancing Treg expansion. They did not examine Tfh or Tfr subsets in this context.

The same authors also tested *wt* Treg infusions in cGvHD, which increased Tfr, while Tfh and GC B-cell frequency, GC size, and tissue pathology scores were significantly reduced in these mice. Thus this implies boosting Tfr can ameliorate cGvHD. They found that these events were CXCR5 dependent since CXCR5KO Tregs given during cGvHD once GC had formed had no effect on lung function, Tfh or GC B cells but did have improve lung function if given prophylactically. The lack of any effect correlated with the Tfr numbers that was not increased but was to a similar level to the one observed in mice with cGvHD. This suggests the importance of targeting Tregs homing to formed GC. Furthermore, the miR-17–92 cluster has been found to facilitate Tfh-cell differentiation and impair Tfr/Tfh balance, thus accelerating the development of cGvHD in mice (74).

Comparatively, Kamihara et al. (75) performed functional assays and flow cytometry on cryopreserved human PBMC of healthy donors and those undergoing allogeneic stem cell transplantation and those with active GvHD on IL2 therapy. Numbers of Tfr were significantly reduced in those who had undergone allo-SCT compared to healthy donors (median 0.08 vs 0.34% of $CD4^+$ T cells respectively). Patients with active cGvHD also had significantly lower Tfr cell frequency compared to matched patients with none or resolved cGvHD. *In vivo* administration of low dose IL-2 therapy for one week led a selective expansion of Tfr which remained stable during the 12 weeks of therapy. The Tfr had increased expression of CD25, FOXP3, CTLA-4, ICOS, Helios, Ki67 Bcl6, and p-STAT5. In contrast, activated $ICOS^+PD\text{-}1^+$ circulating Tfh were suppressed during IL-2 therapy. The selective activation of circulating Tfr and suppression of circulating Tfh provide a mechanism whereby low dose IL-2 therapy can promote both B and T cell tolerance in patients with cGvHD. In summary, these studies suggest a reduction of Tfr correlates with active or chronic GvHD and by boosting numbers of Tfr, either with cell infusion or IL-2, this may prove an effective therapeutic strategy.

Relevance of Tfr in Solid Organ Transplantation

Tfr in Transplantation and Alloantibody Responses

The role of Tfh in transplantation and alloantibody formation has been extensively studied over the last decade (76–78). Some human observational studies have described an increase in bTfh cell numbers in patients with transplant rejection (78, 79), and a reduced proportion of bTfh cells in patients with operational tolerance (80). Tfh cells have been shown in biopsies of patients with acute kidney rejection (77) and in ectopic lymphoid structures in kidney biopsies of acute T cell rejection (81). However, the role of Tfr in the alloimmune context needs to be explored. Some have postulated that Tfr prevent antibody responses in the context of low levels of antigen (36) and/or when low-affinity BCR are produced after somatic hypermutation (12). These situations do not fit with the transplant setting where the antigens are persistent.

Tfr in Acute and Chronic Rejection in Solid Organ Transplantation

Although the exact role of Tfr in transplantation and in antibody production needs further investigation, analysis of phenotype and/or frequency and/or function of these cells might be helpful for diagnostic purpose. Extensive work has been done to link Tfh with rejection (82, 83), or exploring Tfh/Tfr ratio in the context of autoimmune diseases (59). The published data evaluating Tfr in solid organ transplantation other than kidney is scarce. One study in a mouse model of lung transplantation (84) has demonstrated that bronchus-associated lymphoid tissue-resident $FOXP3^+$ T lymphocytes expressing CXCR5 were responsible for the prevention of antibody-mediated rejection. **Table 3** summarize the studies of Tfr in kidney transplant

TABLE 3 | Studies focusing on T-follicular regulatory cells in human and solid-organ transplantation.

Reference	Conditions	Loc	Definition	Major findings	Comments
Wallin EF et al, Blood (2014) (62)	- 5 KTR ttt with rituximab - 21 controls	*Lymph node*	CD4+ CXCR5+ FOXP3+ CD127- CD57+	- Tfr are present in LN of KTR - Rituximab had no impact on Tfr cells - Tfr cells reduce IgA production by B cells *in vitro*	
Xu X et al, Immunol Invest (2016) (85)	- 29 CAR - 5 hyperacute rejection - 12 acute allograft rejection	*Kidney*	CXCR5+ FOXP3+	- Tfr were rarely present in kidney tertiary lymphoid structures	Definition of CAR, acute rejection not clear
Chen W et al, Scientific reports (2017) (86)	- 88 KTR with chronic allograft dysfunction (CAD) incl.40 with biopsy proven ABMR - 30 controls	*Blood* *Kidney*	CD4+ CXCR5+ ICOS+ FOXP3+ CD127- CD4+ CXCR5+ FOXP3+	- ABMR: lower numbers of bTfr and kidney Tfr compared to non AMBR CAD+ - Tfr from ABMR display normal inhibitory function - Sirolimus decrease ratio of Tfr - Tfr inhibit B cell proliferation and differentiation in KTR - Tfr regulation of B cell is dependent on CTLA4	No definition of CAD
Yan L et al, BMC Immunology (2019) (79)	- 34 CAD incl. 21 with biopsy (11 ABMR, 2 TCMR, 9 no rejection) - 33 controls	*Blood*	CD4+ CXCR5+ FOXP3+	- Decreased frequency of bTfr and increased bTfh : Tfr ratio in CAD group - Increased serum CXCL13 and decreased serum TGF-β in CAD - bTfh:bTfr independent risk factor for low GFR and CAD	CAD = eGFR < 60 ml/min/ $1.73m^2$ after 3 months post KT
Niu Q et al, Frontiers Immunol (2020) (61)	- 211 KTR 5-7 years after Tx, inc 24% with background of rejection - 30 controls	*Blood*	CD3+ CD4+ CXCR5+ FOXP3+	- Decreased cTfr to cTfh in transplanted patient compared to controls - No association between anti-HLA antibodies or DSA and cTfr ot Tfh	

KTR, kidney transplant recipients; Tfr, T-follicular regulatory cell; cTfr, circulating Tfr; bTfr, blood Tfr; LN, lymph nodes; IgA, immunoglobulin A; GC, germinal centers; CAD, chronic allograft dysfunction; CAMR, chronic antibody mediated rejection; ABMR, antibody-mediated rejection; TCMR, T-cell mediated rejection; ttt, treated; CAR, chronic allograft rejection; Tx, transplantation; HLA, human leucocyte antigen; DSA, donor-specific antibodies.

patients. The definition of Tfr varies and the markers selected to characterize the Tfr do not allow to distinguish between the different subtypes of Tfr (bTfr vs iTfr or eTfr). However, there seems to be a trend toward lower levels of bTfr and chronic rejection (79, 86) and a potential correlation between low levels (79, 86) of bTfr and reduced kidney function as demonstrated by low estimated glomerular filtration rate (eGFR) (79). However, the number of bTfr in the transplanted patients was not compared to a group of patients with kidney dysfunction. Moreover, the definition of CAD used in these studies was not uniform or not clearly stated. Therefore, it is difficult to know if the decrease in bTfr numbers was secondary to the degree of uraemia (87). As yet Tfr in patients with end-stage renal disease (ESRD) or on haemodialysis has not been assessed. These results could seem inconstant with the findings of high bTfr in the auto-immune settings described above, however there are no definite explanation for these apparent inconstancies. Only two studies managed to collect some lymphoid structures from transplanted patients: Tfr were present in LN from a patient who had a kidney transplantation (62) but rare in kidney tertiary lymphoid organs in patients with either chronic or acute rejection (85). In conclusion, the current evidence is trending toward a decrease of bTfr in patients with CAD, however this needs to be confirmed with bigger numbers of patients and a more consensual definition of CAD.

Tertiary Lymphoid Organs

Tertiary lymphoid organs (TLOs) are ectopic lymphoid aggregates frequently observed in tissues affected by non-resolving inflammation as a result of infection, autoimmunity, cancer, and allograft rejection (88–91). They vary from tight clusters of T and B cells to highly ordered structures resembling the cellular composition of lymphoid follicles typically associated with secondary lymphoid organs (SLOs), such spleen and LN (90). The process whereby inflammatory cells infiltrate chronically rejected grafts and are progressively organised into structures has been termed lymphoid neogenesis (92). Although TLOs within tissues show varying degrees of organization, they frequently demonstrate segregated T and B cell zones, follicular dendritic cell networks, a supporting stromal reticulum, and high endothelial venules. In this respect, they mimic the activities of germinal centers and contribute to the local control of adaptive

immune responses. However, unlike SLOs but akin to mucosa-associated lymphoid tissue, TLOs do not have afferent lymph vessels and are not encapsulated, suggesting they are directly exposed to local antigens or cytokines (93). Studies in various disease settings have described how these structures can contribute to either beneficial or harmful outcomes. In the context of transplantation and DSA production whether lymphoid neogenesis is harmful, beneficial, or simply a bystander occurrence remains to be fully elucidated.

Several groups have reported that TLOs can amplify anti-graft immunity and accelerate tissue destruction. In murine studies of heart and skin transplantation, TLO formation has been associated with rejection (89). Infiltrating lymphocytes were found to be composed of both B cells and follicular-helper like CD4+ T cells in rat aortic allografts, and to be associated with antibody production independent of SLOs, suggesting local antibody production (85). This group went on to find ectopic GCs in all explanted human cardiac (n=5) and renal allografts (n=24) undergoing chronic rejection but not control organs (88). Histological examination revealed B cells near $CD23^+$ follicular DC surrounded by $CD3^+$ T cells, although further phenotyping to identify Tfr was not done.

Other studies suggest that the presence of Tregs in TLO might promote graft tolerance, thereby slowing down the kinetics of chronic rejection (92, 94–98). Xu et al. (85) measured the distribution of TLOs and the expression of FOXP3 and CXCR5 in explanted human renal allografts with chronic rejection. $FOXP3^+$ Tregs were detected in 10/29 chronically rejected grafts and 1/12 acutely rejected grafts and this did not correlate with lymphoid neogenesis or prolonged graft functioning. $CXCR5^+$ $FOXP3^+$ Tfr cells were rare in both chronically and acutely rejected grafts with TLO vs. those without. $CXCR5^+$ $FOXP3^+$ cells were present in 7/29 chronically rejected grafts but none of the acutely rejected grafts, implying Th17 but not Tfh could be involved in lymphoid neogenesis.

In summary, the possibilities remain that 1) lymphoid neogenesis is simply an epiphenomenon related to graft duration as proposed by Thaunat (92), 2) that Treg numbers are diminished in rejected organs in TLO, or 3) that there is another regulatory cell that aids TLO-mediated tolerance.

Effect of Immunosuppression

Regardless of the type of organ transplanted, recipients require some degree of immunosuppression to prevent allograft rejection; this is usually in the form of induction and then subsequent lifelong maintenance immunosuppression. Many of the immunosuppressive agents are T cell targeted and result in disruption of T cell homeostasis. A summary of studies focusing on the effect of immunosuppressive agents on human Tfr is presented in **Table 4**.

TABLE 4 | Summary of studies focusing on the effect of immunosuppressive agents on human Tfr.

Reference	Conditions	Sample	Definition	Major findings
Wallin EF et al, OBM Transplant. (2019)	Alemutuzumab induction for 19 SPK and 23 KTR vs 18 basiliximab treated KTR	Blood	CD4+ CXCR5+ FOXP3+ CD127lo	Tfr and Tfr : Tfh significantly lower in alemtuzumab treated patient up to 24 months post-transplant Trend toward lower Tfr in those developing *de novo* DSA
Wallin EF et al, Front Immunol. (2018) (99)	16 live donor KTR 1 week pre-treated with tacrolimus vs 45 deceased donor SPK or KTR	Blood and lymph node	CD4+ CXCR5+ FOXP3+ CD127lo	Decreased bTfh and lymph node Tfh. Trend towards fewer Tfr Co-culture of memory B cells and Tfh with tacrolimus showed lower plasmablast differentiation and antibody production
Chen W et al, Scientific reports (2017) (86)	30 controls 88 KTR with CAD incl.40 with biopsy proven ABMR	Blood	CD4+ CXCR5+ FOXP3+ CD127– ICOS+	In vitro tacrolimus increased Tfh1, decreased Tfh2 and Tfh17, no change in Tfr In vitro rapamycin reduced ratio of Tfr, no effect on Tfh1, Tfh2, and Tfh17 cells Overexpression of CTLA4 increased Tfr proportion and associated with less B cell proliferation
Niu Q et al, Expert Rev Clin Immunol. 2019 (100)	KTR on Tac, MMF and steroids	Blood	CD3+ CD4+ CXCR5+ FOXP3+	Lower numbers of bTfr associated with anti-HLA antibodies and worse renal function
Niu Q et al, Front Immunol. (2020) (61)	211 KTR 5-7 years after Tx, inc 24% with background of rejection 30 controls	Blood	CD3+ CD4+ CXCR5+ FOXP3+	Lower bTfr, no difference in bTfh, thus decreased bTfr : Tfh in KTR vs HC Previous rejection had reduced Tfh and Tfr – Tfr : Tfh same No association between anti-HLA antibodies or DSA and bTfr to Tfh Alemutuzumab and MP treated patients had significantly lower bTfr and bTfh

SPK, simultaneous pancreas kidney transplant; KTR, kidney transplant recipients; Tfr, T-follicular regulatory cell; bTfr, blood Tfr; CAD, chronic allograft dysfunction; ABMR, antibody-mediated rejection; Tx, transplantation; HLA, human leucocyte antigen; DSA, donor-specific antibodies; MP, methylprednisolone; Tac, tacrolimus; MMF, mycophenolate mofetil; HC, healthy controls.

Early Transplant Immunosuppression

Induction T cell depleting agents include anti-thymocyte globulin (ATG) and alemtuzumab which is a monoclonal antibody against CD52 expressed by most lymphocytes. Comparatively, another induction agent basiliximab, is a non–T cell depleting monoclonal antibody specific for the IL-2Rα receptor (CD25). Blockade of CD25 is designed to prevent T cells activation, in part by blocking the effect of autocrine IL-2 production and also to reduce T cell activation of B cells (101). Whether it affects Tfh and Tfr equally is unclear.

Lymphopenia-induced proliferation after depletion initiates repopulation of $CD4^+$ T cells with a phenotype skewed toward effector memory pool and a significant decrease in the naïve pool (102). These cells have a lower threshold for activation, can circulate to the graft and are less dependent on costimulation for activation (56). Thus, a predominance of memory T cells could contribute to graft injury and rejection. Conversely, the frequency and memory differentiation of $CD4^+$ T cells post-basiliximab induction remains unchanged (103).

In an ATG treated group of renal transplant patients, Macedo et al. (104) found that the absolute numbers of circulating Tfh ($CD45RO^+CXCR5^+CD4^+CD3^+$) were significantly decreased at all time points up to 360 days post induction. The percentage of Tregs was overall decreased in the ATG group, although they did not evaluate the Tfr numbers. Furthermore, the Tfh repopulation following ATG was found to be skewed toward Th1 polarization and effector memory. There was also a correlation of higher Th1 polarized cTfh cells relative to Tregs numbers in those that developed DSA. This suggests that rising Tfh numbers post-transplant may associate with DSA occurrence in the context of ATG induction.

Wallin et al. (105) compared induction with alemtuzumab in simultaneous pancreas-kidney (SPK) vs. renal transplant patients receiving basiliximab. $CXCR5^+IL\text{-}7R^{lo}FOXP3^+CD4^+$ bTfr cells remained significantly lower in alemtuzumab treated patients, both in proportion of total CD4 population and absolute cell count (compared to basiliximab patients at almost all time points up to 24 months post-transplant) despite being significantly higher in this group prior to transplant. There was also a trend toward a lower proportion of $CXCR5^+IL\text{-}7R^{lo}FOXP3^+CD4^+$ Tfr cells in patients developing *de novo* DSA compared to those who did not, but this was not statistically significant owing to low patient numbers developing *de novo* DSA.

Overall, the ratio of circulating Tfr : Tfh between treatment groups was significantly lower in all alemtuzumab patients compared to basiliximab treated patients up to 24 months post-transplant, reflecting the persistent low levels of Tfr cells in alemtuzumab treated patients despite a recovering bTfh population. The fact that both alemtuzumab use and *de novo* DSAs associates with a pattern of low bTfr:cTfh is one putative explanation of why alemtuzumab patients develop higher rates of *de novo* DSA post-transplant (106), but necessitates further elucidation to establish causality.

Glucocorticoids are frequently used at least in the early stages of post-transplant immunosuppression or during rejection. They act *via* inhibition of cytokines such as IL-1, TNF alpha, IFNγ and IL-6. A study by Wen et al. (107) found a correlation between 3 months of glucocorticoid use in thirteen previously untreated myasthenic patients and an increase in circulating Tregs and Tfr cells with a reduction in circulating Tfh. They inferred that treatment with steroids can attenuate the symptoms of myasthenia gravis by restoration of the imbalances between circulating Treg, Tfr, and Tfh and maintaining immune homeostasis. This suggests that steroids may exert a similar effect in transplant recipients by promoting a positive ratio of Tfr : Tfh. This was also seen in autoimmune treatment with 5mg prednisolone causing an increase in Tfr compared to pre-treatment.

In the context of renal transplant rejection Seissler et al. (108), found methylprednisolone (125-250mg) for 3 days did not affect the percentage of Treg numbers ($CD4^+FOXP3^+CD127^{lo+/-}$) within the total $CD4^+$ T cell population. However, the ratio of different Treg subsets changed such that $DR^+CD45RA^-$ Tregs increased significantly, while the naïve $DR^-CD45RA^+$ Tregs decreased significantly. Moreover, they observed a disproportionally strong expansion of the $DR^{high+}CD45RA^-$Tregs which have been shown to have maximal suppressive properties (108). However, this proportional increase in $DR^{high+}CD45RA^-$Tregs was not sustained beyond 3 days and may be confounded by the effects of increasing other immunosuppressive doses.

Longer-Term Immunosuppression

For maintenance immunosuppression, post-transplant patients are commonly on calcineurin inhibitors which remain a cornerstone of immunosuppression regimes. Tacrolimus (Tac) and cyclosporin (Csa) are often used and exhibit their action *via* blockade of the dephosphorylation of the nuclear factor in activated T cells (NFAT) (109). This prevents translocation into the DNA promoter region in the nucleus, thereby selectively suppressing the cytokine gene transcription for IL-2 (110), TNF-a, IL-3 and IL-4 (111). This affects T cell proliferation and activation (112). Vaeth et al. (20), found that Tfr are highly dependent on NFAT signaling indicating that CNI could plausibly impair the function of these subsets (113).

Wallin et al. (99), compared the effect of tacrolimus on paired blood and lymph node samples from transplant recipients. Living-donor kidney transplant recipients were treated with tacrolimus for a week prior to transplantation while the deceased-donor recipients received no pre-transplantation tacrolimus. One week of treatment reduced the frequency of both circulating and lymph node Tfh cells in the transplant recipients. At the same time, Treg remained the same in both tacrolimus treated and untreated recipients. There was a trend to toward fewer Tfr ($CXCR5^+IL\text{-}7R^{lo}FOXP3^+CD4^+$ cells) numbers and as a proportion of total $CD4^+$. Comparatively, 11 days of tacrolimus administered to Tfh in co-culture with memory B cells lead to lower PD-1 expression, plasmablast differentiation and antibody production compared to control treated cells.

Further *in vitro* evidence indicates Tac administration to Tfh-B co-culture prevented plasmablasts and IgG formation in cells from renal transplant patients, suggesting it is Tac targeting of Tfh that prevents DSA formation (114). Similarly, in healthy volunteers, Chen et al. found that Tfr cell proportions were unaffected by treatment with CNI but the Tfh1 percentage

increased while the IL-21 producing Tfh2 and Tfh17 cells decreased (86). The results from these studies may be influenced by the use of healthy volunteers in Chen's study vs. transplant recipients. Thus, CNIs are likely to have a dominant effect on Tfh rather than Tfr. This may be explained by higher expression of NFAT in Tfh cells than in other CD4 subsets (20). Alternatively, suppression of Tfr cells, like Tregs, may require higher doses or longer duration of CNI treatment than required for suppression of Tfh cells.

Antiproliferative agents, such as mycophenolate mofetil (MMF), are another commonly used immunosuppressive drug. This is a pro-drug which undergoes hydrolyzation by gut esterases to give the active mycophenolic acid. This acts by inhibiting inosine monophosphate dehydrogenase, which is crucial for purine synthesis in T and B cells. However, there are conflicting reports of MMF promoting induction of Tregs from Tconvs (115), while others suggest a dose dependent reduction in Treg viability and proliferative capacity (116). The specific effect on Tfh and Tfr has not been examined in the literature.

A less commonly used agent is sirolimus, a mammalian target of rapamycin inhibitor (mTOR). The mTOR signaling pathway plays a crucial role in dictating T cell fate through the interaction and balance of two mTOR containing complexes, mTORC1 and mTORC2. Xu et al. (117) found mTORC1 was expressed at high levels in mouse Tfr cells. By deleting the essential components of mTORC1 and mTORC2 they demonstrated that mTORC 1 but not mTORC2 was essential for Tfr differentiation, which was *via* the p-STAT3-TCF-1-Bcl-6 pathway. Essig et al. also found mTOR inhibitors suppressing PI3K-mTOR signaling inhibits the conversion of Treg to Tfr cells (118). Xu et al. showed that Tfr differentiated from mouse Tregs in the presence of rapamycin had lower expression of CXCR5, GITR and CTLA-4 compared to vehicle treated precursors. Tfr derived in the presence of rapamycin also had reduced suppressive function as indicated by an increased proportion and total number of GC B cells in spleens compared with the spleens of mice that had received *wt* Tfh and vehicle-treated Tfr cells. Essig et al. (118) used Roquin, an RNA-binding protein, to inhibit the PI3K-mTOR pathway at several levels, noting that differentiation toward Th17 and Tfh ($PD1^{int}CXCR5^{int}CD4^{+}$) was inhibited as well as Treg to Tfr ($CXCR5^{hi}$ $PD\text{-}1^{hi}$ FOXP3 $CD4^{+}$).

Chen et al. (86) found 48-h *in vitro* culture with rapamycin could reduce the ratio of $CD4^{+}CXCR5^{+}ICOS^{+}FOXP3^{+}CD127^{-}$ Tfr cells in healthy volunteers but had no effect on Tfh1, Tfh2 and Tfh17 cells. Thus, mTOR inhibitors may adversely affect Tfr cell function, skewing the balance toward Tfh cells. This is supported by mouse models suggesting that the use of mTOR inhibitor rapamycin after alemtuzumab induction increased the proportion of Tfh cells while significantly reduced the number of Tregs 2 weeks post cardiac transplantation and was associated with an increase in DSAs (119).

Another key mediator of Treg function is CTLA-4, which contributes to the suppressive function of Tfr by downregulating the expression of CD80/86 on antigen presenting cells and consequently reducing CD28 engagement (120). Treg-specific deletion of CTLA-4 results in a massive increase in antibody production, pointing to a crucial role for CTLA-4 on Treg cells in limiting B cell responses (120). Wing et al. subsequently (71) showed that murine CTLA-4 deficient Tfr ($CXCR5^{+}$ $Bcl6^{+}$ $FOXP3^{+}$) either from CTLA-4 KO mice or using anti-CTLA Fab were less able to reduce the expression of CD80 and CD86 on B cells. They also were less able to prevent effector T cell proliferation when purified B cells were used as stimulators. Thus, CTLA-4-deficient Tregs and Tfr exhibited significantly reduced, but not a total loss of, suppressive function *in vitro*.

Sage et al. (47) demonstrated murine Tfr ($CD4^{+}CXCR5^{+}ICOS^{+}$ $FOXP3^{+}CD19^{-}$) had very high expression of CTLA-4 compared to Tfh $CD4^{+}CXCR5^{+}ICOS^{+}FOXP3^{-}CD19^{-}$. In their mouse model where CTLA-4 is conditionally deleted on $FOXP3^{+}$Tregs upon tamoxifen administration, they found substantial increases in total Treg and Tfr numbers, ICOS expression and an increase in Tfr : Tfh after tamoxifen. However, these Tfr showed diminished suppressive capacity of B cell function in suppression assays both *in vitro* and *in vivo*, as indicated by a substantial increase in antigen specific IgG. Chen et al. (86) used a lentivirus to overexpress CTLA-4 in Tregs from renal patients with CAD finding an increased proportion of human Tfr ($CD4^{+}CXCR5^{+}ICOS^{+}FOXP3^{+}CD127^{-}$) and significantly less B cell proliferation. ELISA results showed Tfr inhibited IgG and IgA production from plasma cells. Conversely, there was increased proliferation and differentiation to plasma cells in the Tfr deleted group. This suggests that selectively increasing Tfr *via* CTLA-4 may be a good strategy to treat AMR by preventing B cell proliferation and differentiation.

Belatacept is a CTLA-IgG fusion protein which binds to the CD80 and CD86 molecules on antigen presenting cells preventing T cell co-stimulation and promoting expression of indoleamine 2,3-dioxygenase. Oh et al. (114) showed CTLA-Ig in addition to rapamycin increased Tfh ($ICOS^{+}$ $PD\text{-}1^{+}$ $CD4^{+}$) cells but not Tregs ($CD4^{+}$ $CD25^{+}$ $FOXP3^{+}$) in a murine cardiac transplant model with alemtuzumab induction. In clinical transplantation, the impact of belatacept on Tregs has been difficult to assess because it is administered in combination with blocking antibodies targeting IL-2Rα and cyclosporin (121). Despite this, it is currently widely accepted that the use of high doses of CTLA4 Ig is detrimental to Treg survival, whereas low doses of CTLA4 Ig, unable to saturate CD80 and CD86, may favour Treg expansion to some extent in the long term (122). Kim et al. (123) in a primate renal transplant model found $CXCR5^{+}Bcl6^{+}PD\text{-}1^{hi}CD4^{+}$ T cells were greatly reduced in lymph nodes of costimulatory blockade treated (belatacept or anti-CD40 mAb) animals with AMR that had lower levels of DSA compared to primates with AMR receiving the control immunosuppression regimen.

While these studies involving immunosuppression are confounded by small patient numbers, differences in HLA typing and variation in immunosuppression regimes, there is a consistent finding that a positive ratio of Tfr : Tfh correlates with immune regulation and a trend toward less DSA formation. Niu et al. (100) observed that kidney transplant recipients on immunosuppressive therapy with tacrolimus (Tac), mycophenolate mofetil (MMF), and steroids, with anti-HLA antibodies including DSA had lower numbers of circulating Tfr ($CD3^{+}$ $CD4^{+}$ $CXCR5^{+}$ $FOXP3^{+}$) cells than patients without

anti-HLA antibodies. Inverse correlations between the kidney function parameters (serum creatinine level), and number of circulating Tfr cells and number of Helios+ circulating Tfr cells were found, indicating that the reduction of number of bTfr cells might reflect the lack of regulation of active B cell immunit directed against the allograft in kidney transplant recipients.

Niu et al. (61), evaluated Tfr in the clinical context of long term multiple agent immunosuppression in 211 patients with a functioning renal transplant over 5 years post transplantation. They found absolute numbers of Tfr (total $CD3^+CD4^+CXCR5^+FOXP3^-$) including subsets that were PD1+ and Helios+, were lower in transplant recipients compared to healthy controls. There was no difference in total Tfh ($CD3^+CD4^+CXCR5^+FOXP3^-$), meaning consequently there was also a decrease in the Tfr : Tfh. Although different induction agents were used according to clinical indication, the majority being anti-CD25 mAb, most patients had similar maintenance regimes; tacrolimus, MMF and tapering prednisolone to 0 at 4–5 months. They compared 162 patients with no history of rejection vs 49 with rejection (5 presumed and 44 with biopsy proven), noting that those with previous rejection had lower numbers of Tfr and Tfh such that the Tfr : Tfh remained the same as those without rejection. However, blood samples were taken at a median of 4.9 years post rejection episode thus cell numbers may be as a consequence of specific anti-rejection treatment, enhanced overall immunosuppression or reflecting a pattern that predated rejection. Of the anti-rejection treatments, Alemtuzumab in combination with methylprednisolone was the only one associated with significantly lower numbers of both bTfr and bTfh cells, including their subsets.

One of the limitations of this study was the use of healthy controls as a comparison without CKD. Although the transplant recipients had functioning grafts, their eGFR was <40ml/min and they demonstrated that lower eGFR correlated with lower Tfr. Thus, a comparison to CKD patients with a GFR may have controlled for the confounding effect of uraemia rather than contrasting with healthy controls.

Overall, there appears to be a reduction of Tfr associated with most immunosuppression regimens. However, elucidating each agent's effect is substantially confounded by the use of other drugs and frequent clinician adjustment in the context of rejection, infection etc. There has also been a trend in some studies (100) of a positive Tfr : Tfh associating with less DSA formation. However, Niu et al. (61), found patients with anti-HLA antibodies or DSA at 5–7 years post-transplant or any form of rejection had similar bTfr and bTfh cell numbers as those without antibodies or rejection. This is in opposition to Macedo et al. (104), who demonstrated lower Tfh. Similarly, a longitudinal study by Cano-Romero et al. (124), indicated that cTfh ($CD4^+CXCR5^+PD1^{hi}CCR7^{lo}$) expanded significantly more after transplantation in patients who developed *de novo* anti-HLA antibodies than in patients who remained unsensitised. Thus, a contemporaneous longitudinal study of both Tfr and Tfh numbers and their functional assessment may indicate which patterns associate with immunosuppression changes, rejection and *de novo* DSA development.

CELL THERAPY USING TFR

Tregs Therapy

Over the last decade, clinical trials using autologous Tregs have grown exponentially, with not less than 52 current Treg clinical trials registered in January 2020 on ClinicalTrials.gov, two of which have involved our group. In solid organ transplantation, two trials in liver transplantation have been published (125, 126). One was suspended because of rejection in three patients even if seven were successfully weaned of immunosuppression (125), though there were some concerns about the potential presence of antigen-specific effector cells in the cell product used in this study. The ThRIL study (126) demonstrated that Treg infusion in liver transplant recipients was safe. It was easier to achieve the aim of delivering 4.5 millions of Tregs per kilogram after expansion if the Tregs were isolated 6–12 months after liver transplantation, in comparison to trying to identify patients to treat while they were awaiting their transplant. Treg infusion resulted in a transient increase of the pool of circulating Tregs. Patients who received the full 4.5 million/kg Tregs product displayed a decrease in T cell responses against donor cells (assessed by the upregulation of CD154 on memory $CD8^+$ Tcells).

The One study (127) presented the results of seven trials using different types of regulatory cell products, used in place of induction treatment for kidney transplantation. The results were not individualized for each type of cell products but demonstrated that the use of regulatory cell products is safe and could even lead to a decrease to post-transplant viral infections. Patients receiving Tregs products displayed more stable Treg-specific demethylated region compared to those receiving standard of care. However, those studies have used autologous polyclonally expanded Tregs and the proportion of Tfr in these products was not investigated.

These have led to the development of an ongoing Phase IIb study in kidney recipients (TWO Study, https://doi.org/10.1186/ISRCTN11038572) and to a Phase IV study in liver recipients, with the association of low dose IL-2 infusions (LITE study: NCT02949492). The latter, which tested the efficacy of low dose IL-2 infusions in liver transplant recipient as potential therapy to increase autologous Tregs *in vivo* was stopped prematurely, as some patients developed rejection on protocol biopsies.

Access to Tfr and Cell-Engineering

The biology and role of different subsets of Tfr remains to be further elucidated in humans. However, bTfr seem to be good candidates for autologous therapy. First, they are accessible by leukapheresis (in contrast to their lymph nodes counterparts) and display a memory-type phenotype (36). Moreover, they are easily identifiable and could potentially be sorted from fresh blood as they could be defined as $CD4^+CD25^+$ $CXCR5^+$ cells. However, their suppressive capacity, in comparison to mTfr has not been tested and it remains unknown whether they will be recruited into GC and become fully mature. Moreover, the frequency of bTfr in human blood and whether they can be expanded *in vitro* are both unclear.

As Tfr are derived from Tregs, it could be possible to use general Tregs, and modify them so that they become Tfr. Kim et al. (128), used retroviral transduction of the CXCR5 gene in FOXP3$^+$ Tregs and demonstrated stable expression of functional CXCR5 on transduced-Tregs. CXCR5-transduced Tregs maintained a Treg signature and suppressive activity *in vivo* after adoptive transfer in mice. Moreover, using a transwell culture system, Kim et al. demonstrated that they migrated efficiently down a CXCL13 gradient and suppressed antibody production by B cells.

The field of tailored cell therapy has advanced enormously in recent years thanks to new engineering techniques including CRISPR-Cas9 (129) and chimeric antigen receptor (CAR) technologies (130, 131). The latter involves synthetic fusion proteins which typically combine an extracellular antibody-derived antigen targeting moiety and an intracellular TCR complex-derived signaling domain (or domains). The resultant protein is consequently able to bind designated target antigens, in an MHC-independent manner, and translate this engagement into activation of customized T cell signaling cascades [reviewed by us (132)]. The first Phase 1/2 human clinical trial using CAR-Tregs (in a setting other than cancer) will launch in 2020 (STEADFAST study, Sangamo Therapeutics) and will test CAR-Tregs in prevention of immune-mediated rejection following HLA-A2 mismatched kidney transplantation. Using a CXCR5-CAR on Tregs could help them to migrate to the lymph node and potentially inhibit antibody responses. Fine tuning the expression of CXCR5 seems to be more appropriate and as the Bcl-6 dependant CXCR5 expression is a dynamic process, using CRISPR-Cas9 technology to incorporate Bcl-6 to Tregs genome might be another track to explore.

Although promising and full of potential, cell-engineering might not be appropriate for Tfr production, as these cells cannot be defined by the presence of one parameter. More than producing Tfr through an engineering process, expanding Tfr from isolated Tregs might be another tempting option.

Applicability of Treg Expansions Protocols to Tfr: IL-2/Rapamycin

The production and expansion of polyclonal autologous Tregs for cell therapy has been extensively described over the last few years, with a few protocol variations between centers (126, 127, 133–137). Others groups have been focusing on delivering autologous donor-antigen reactive Tregs to prevent transplant rejection (138, 139). In order to obtain a pure cell product which complies with Good Manufacturing Product (GMP) regulations before being reinfused to patients, experts have made a step forward standardization of required tests in a recent publication (140). The different options from isolation to culturing these cells are presented in **Figure 2**.

The communally accepted GMP-compliant process to expand polyclonal autologous Tregs developed in our laboratory which has been in use and demonstrated to be safe during the Phase I ONE Study (127) and ThRIL (126) includes harvesting Tregs from the (potential) recipient using CD8$^+$ depletion and CD25$^+$ positive selection (Miltenyi), followed by polyclonal expansion using CD3/CD28 beads (Dynabeads, Thermofisher) in the presence of Rapamycin (116, 135) and

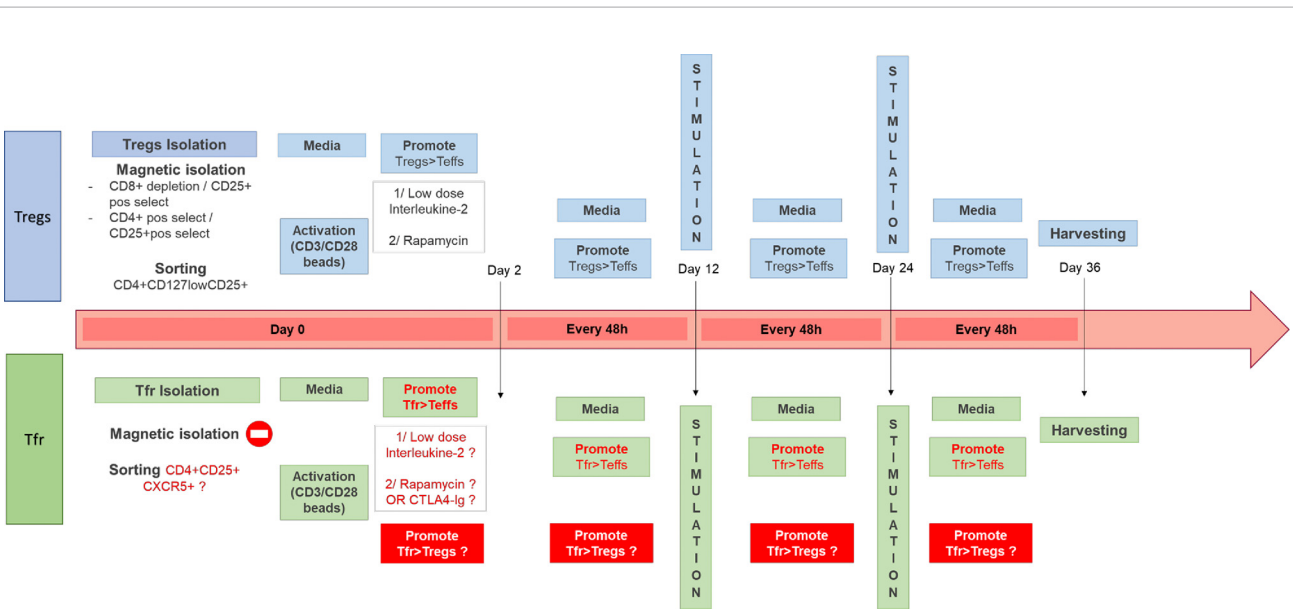

FIGURE 2 | Different Tregs Good Manufacturing Product (GMP)-compatible expansion protocols and potential areas to improve for Tfr cell therapy. In the top diagram are described some of the protocols in use in our GMP facility (126, 127, 135) for the expansion of Tregs from the isolation step (using either magnetic beads or cell sorting) to the other steps such as stimulation every 12 days (with anti-CD3/CD28 beads) and feeding every 48 h. The use of IL-2 and rapamycin has been shown necessary to expand functional and stable Tregs in the absence of T effectors. In the bottom diagram is described a similar process for the generation of a Tfr product and in red are all the different steps which remains to be optimized.

Interleukin(IL)-2 (141). This protocol allows the preferential expansion of Tregs compared to other T cell population. The aim of the expansion is to increase the numbers of cell present while maintaining phenotype, purity, and suppressive abilities. Other centers have used sorted cells as the initial Treg product (142), followed by expansion with or without Rapamycin present (138, 142, 143). As discussed previously the mTOR pathway seems to be essential for Tfr functions, therefore using Rapamycin to expand Tfr *ex vivo* might not be appropriate (86). Li et al. (144) demonstrated that patients on Sirolimus had lower numbers of $CD4^+CXCR5^+$ cells, but in this study Tfr and Tfh were not differentiated. Therefore, further exploratory work is needed before using Rapamycin in Tfr culture. Guinan et al. (139) have developed a technique using *ex vivo* costimulatory blockage with Belatacept to support the expansion of potent allo-specific Tregs. They perform a mixed lymphocyte reaction using T cells and allogenic PBMC stimulators in the presence of co-stimulatory blockade for 72 h. The cell product is then washed and the Treg fraction is isolated using CD8 and CD19 depletion, followed by CD25+ isolation prior to administration. These Tregs may represent induced Tregs, by co-stimulatory-deficient activation trough the TCR. This approach has been demonstrated to be safe, as part of the recently published ONE Study and is currently under investigation in a Phase I/II liver transplant study (NCT03577431). Using Belatacept to promote Tfr might be an attractive alternative to Rapamycin.

IL-2 is a crucial cytokine for T cell expansion. However, as Tregs constitutively express CD25, which is the α subunit of the IL-2 receptor, Treg proliferation requires less IL-2. Using low-dose IL-2 is therefore a way to foster Treg expansion in preference to Teffs, which require higher doses of IL-2 to expand. Low dose IL-2 has been used in all GMP-compatible Treg expansion protocols (126, 127, 134, 142) except in the antigen-specific Treg expansion protocol published by Guinan et al. mentioned above (where there is no expansion per se). The sensitivity of Tfr to IL-2 remains to be determined. As described earlier, low dose IL-2 in the context of GvHD seems to positively influence Tfr. On the other hand, some mouse and human (30, 56) bTfr may express lower levels of CD25 than other Tregs, and thus may not respond as well to lower doses of IL-2 compared to other Tregs. Moreover, high levels of IL-2 may be detrimental for Tfr expansion (30), as described by Botta et al. using a mouse model of influenza, although Tfr in this model seem to be differentially regulated by IL-2 during the early and later stages of infection. Therefore, the relevance and importance of low-dose IL-2 in protocols to isolate and expand autologous Tfr remains to be determined.

It remains to be explored if current protocols in place for Treg expansion could be extrapolated to Tfr expansion.

POTENTIAL APPLICATIONS IN TRANSPLANTATION

Despite having a non-clear potential diagnostic and prognostic applications in the transplant settings, cell therapy using Tfr could have a high potential both in prophylactic and therapeutic applications. Assuming the challenges associated with isolation and expansion of Tfr are solved, there are still multiple uncertainties regarding optimal indication (tolerance induction, treatment of CAD secondary to DSA), dose (in relation to number of Tfh), timing (in relation to the transplant and the need to re-dosing), and antigen specificity (to promote specific suppression of DSA production).

In summary, improving the understanding of Tfr-Tfh interactions might be the relevant to enhance long-term outcomes after transplantation. While Tfr have just started to be explored in the transplantation setting, their role remains to be further defined in human. Cell therapy using autologous Tfr has never been done, but should be technically feasible, with some refinement of existing protocols. Work to define the exact potential of such therapy is required.

AUTHOR CONTRIBUTIONS

CD participated in manuscript writing, editing, and coordination of its submission. SB contributed to manuscript writing. AD, GL, and CS contributed to manuscript writing and editing. AD and GL are co-last author of the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Mesenchymal Stromal Cell Therapy in Solid Organ Transplantation

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Transplantation is the gold-standard treatment for the failure of several solid organs, including the kidneys, liver, heart, lung and small bowel. The use of tailored immunosuppressive agents has improved graft and patient survival remarkably in early post-transplant stages, but long-term outcomes are frequently unsatisfactory due to the development of chronic graft rejection, which ultimately leads to transplant failure. Moreover, prolonged immunosuppression entails severe side effects that severely impact patient survival and quality of life. The achievement of tolerance, i.e., stable graft function without the need for immunosuppression, is considered the Holy Grail of the field of solid organ transplantation. However, spontaneous tolerance in solid allograft recipients is a rare and unpredictable event. Several strategies that include peri-transplant administration of non-hematopoietic immunomodulatory cells can safely and effectively induce tolerance in pre-clinical models of solid organ transplantation. Mesenchymal stromal cells (MSC), non-hematopoietic cells that can be obtained from several adult and fetal tissues, are among the most promising candidates. In this review, we will focus on current pre-clinical evidence of the immunomodulatory effect of MSC in solid organ transplantation, and discuss the available evidence of their safety and efficacy in clinical trials.

Keywords: mesenchymal stromal cells, regulatory cells, tolerance, kidney, liver, lung

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INTRODUCTION

Solid organ transplantation has been established as the standard of care for end-stage disorders affecting the kidneys, liver, heart, lungs and small bowel. Advances in our understanding of the adaptive host-versus-graft immune response have led to the development of potent immunosuppressive agents that have improved graft and patient survival in early post-transplant stages substantially (1). Despite these breakthroughs, the current immunosuppressive regimen is associated with detrimental side effects, such as cardiovascular diseases (2), metabolic complications (3), cancer (4), and infections (5), which cause significant morbidity and mortality. Moreover, immunosuppressants are ineffective in preventing the development of chronic rejection, which causes 10% of kidney allograft loss every year (6) and affects 50% and 75% of lung transplant recipients at 5 and 10 years post-transplant (7). Therefore, there is an urgent need for alternative strategies to enable the minimization of immunosuppression and to improve long-term graft

survival. Among these, the use of live suppressor/regulatory cells is emerging as the most promising tool. The use of mesenchymal stromal cells (MSC) is gaining particular attention due to their potential to inhibit the host-versus-graft immune response at the different key steps involved in acute and chronic graft rejection.

In this review we provide a summary of the immunomodulatory features of MSC in pre-clinical models of solid organ transplantation and analyze the results of clinical studies using MSC-based cell therapies in patients with kidney, liver, lung, and small bowel transplantation.

Mesenchymal Stromal Cells

Mesenchymal stromal cells are plastic-adherent, non-hematopoietic, fibroblast-like cells with the capability to differentiate into osteoblasts, adipocytes and chondrocytes. Traditionally, bone marrow (BM) was the main source of MSC considered; however, alternative sources, such as adipose tissue (8), the umbilical cord (9), or the placenta (10) are now widely used as sources of MSC due to their higher yield and the less invasive procurement strategies involved.

MSC are isolated and expanded in culture from whole cell preparations by using their ability to adhere to cell culture plastic and to proliferate for several weeks. This approach yields a population of fibroblast-like cells that are relatively homogenous morphologically, but it inevitably contains a heterogeneous population of cells with distinct phenotypes and biological properties. In 2006, the International Society for Cell & Gene Therapy established a non-ambiguous and broadly accepted set of minimal criteria for defining "mesenchymal stromal cells": plastic-adherence, expression of CD105, CD90, and CD73 surface markers, negativity for CD45, CD19, and CD14 hematopoietic antigens, and stimulus-induced tri-lineage differentiation *in vitro* into osteoblasts, adipocytes and chondrocytes (11).

The lack of specific markers and the retrospective characterization of MSC (which is still performed after long-term culture) have long precluded a deeper understanding of their native origin and physiological functions (12). Studies conducted during the last decades showed that MSC represent a fundamental component of the BM stroma, where they control maintenance, self-renewal and differentiation of hematopoietic stem cells (12, 13). Impaired functional, replicative, and regenerative capacities of BM-MSC have been implicated in development of hematological malignancies (14), such as myelodysplastic syndromes (15, 16), leukemia (17), and multiple myeloma as well as in BM failure syndromes (18, 19). More recent evidence suggests that MSC reside in the vascular niches, being either identical to or deriving from pericytes (20). Here, MSC stabilize the vascular network, contribute to the normal tissues and immune homeostasis, and modulate osteoclast formation. In response to injury, MSC participate in tissue repair and might inhibit overaggressive autoimmune reaction against the injured tissue (21).

Despite arguments about heterogeneity and *in vivo* counterparts (22), a wealth of data has provided irrefutable evidence that MSC have unique and highly potent immune-dampening, immune-regulatory, anti-inflammatory, and pro-reparative properties. This evidence, coupled with simple and cost-effective cell production, have stimulated intense investigation of MSC as a novel therapy for numerous clinical indications (23), including solid organ transplantation (24).

Immunomodulatory Features of MSC on Adaptive Immunity

One of the first pieces of evidence of the immunomodulatory effect of MSC was provided—almost 20 years ago—in a baboon skin allograft model (25). In this study, MSC were shown to suppress allogeneic T-cell proliferation in a mixed lymphocyte reaction and to delay skin allograft rejection (25). Since then, numerous *in vitro* and *in vivo* studies have demonstrated the capability of MSC to inhibit the activation and proliferation of $CD4^+$ T cells (26), preventing their differentiation into T_H1 and T_H17 effector cells (27), and to reduce $CD8^+$ T-cell cytotoxicity in response to allogeneic stimuli (28). MSC were shown to also suppress the activation of memory T cells induced by cytokines (29) or by alloantigens from both minor and major histocompatibility complexes (30, 31).

Of particular interest, MSC exhibited a remarkably potent ability to convert not only naïve (32, 33) but also effector/memory $CD4^+$ T (34, 35) and $CD8^+$ T cells (36–38) toward a regulatory phenotype. Indeed, in *in vitro* studies, human BM-MSC expanded Tregs from $CD3^+CD45RO^+$ human memory T cells (34) and from collagen-reactive human T cells, including $CD8^+$ T cells (36, 39). MSC-induced $CD4^+$ Tregs maintained a regulatory phenotype and function over time (34) and suppressed the *ex vivo* proliferation of T cells from patients with rheumatoid arthritis in an antigen-specific manner (39). The mechanisms at the basis of this Treg-inducing capacity are incompletely understood, but likely involve cell-to-cell contact (40, 41), the release of soluble mediators such as Transforming Growth Factor (TGF)-β1 (40, 41) and Prostaglandin E2 (PGE2) (41), as well as the induction of regulatory phenotype in antigen presenting cells (37, 41). PGE2 (35) and Hepatocyte Growth Factor (HGF) (42) had a key role in the induction of a Treg phenotype in differentiated Th17 cells, either after *in vitro* polarization (42) or isolated from inflamed tissues from patients with psoriasis vulgaris or active Crohn's disease (35). A very recent study described that the transfer of mitochondria from MSC to $CD4^+$ T cells may be a mechanism capable of driving Treg differentiation by itself (43).

Regardless of the underlying mechanisms, Treg induction by MSC has so far been observed consistently in several animal models of immunological diseases (41), in different human autoimmune disease conditions (44–46), as well as in acute and chronic graft-versus-host disease (GVHD) (47, 48).

Recent studies in autoimmune disorders have identified follicular T helper cells (T_{FH}) as an additional target of MSC immunomodulation. MSC downregulated the proliferation and differentiation of T_{FH} cells during *in vitro* polarizing conditioning of $CD4^+$ T cells isolated from patients with rheumatoid arthritis (49) and Sjogren syndrome (50), or from lupus-prone mice (51–53). Indoleamine 2,3-dioxygenase (IDO) (49) and inducible Nitric Oxide Synthase (iNOS) (52) expression

or cell-contact (51) have been reported as possible mechanisms. MSC infusion in NZB/W (51) or MRL/lpr (52, 53) lupus-prone mice or in mice with collagen-induced arthritis (CIA) (49) attenuated disease severity, reduced autoantibody levels and was associated with a decrease in the frequency of T_{FH} cells. A very recent study in a mouse model of chronic GVHD (54) showed that extracellular vesicles isolated from human umbilical cord (UC)-MSC alleviated disease manifestation by reducing germinal center B cell and T_{FH} cell number in the spleen (54). Moreover, T_{FH} cells isolated from UC-MSC treated CIA mice inhibited *ex vivo* proliferation, differentiation and IgG production from B cells (49). This evidence suggests that MSC could indirectly regulated the B cell responses in autoimmune diseases by exerting their inhibitory action on T_{FH} cells.

Conflicting results have been reported on the direct effects of MSC on B cells. Some groups found that MSC may inhibit *in vitro* proliferation of B cells and their differentiation into plasma cells (55), while other authors described an opposite effect (56). These discrepancies can be explained by the different experimental conditions used in these studies, such as the starting B cell population—whether purified B cells or total lymphocytes—as well as the type of stimuli used for activating B cells, and the effects these stimuli could directly exert on MSC (57). Nevertheless, recent findings indicate that MSC inhibit proliferation of and IgG production of B cells in the presence of activated by T cells (58) or inflammatory cytokines (59, 60). In contrast, direct interaction between MSC and B cells mainly affected B cell differentiation, resulting in reduced plasmablast formation and increased generation of IL-10–secreting regulatory B cells (59, 60).

The generation of Bregs by MSC have been confirmed in *in vivo* studies. In mouse models of multiple sclerosis (61) and lupus (62), treatment with MSC suppressed the severity of the disease by increasing the frequency and activity of Bregs along with an enhanced secretion of IL-10. In a clinical study, patients with refractory chronic GVHD given MSC infusions had clinical improvements associated with increased proliferation and IL-10 production by Bregs (63).

Overall, these studies indicate that MSC have a broad immunomodulatory actions on cells of the adaptive immune system, modulating effector functions and promoting regulatory properties.

Immunomodulatory Features of MSC on Innate Immunity

Another fundamental immunoregulatory property of MSC is their effect on antigen-presenting cells.

In *in vitro* experiments, MSC impaired dendritic cell maturation, downregulating their expression of MHC-II and costimulatory molecules (64, 65) and preventing the secretion of the pro-inflammatory cytokines IL-12, IFNγ, and TNFα (66). Consequently, DC exposed to MSC exhibited impaired alloantigen presentation and inefficient effector T-cell activation (65). These effects, coupled with enhanced secretion of the anti-inflammatory cytokine IL-10, resulted in sustained expansion of regulatory T cells (Tregs) (67). In addition, MSC inhibited *in vivo* DC migration toward lymphoid organs by downregulating CCR7 expression (65).

Among the cells of the innate immune system, macrophages are the main target of MSC immunoregulation, as highlighted by several recent studies. MSC promote macrophage polarization toward the anti-inflammatory M2 phenotype (68), downregulating the secretion of pro-inflammatory cytokines while upregulating phagocytic activities and the release of IL-10 (69). MSC, either by inducing (70) or undergoing (71) apoptosis, enable macrophages to produce TGFβ and to promote the induction of Tregs. In addition, by releasing trophic factors, MSC play an important role in educating macrophages to promote tissue repair and inflammation resolution (72).

The mechanisms through which MSC exert these effects on the multiple adaptive and innate immune effector cells are incompletely understood. However, paracrine effects mediated by their plentiful secretome, which includes cytokines, growth factor, and miRNA directly transferred to close target immune cells or encapsulated in extracellular vesicles, appear to be among the main mechanisms of MSC immunomodulation. Key mediators include TGFβ (73); HGF (39); PGE2 (69); IDO (74); iNOS (75); leukemia inhibitor factor (LIF) (76); HLA-G1 (77); TNF-stimulated gene 6 (TSG-6) (78); galectin-1, -3 and -9 (79); purinergic signals (80), as well as miRNA targeting TLR-associated pathways and the inflammasome (81) and mitochondrial transfer (82).

Overall, it is now clear that it would be impossible to identify a single mechanism responsible for the effect of MSC: different mediators released by MSC or surface molecules expressed on these cells are likely to act in concert to inhibit the alloimmune response at several crucial points, inducing the differentiation and proliferation of Tregs, Bregs and immature DC and M2 macrophages to dominate the anti-graft immune response. The establishment of a regulatory cell network could resolve the long-standing conundrum of the long-term effects of MSC in spite of their very short-term engraftment and *in vivo* survival (83).

Insights From Experimental Models of Solid Organ Transplantation

MSC have been the subject of vigorous investigation as a potential tolerogenic cell therapy in pre-clinical transplant models of the kidney, heart, liver and lung (**Figure 1**).

Kidney Transplantation

In murine models of acute transplant rejection following kidney transplantation, an intravenous injection of MSC derived from either donor mice (84) or syngeneic recipient mice (85, 86) induced graft tolerance, mediated by the generation of donor-specific $FOXP3^+$ Tregs (84–86) and tolerogenic dendritic cells (84, 87). The main mediator involved appeared to be IDO, since MSC from IDO knock-out mice failed to prolong graft survival (84). These findings have been confirmed in a rabbit model of kidney transplantation, where the induction of donor-specific Tregs and graft tolerance mediated by bone marrow-derived MSC (BM-MSC) were strengthened by transgenic IDO overexpression (88). The Treg-inducing property was found to be dependent on MSC localization in secondary lymphoid

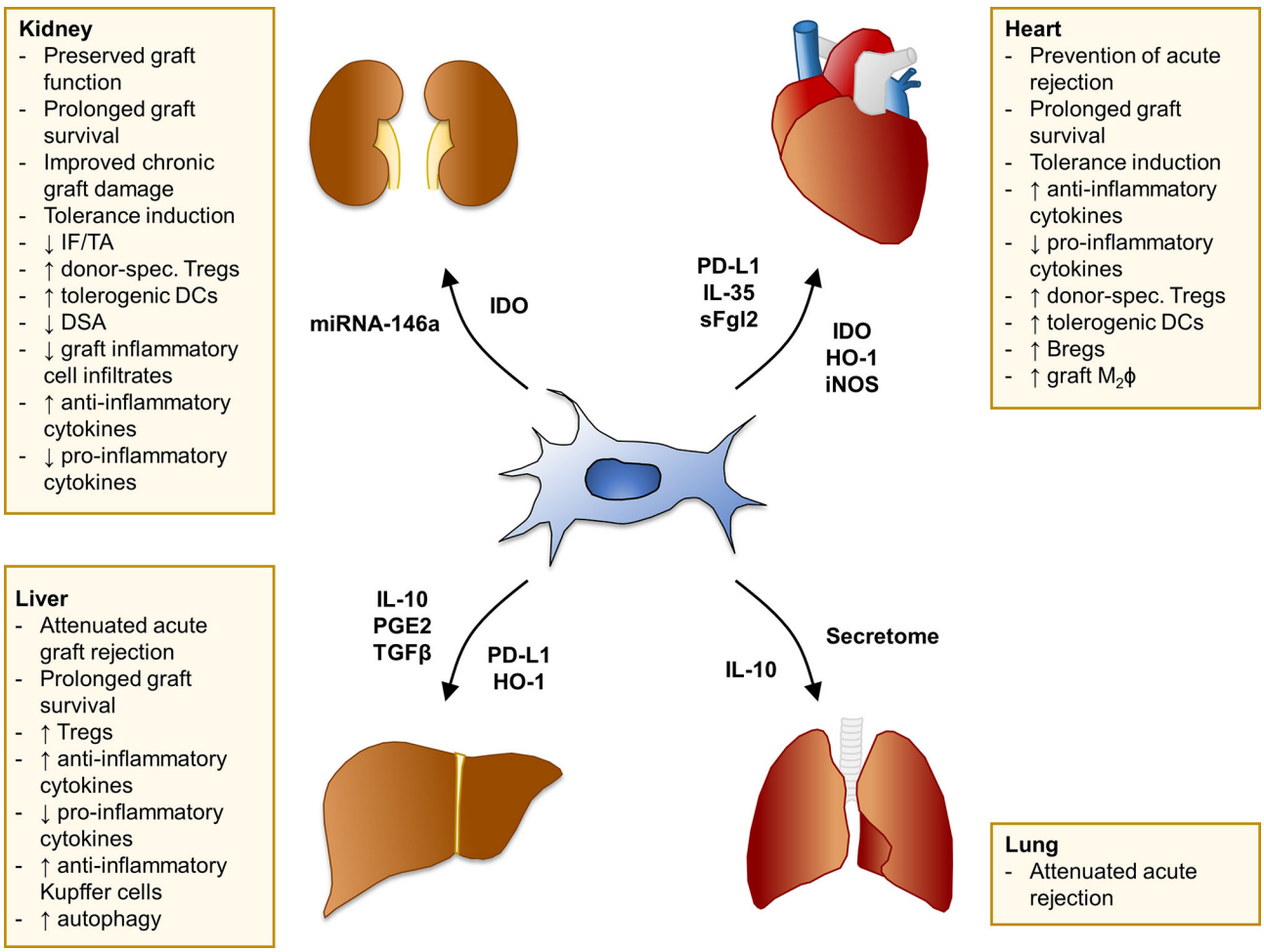

FIGURE 1 | Summary of MSC effects in pre-clinical models of solid organ transplantation. Main findings of studies with MSC in experimental models of kidney, heart, liver, and lung transplantation. The mediators involved in MSC-induced pro-tolerogenic effects and/or specifically overexpressed in selected MSC cell-lines through genetic engineering are listed next to each arrow. Bregs, regulatory B cells; DCs, dendritic cells; DSA, donor-specific antibodies; HO-1, heme oxygenase-1; IDO, indoleamine 2,3-dioxygenase; IF/TA, interstitial fibrosis/tubular atrophy; IL-, interleukin-; iNOS, inducible nitric oxide synthase; $M_2\phi$, M2 macrophages; PD-L1, programmed death-ligand 1; PGE2, prostaglandin E2; sFgl2, soluble fibrinogen-like protein 2; TGFβ, transforming growth factor β; Tregs, regulatory T cells.

organs, before (85) or at the beginning (86) of the immune response, since MSC injection 2 days after transplant failed to expand Tregs and to induce long-term graft acceptance (85, 86).

Similarly, the administration of BM-MSC as multiple (89) or single (90) intravenous injections in rats undergoing kidney transplantation preserved renal function in the early post-transplant and reduced graft mRNA levels of inflammatory cytokines and the number of infiltrating macrophages and dendritic cells, while increasing graft $FOXP3^+$ Tregs. These positive effects increased when BM-MSC were induced to overexpress CXCR4, a procedure that upregulated the MSC expression of anti-inflammatory factors (90).

MSC have also shown the potential to improve chronic kidney graft damage (91–94). In rat models of chronic graft injury, the administration of BM-MSC at both early (91, 93, 94) and late (92) post-transplant time points reduced T-cell and macrophage graft infiltration, inhibited the mRNA expression of inflammatory cytokines and prevented the development of interstitial fibrosis, tubular atrophy and glomerulosclerosis, as well as of donor-specific antibodies (91–94).

Despite these very promising results, a number of reports have described severe complications following MSC infusion, raising concerns about the safety of this cell therapy. We observed that MSC infusion 2 days after kidney transplantation was associated with transient graft dysfunction characterized by increased complement C3 deposition and neutrophil infiltration. In rat kidney transplant models, the injection of MSC from either syngeneic bone marrow (95) or donor adipose tissue (96) was associated with increased mortality of recipient rats due to thrombotic microangiopathy, renal infarctions and infection (95) or to premature graft loss (96).

Heart Transplantation

In heterotopic heart transplant models, the administration of MSC from bone marrow or adipose tissue, either of donor or recipient origin, mildly but significantly prolonged heart graft survival (97–100). The beneficial effect of MSC on graft survival prolongation translated into long-term graft acceptance when cell infusion was associated with a short post-transplant course of mycophenolate mofetil (100, 101) or rapamycin (99, 102). The infusion of MSC before transplantation achieved better results than post-transplant administration, and the maintenance of MSC-mediated tolerance was noticeably dependent on the generation of donor-specific regulatory T cells (99, 102), as we first demonstrated in a semi-allogeneic heart transplant model (103). The transfection of MSC with IL-35 (104) or soluble fibrinogen-like protein 2 (105), two molecules involved in Treg generation and function, was able to boost the immunomodulatory effect of MSC in preventing acute rejection. The increase in Treg cells following MSC administration was also associated with the development of tolerogenic DC (99, 102) and regulatory B cells, effects that were mediated mainly by programmed death-ligand 1 (PD-L1) expression (102) and an increased proportion of graft M2 macrophages (105).

Liver Transplantation

MSC therapy has been tested extensively in pre-clinical liver transplant models. All of the studies demonstrated that MSC, isolated from the bone marrow of both donor and recipient origin (106, 107), as well as from adipose tissue (108, 109), and injected on the day of transplantation, can attenuate acute graft rejection, prolong graft survival, inhibit T_H1 activation and reduce the release of pro-inflammatory cytokines while promoting anti-inflammatory cytokine generation and the emergence of FOXP3 regulatory T cells (106, 107, 109). MSC were also found to be effective in a large animal model (108), and in small-for-size (109) and non-heart beating donor (110) liver transplant models.

The Treg-generating ability of MSC was also beneficial in preventing—but not in reversing—the development of post-liver transplant acute GVHD (111). Indeed, the administration of either donor-derived or syngeneic BM-MSC into LEWxBNF1 recipients during the first 7 days after LEW liver transplant prevented the onset of acute GVHD mediated by LEW splenocytes injected post-operatively. The injection of MSC between 8 and 14 days after transplantation failed to reverse GVHD symptoms, suggesting, also in this setting, the importance of the timing of cell administration in order to fully take advantage of MSC immunomodulation (111).

In the setting of liver transplantation, different key immunomodulatory molecules have been overexpressed in MSC to enhance their tolerogenic properties and improve liver transplant outcomes. MSC overexpressing IL-10 (112), PGE2 (113), TGFβ (114), and HO-1 (115, 116) increased the capability to skew the Treg/T_H17 balance (112, 116), to promote the development of induced-Tregs (2) and to convert Kupffer cells toward an anti-inflammatory phenotype (113). HO-1 overexpression conferred a higher cytoprotective effect on MSC by promoting autophagy (117) and by improving hepatic sinusoidal microcirculation and energy metabolism (118). Notably, MSC transfected with PDL1-Ig were found to induce long-term graft tolerance in a rat model of liver allotransplantation (119).

Lung Transplantation

In rat models of orthotopic left lung transplantation, human BM-MSC given as a double injection of 3×10^6 cells *via* the left pulmonary artery at day 0 and intravenously at day 3 post-transplantation decreased lymphocytic infiltrates, edema and hemorrhage at the histological examination 6 days after transplant, even though the total acute rejection score was reduced only mildly (120). A more remarkable effect was achieved when MSC were associated with conventional immunosuppression. The co-administration of MSC isolated from autologous adipose tissue with tacrolimus significantly reduced rejection scores at day 7 post-transplantation, and this effect was associated with a reduced frequency of proliferating cell nuclear antigen (PCNA)-positive cells in bronchus-associated lymphoid tissue cells (121), suggesting that MSC could inhibit the local early rejection process (122).

Similarly, the administration of IL-10 overexpressing-BM-MSC together with CsA improved graft function and alleviated 5-day acute rejection (123), an effect that could be reproduced through the use of daily intratracheal injections of conditioned media from unmanipulated BM-MSC (124), suggesting the MSC secretome plays a major role in inhibiting the early phase of acute lung allograft rejection.

Overall, these studies in pre-clinical transplant models have demonstrated that MSC have a powerful capacity to skew the host-versus-graft immune response toward a regulatory phenotype, promoting a pro-tolerogenic environment dominated by donor-specific Tregs (**Figure 1**). How MSC, regardless of their origin (i.e., autologous, donor- or third party-derived) can promote the expansion of donor-specific Tregs and the development of tolerance is not completely understood. Several studies showed that MSC potently induce Tregs (41), mainly by converting conventional T cells into Tregs (33, 40). This likely results in the expansion of a broad repertoire of polyclonal T cells with different specificities. The leading hypothesis is that the antigen pressure deriving from the graft could lead to the selection of Tregs able to recognize donor antigen, therefore receiving the correct TCR signaling for survival advantage and long-term dominance. Moreover, MSC can sense the microenvironment and, depending on the prevailing immunological milieu they encounter *in vivo*, may modulate both their phenotype and the function of immune cells from the host. The timing of cell infusion and the degree of T-cell activation are the most crucial factors in determining the beneficial effect of MSC in the transplant setting. Highly activated T cells and an inflammatory environment can hamper MSC-mediated immunosuppression or even promote their conversion into pro-inflammatory cells.

Clinical Studies

Kidney Transplantation

After encouraging results were obtained in animal models and following reports of the efficacy of MSC in treating graft-versus-host disease in bone marrow transplant recipients (125), our group was

the first to translate MSC therapy to clinical trials in solid organ transplantation (126). Since then, several research groups have tried to determine the extent of the immunomodulatory effects that MSC have in clinical settings. In renal transplantation, MSC have been used with different aims: to induce operational tolerance to the allograft, to treat subclinical rejection, thus preventing the development of chronic tissue damage and renal function deterioration, or to reduce the overall dose of induction and/or maintenance immunosuppression.

In the pursuit of immune tolerance, our group designed a phase 1 clinical study to assess the safety and feasibility of MSC administration in two recipients of living-donor kidney transplants, whose preliminary results were first reported over ten years ago. Autologous BM-MSC at a dose of 1 to 2×10^6 cells/kg were infused seven days after transplantation, following induction with low-dose anti-thymocyte globulins and basiliximab (126). Immune monitoring revealed a progressive increase in the Treg fraction and a marked reduction in the percentage of circulating $CD8^+$ memory T cells, coupled with reduced donor-specific T-cell alloreactivity. However, due to the occurrence of transient renal dysfunction without evidence of rejection in both patients, the MSC infusion schedule was reconsidered; indeed, post-transplant MSC administration was shown to be associated with MSC intra-graft migration and pro-inflammatory polarization, resulting in severe neutrophilic infiltration and C3 deposition. Consistent with studies in animal models (85, 127), this engraftment syndrome was completely abrogated by infusing MSC the day before renal transplantation (128).

Long-term follow-up highlighted a sustained increase in the ratio between Treg and $CD8^+$ effector T cells in one of these patients, which was associated with a B-cell profile consistent with the pro-tolerogenic signature identified in other cohorts of spontaneously tolerant kidney transplant recipients (129). This patient consented to gradual tapering of immunosuppression, which was successfully completed without any evidence of rejection (the patient has been off immunosuppression for over two years), thus supporting the hypothesis that a single administration of MSC may induce a long-term, self-sustaining immunoregulatory process responsible for tolerance induction (130). Other groups reported similar immunomodulating effects after the administration of autologous BM-MSC, which were safe and induced an increase in Treg frequency and a reduction in T-cell proliferation (131); nevertheless, so far immunosuppression withdrawal has not been attempted in any other study on renal transplant recipients.

Delayed administration (i.e., over 4 weeks and up to 6 months after transplantation) of autologous BM-derived MSC was instead used by Reinders and colleagues to treat patients who exhibited signs of subclinical rejection or interstitial fibrosis/tubular atrophy on protocol biopsies (132). Most of these recipients displayed donor-specific hypo-responsiveness in T-cell proliferation assays, and the resolution of tubulitis was reported in the two patients who underwent repeat renal biopsy.

Several investigators also exploited MSC immunomodulation to safely reduce, but not completely withdraw, induction and/or maintenance immunosuppression. The efficacy of peri- and post-transplant infusion of autologous BM-derived MSC as a replacement of induction therapy with basiliximab was assessed in a randomized controlled trial involving 159 patients (133). Independent of the maintenance immunosuppression dose, patients allocated to MSC had a significantly lower incidence of acute rejection and renal function decline.

Similar results were obtained with the use of allogeneic, donor-derived MSC, which reportedly allowed a 50% dose reduction of calcineurin inhibitors without having an impact on the incidence of rejection episodes, graft function or survival (134, 135). Despite these results, these studies did not find any difference in the immunophenotype of MSC recipients over time, underscoring that a certain degree of variability in results due to the heterogeneity of MSC preparations, timing of infusion, concomitant immunosuppression and patient selection needs to always be considered in these trials.

These initial experiences with non-autologous MSC paved the way for the use of off-the-shelf third-party allogeneic MSC, which have the invaluable advantage of prompt availability for use in deceased-donor renal transplantation. Sun and colleagues first reported that pre-transplant infusion of third-party umbilical cord-derived MSC (UC-MSC) under standard immunosuppressive therapy (including anti-thymocyte globulins) was safe and well tolerated in deceased-donor renal transplant recipients (136).

Early post-transplant administration of third-party BM-MSC obtained consistent results, and immunophenotype monitoring showed increased frequency of Treg compared to the control group (137). However, the same study also indicated that 40% of patients developed *de novo* donor-specific antibodies (DSA) against MSC or shared graft-MSC HLA, whose long-term relevance is still largely unknown.

Dreyer and colleagues recently reported the results of a clinical trial assessing the safety of a single third-party BM-MSC infusion 6 months after transplantation with a concomitant reduction of maintenance immunosuppression (138). To reduce the risk of sensitization against graft-relevant antigens, the investigators designed an allocation strategy to avoid repeated mismatches between the graft and the MSC product. At variance with the aforementioned study, none of the patients developed *de novo* DSA, possibly due to the more quiescent immunologic state at the time of MSC infusion compared to the peri-transplant period. Notably, no significant change in leukocyte subsets was observed after MSC infusion, suggesting that delayed administration may have limited immunomodulatory effects in this setting.

Liver Transplantation

Similarly to renal transplantation, MSC immunomodulatory properties were exploited for heterogeneous purposes in liver graft recipients, including the induction of operational tolerance, inhibition of acute rejection and treatment of ischemic biliary lesions.

The safety and feasibility of early post-transplant infusion of third-party BM-MSC ($1.5–3.0 \times 10^6$ cells/kg) was assessed in ten liver transplant recipients participating in a controlled, open-label, non-randomized clinical trial (139). Within the limits of

the short follow-up, MSC did not increase the risk of infection or malignancy, and the rates of graft rejection, survival and histologic analysis of 6-month protocol biopsies were similar between patients who received MSC and controls. However, MSC failed to induce changes in the immunophenotype, and weaning MSC recipients off immunosuppression was not successful.

Intriguing results were reported with the use of MSC for the treatment of biopsy-proven acute liver allograft rejection. Twenty-seven patients were randomly allocated to receive conventional immunosuppression with or without UC-MSC infusion. At the end of the 12-week follow-up, the patients who received MSC exhibited lower liver enzyme levels, increased frequency or circulating Treg and improved histology compared to controls (140).

The therapeutic potential of six doses of UC-MSC (1.0×10^6/kg each) was also assessed in 12 liver transplant recipients with ischemic-type biliary lesions (141). Compared to a group of patients treated with a traditional protocol, those who received MSC had a significantly lower need for interventional therapeutic procedures, lower mortality and higher graft survival.

Lung Transplantation

In lung transplant recipients the use of MSC has focused on treating chronic allograft dysfunction, the main limitation to long-term graft and patient survival in this setting. A single-arm, phase 1 trial assessed the safety and feasibility of four infusions of allogeneic third-party BM-MSC (2×10^6 cells/kg) in 10 patients with progressive chronic lung allograft dysfunction (142). The therapy was well tolerated, and no adverse events involving hemodynamics or gas exchanges were reported. The authors observed a trend toward a slower rate of decline in forced expiratory volume in one second in MSC-treated patients. Nonetheless, two patients died during follow-up due to progressive graft dysfunction, suggesting that the effect of MSC may be heterogeneous in this context as well.

The therapeutic potential of a single infusion of third-party BM-MSC was also assessed by Keller and colleagues in a dose-escalation trial that enrolled a relatively homogenous cohort of 9 patients with moderate chronic lung allograft dysfunction (143, 144). Gas exchanges and pulmonary function tests did not change significantly immediately after infusion or during the first month of follow-up. However, lung function parameters stabilized after MSC infusion and did not significantly decline at one year of follow-up, a finding consistent with a possible beneficial effect of MSC on the progression of chronic lung allograft dysfunction.

Small Bowel Transplantation

The properties of MSC have also been assessed in a few cases of small bowel transplantation. A preliminary report described the case of an HLA-matched small bowel graft recipient who developed severe refractory bowel dysfunction (145). The patient was treated with a single infusion of allogeneic BM-MSC (1×10^6 cells/kg) as rescue therapy with the dual intent of providing immunosuppression and support for tissue regeneration. An early, marked functional and histological improvement was noted in the first two weeks after treatment, and the patient remained stable up until 2 months of follow-up.

Peri- and post-transplant intra-graft administrations of autologous BM-MSC (three doses, 1×10^6 cells/kg each) were also employed in a case series of 6 patients who underwent small bowel transplantation (146). Half of these patients experienced severe acute rejection, an event rate that is similar to other patient series of small bowel transplantation (147), and died due to complications within 3 months of surgery. The results of this study indicate that MSC are safe in small bowel transplantation as well, but the small number of patients treated so far mandates further studies before definitive conclusions on their effects can be drawn.

OPEN ISSUES AND FUTURE PERSPECTIVES

Pre-clinical studies have clearly demonstrated the potential of MSC to substantially improve outcomes in solid organ transplantation. On the other hand, the clinical studies conducted so far were mainly phase 1 trials, which were designed to assess the feasibility and safety of MSC therapy.

In our opinion, original concerns regarding a potentially higher risk of infections and malignancy in MSC recipients have been progressively debunked by these trials. Indeed, one of the first studies in kidney transplant recipients raised the issue of increased incidence of opportunistic infections in patients who received MSC (132), but these results were not confirmed by other studies (133, 137, 148). Similarly, human MSC did not demonstrate any potential of malignant transformation, even after long-term *in vitro* expansion, and no association between MSC and cancer has been reported in any of the trials conducted so far (149, 150). Overall, this provides a strong signal regarding MSC safety, even in this context, but long-term surveillance still needs to be implemented, as most of these trials reported results during a limited follow-up period.

Despite the inherent design limitations of phase I studies, MSC have shown some degree of efficacy in protecting the graft from chronic rejection and in promoting a pro-tolerogenic environment, even in this setting. Nonetheless, these effects are not as robust as those demonstrated in pre-clinical studies.

Several factors are at the basis of the limited success of MSC therapy in humans. First, despite decades of intense research, the precise mechanism through which MSC interact with the host immune system has not been completely understood yet. An improved understanding of the mechanism of action of MSC will be crucial in allowing the set-up of assays for selecting the most effective cell preparation *a priori*, in enabling the standardization of cell manufacturing processes in cell factories, and in establishing the appropriate dose, timing, source and concomitant immunosuppressive therapy to favor the beneficial effects of MSC. Identifying the most important mediator(s) of MSC-induced immunomodulation will also

make it possible to clarify whether engineering MSC could provide additional benefits *in vivo* compared to standard preparations, or whether MSC secretome could replace live cells for cell-free tolerogenic therapy.

Research should also aim to develop methods to identify biomarkers of response to MSC therapy in transplant patients. This will make it possible to identify factors that can influence MSC therapeutic efficacy *in vivo*, such as recipient age, medical history, underlying diseases and type of solid organ transplant. These factors would enable the selection of candidates who would benefit from MSC therapy and the tailoring of MSC therapy to each solid organ transplant recipient.

Once these outstanding challenges are addressed adequately, we might finally be able to make a major breakthrough in the induction of tolerance to solid organ transplantation.

AUTHOR CONTRIBUTIONS

FC and MP searched the literature and wrote the first draft of the manuscript. GR critically revised the work. All authors contributed to the article and approved the submitted version.

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Regulatory Cell Therapy in Organ Transplantation: Achievements and Open Questions

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The effective development of innovative surgical applications and immunosuppressive agents have improved remarkable advancements in solid organ transplantation. Despite these improvements led to prevent acute rejection and to promote short-term graft survival, the toxicity of long-term immunosuppression regiments has been associated to organ failure or chronic graft rejection. The graft acceptance is determined by the balance between the regulatory and the alloreactive arm of the immune system. Hence, enhance regulatory cells leading to immune tolerance would be the solution to improve long-term allograft survival which, by reducing the overall immunosuppression, will provide transplanted patients with a better quality of life. Regulatory T cells (Tregs), and regulatory myeloid cells (MRCs), including regulatory macrophages and tolerogenic dendritic cells, are promising cell populations for restoring tolerance. Thus, in the last decade efforts have been dedicated to apply regulatory cell-based therapy to improve the successful rate of organ transplantation and to promote allogeneic tolerance. More recently, this approach has been translated into clinical application. The aim of this review is to summarize and discuss results on regulatory cell-based strategies, focusing on Tregs and MRCs, in terms of safety, feasibility, and efficacy in clinical studies of organ transplantation.

Keywords: regulatory T cells, tolerogenic dendritic cells, myeloid regulatory cells, cell therapy, solid organ transplantation

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INTRODUCTION

Solid organ transplantation (SOT) is a life-saving treatment for patients with end-organ dysfunction. Thanks to advances in the surgical techniques and in the use of effective immunosuppressive drugs, acute transplant rejection has been declined. Unfortunately, toxicity of immunosuppressive regimens and chronic rejection remain the main limiting factors for organ acceptance and patient survival (1). Current research focused on preventing the activation of the alloreactive responses and inducing immune tolerance (2, 3).

In the last two decades adoptive transfer of regulatory T cells (Tregs), regulatory myeloid cells (MRCs) or mesenchymal stromal cells, has become one of the most promising approach to promote/restore immunological tolerance. In the context of SOT these cell-based approaches in pre-clinical animal models demonstrated their ability to modulate alloreactive immune responses, to prevent organ rejection, and to promote long-term tolerance (4–6). These results prompted the development of protocols to expand or generate regulatory cell products for clinical

application in allogeneic transplantation with the aim at preventing/modulating graft vs. host disease (GvHD) or organ rejection and at promoting tolerance. Results demonstrated the feasibility, safety, and efficacy of several regulatory cell products. An overview on tested cell-based strategies and future perspectives in SOT will be presented.

TREG CELL-BASED THERAPY IN ORGAN TRANSPLANTATION

The aim of Treg cell-based therapy is to promote tolerance without interfering with the normal function of the immune system. In pre-clinical models, administration of Tregs have been used to control GvHD and organ rejection (1, 7, 8). The development of good-manufacturing-practice (GMP)-compliance protocols to isolate and expand human Tregs *ex vivo* and to generate donor-specific Tregs allowed the translation of the two main subsets of Tregs, the Forkhead box P3-expressing Tregs ($FOXP3^+$ Tregs) (9) or the IL-10-producing T regulatory type 1 (Tr1) cells (10), in to clinical testing.

Ex vivo Isolated, Expanded, or Induced Tregs in Allogeneic Transplantation

After the seminal work in 2009 demonstrating that adoptive transfer of *ex vivo* expanded Tregs modulated symptoms and allowed tapering immunosuppression in chronic GvHD (11), several clinical trials provided evidence of Treg effectiveness in this context (6), and prompted investigators to apply Treg cell-based therapy in the context of SOT (**Figure 1**) (12).

The first application of Tregs in SOT was conducted in patients undergoing living-donor liver transplantation treated with autologous Tregs cultured with irradiated donor cells in the presence of anti-CD80/86 agonists (13). This study demonstrated that Treg infusion led to taper immunosuppression starting from 6 months, with complete withdrawal achieved by 18 months. Similar studies, focused on the safety of the approach, have been then conducted using *ex vivo* induced donor-specific Tregs or *ex vivo* expanded Tregs in SOT (14). The ONE study, the first study aimed at comparing different cell products and at generating consensus on the standardization of the outcome of the trials (http://www.onestudy.org/), demonstrated that Treg administration in living-donor kidney transplanted patients is safe, and is associated to lower infectious complications compared to standard immunosuppressive treatments, but an overall similar rejection rates in the first year post-transplantation was observed (15). Beside the ONE study, a number of clinical trials with *ex vivo* expanded Tregs in SOT have been completed or are ongoing (NCT02166177; NCT02145325; NCT02088931; ISRCTN11038572; NCT01446484; NCT03284242; NCT01624077). Overall, these studies demonstrated that Treg-cell based therapy is a potentially useful therapeutic approach in recipients of organ transplantation to minimize the burden of general immunosuppression (16–20). Moreover, the safety profile of the treatment opened the possibility to improve its efficacy by tailoring immunosuppression regiment to favor Treg survival upon *in vivo* injection, or by combining Treg administration with low dose of IL-2, which supports Treg survival *in vivo* (21).

In line with pre-clinical data revealing that donor-specific Tregs better suppress alloreactive T cells than polyclonal Tregs (22), a protocol to generate donor-specific Tregs generated with CD40L-activated allogenic B cells (darTregs) has been established (23) and tested in liver transplantation (NCT02244801 and NCT02091232). Results showed that infusion of darTregs is safe and lowers the incidence of serious adverse effects related to infections after transplantation (15). Other clinical studies are ongoing to test safety and efficacy of donor-specific Tregs administration alone or in conjunction with costimulatory blockade therapy (NCT03577431 and NCT03654040). Alternatively, trials in which donor-specific Tregs are administered at different time points post-transplantation (ARTEMIS trial, NCT02474199) or at different cell doses (dELTA trial, NCT02188719) are ongoing.

Tr1 cells are phenotypically defined as memory T cells that co-express CD49b and LAG-3 (24), and suppress immune responses *via* an IL-10-mediated mechanism (25). Tr1 cells were identified in patients treated with allogenic-HSCT who developed immunological tolerance with mixed chimerism (26, 27). Several GMP compatible protocols have been established to generate human allo-specific Tr1 cells (28). Originally, allo-specific Tr1 cells, differentiated by culturing human PBMC (or purified $CD4^+$ T cells) with allogeneic monocytes in the presence of exogenous IL-10 (29), prevented GvHD after haploidentical HSCT in adult patients affected by hematological malignancies, the ALT-TEN trial (30). The discovery of DC-10, a subset of monocyte-derived human DC that secrete IL-10 and express the tolerogenic molecules ILT4 and HLA-G (31), allowed the improvement of the protocol to generate allo-specific Tr1 cells leading to a population, which contains up to 15% of differentiated Tr1 cells (29) (**Figure 1**). A phase I trial was initiated (NCT03198234) in which the improved Tr1 cell product, termed T-allo10, generated by culturing patient-derived $CD4^+$ T cells with donor-derived DC-10 in the presence of IL-10, is administered at the time of allogeneic HSCT. Thus far, results indicate that the therapy is well-tolerated, but effects on GvHD and long-term tolerance are under investigation (Roncarolo M.G., personal communication). An alternative protocol to generate a Tr1 cell product, named T10 cells, suitable for cell-based approaches in SOT, has been established by culturing donor-derived $CD4^+$ T cells with patient-derived DC-10 in the presence of IL-10 (32). T10 cells have been planned to be tested in a clinical trial to prevent graft rejection after living-donor kidney transplantation (15), but they have not been tested yet. Finally, a protocol to expand DC-10-induced allo-specific Tr1 cells with stable phenotype and suppressive activity have been recently presented (Arteaga S. et al., FOCIS 2020). This protocol opens the window for establishing a Tr1 cell-based therapy in preventing allograft rejection.

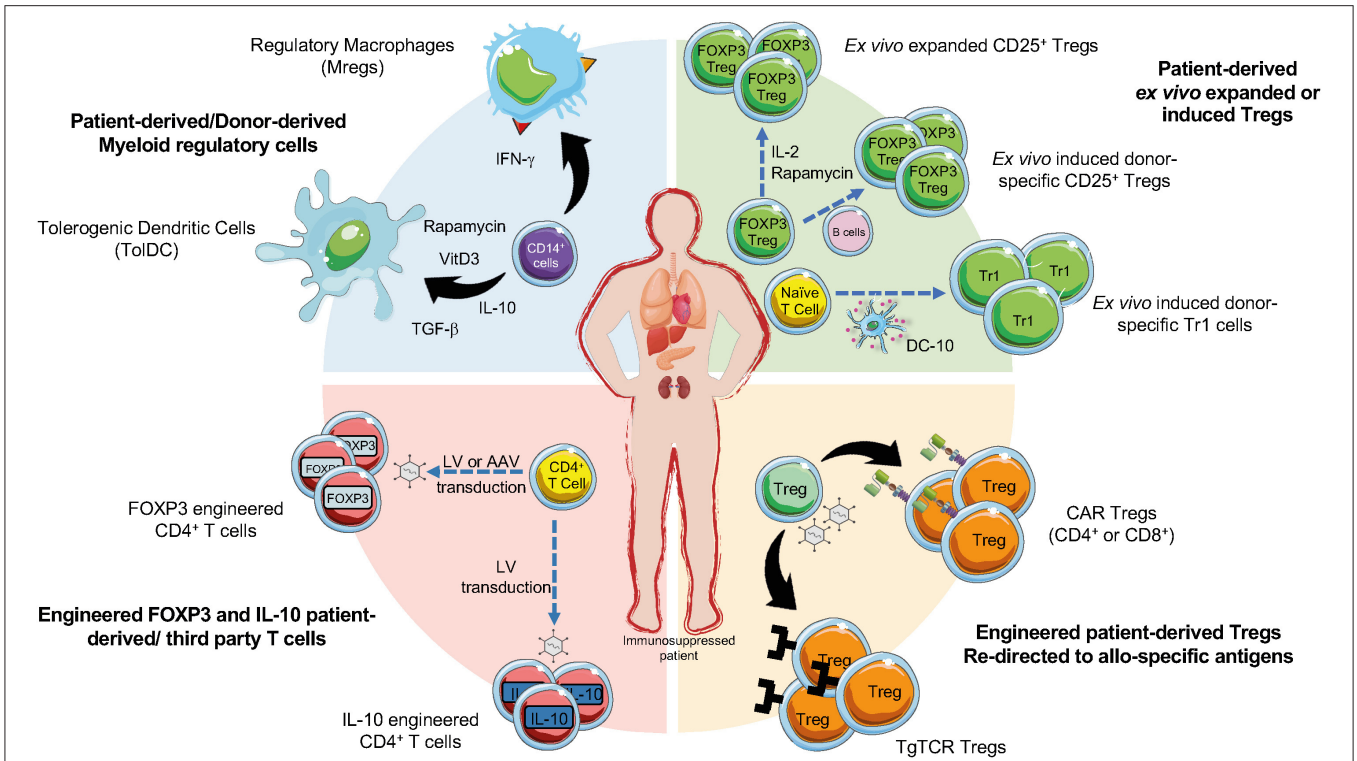

FIGURE 1 | Current cell-based strategies in organ transplantation. Regulatory T cells (Tregs), regulatory myeloid cells (MRCs), and engineered Tregs have been applied as cell-based therapy to promote tolerance in pharmacological immunosuppressed patients undergoing organ transplantation. *Ex vivo* expanded Tregs can be generated in presence of different agents (e.g., IL-2, or Rapamycin). Donor-specific Tregs can be generated upon activation with CD40L-activated B cells and then expanded, $CD4^+$ T cells co-cultured with allogeneic DC-10 differentiate into allo-specific Tr1 cells (green sector). Allo-specific redirected Tregs can be induced through the transduction with CARs or transgenic TCR (orange sector). The production of engineered FOXP3 and IL-10 overexpressing Tregs can be obtained by the transduction of $CD4^+$ T cells with lentiviral vectors (LV) or adenoviral vectors (AAV) encoding for IL-10 or FOXP3 (red sector). MRCs, tolerogenic DC (TolDC) or regulatory macrophages (Mregs), are differentiated from $CD14^+$ cells through exposure to immunomodulatory agents (e.g., IL-10, TGF-β, Rapamycin, Vitamin D3) (blue sector).

Engineering Tregs in Allogeneic Transplantation

Among various options to confer target specificity to Tregs, genetic engineering is highly appealing. Transduction of chimeric antigen receptors (CARs) (33) or synthetic T cell receptors (TCRs) (34) in Tregs have been demonstrated to be effective in pre-clinical studies *in vitro* and *in vivo* and are currently under intensive investigation.

CARs are synthetic proteins created by combining a single-chain antigen–binding domain derived from an antibody, fused to trans-membrane and intracellular signaling domains, usually encoding components of CD3ζ of a TCR and one or more costimulatory domains relevant for T cell activation (35). First developed for cancer immunotherapy, CARs demonstrated their feasibility in early pre-clinical studies in which $CD4^+FOXP3^+$ Treg specificity was redirected against antigen relevant to autoimmunity (36, 37). In the context of organ transplantation three groups developed Tregs expressing CAR targeting HLA-A2 (A2-CAR) to control alloreactive T cells after SOT. A seminal work in 2016 proved that A2-CAR expression in $CD4^+FOXP3^+$ Tregs enabled allo-specific recognition, proliferation, and preserved suppressive function *in vitro*. Despite this relatively strong CAR-mediated activation, A2-CAR Tregs retained high expression of FOXP3 without any significant induction of cytotoxic activity. In a humanized mouse model, A2-CAR Tregs prevented xeno-GvHD (38). Subsequently, other groups confirmed this approach, showing that A2-CAR Tregs suppress allo-responses better than polyclonal Tregs both *in vitro* and in humanized mouse models of $A2^+$ skin xenografts (39, 40). A2-CAR Tregs controlled *de novo,* but not memory, alloreactivity in skin allograft immunocompetent recipients (41). A panel of humanized A2-CARs was then generated and tested in $CD4^+FOXP3^+$ Tregs showing different degree of CAR expression, ability to bind A2, and induction of Treg-mediated suppression *in vitro* and *in vivo* (42). CAR encoding the wild type form of CD28 was superior to all other CARs *in vitro* and *in vivo* in terms of proliferation, suppression, and delay of GvHD (43). Despite the need for optimization, early success with CAR Tregs already brought the authorization of the first-in-human clinical trial to evaluate A2-CAR Treg therapy (TX200) for the prevention of rejection following A2-mismatched kidney transplantation (https://sangamo.com) (**Figure 1**). CAR

technology has been also applied to $CD8^+CD45RC^{low/-}$ Tregs, which delay allograft rejection in humanized mice (44), and are currently under clinical development for kidney transplanted patients (45) (https://www.reshape-h2020.eu/) (**Figure 1**). Pre-clinical results showed that A2-CAR $CD8^+$ Tregs were significantly more effective than polyclonal $CD8^+$ Tregs in preventing human skin transplant and xeno-GvHD in mice (46) (**Figure 1**).

Ectopic expression of a TCR, used to engineer T cell specificity in the field of cancer immunotherapy (47), has been applied also to Tregs. It has been reported that $CD4^+FOXP3^+$ Tregs expressing a transgenic TCR with direct allo-specificity were superior to polyclonal Tregs at prolonging heart allograft survival in mice (48, 49) (**Figure 1**). Although the development of human allo-specific TCR engineered Tregs has not been yet reported, this represents a promising approach because it recapitulates a more physiologic activation process, confers specificity for either extracellular or intracellular antigens, but limitations occur due to MHC restriction that implies matching of patients MHC genotype (12).

An alternative strategy to generate allo-specific Tregs, is the conversion of conventional T cells into Tregs by the overexpression of FOXP3 (50, 51). Lentiviral (LV)-mediated FOXP3 gene transfer into naïve $CD4^+$ T cells lead to $CD4^{FOXP3}$ T cells (**Figure 1**), with a stable phenotype, even in inflammatory conditions, and suppressive function *in vitro* and *in vivo* in several models. Moreover, $CD4^{FOXP3}$ T cells do not affect immune responses to pathogens or tumor clearance in xeno-GvHD model (50, 52). Alternative FOXP3 over-expressing $CD4^+$ T cells can be generated by the insertion of an enhancer/promoter proximal to the first coding exon of FOXP3 by passing epigenetic silencing of the gene. The edited cells exhibited a phenotype and cytokine profile superimposable to Tregs and showed strong immunosuppression *in vitro* and *in vivo* (51). Converted polyclonal $CD4^+$ T cells into $FOXP3^+$ Tregs can be used in the context of autoimmunity or allogeneic responses. Finally, to generate a more homogeneous population of IL-10 producing $CD4^+$ T cells ($CD4^{IL-10}$ cells) (**Figure 1**) an efficient protocol based on the use of LV encoding for human *IL-10* has been developed (53, 54). $CD4^{IL-10}$ cells are phenotypically and functionally superimposable to Tr1 cells and suppress xeno-GvHD *in vivo* (54). These findings pave the way for the improvement of the adoptive cell therapy with IL-10-engineered T cells in patients undergoing SOT and HSC transplantation.

Treg-Cell Based Therapy Conclusions and Future Perspectives

Clinical trials have proved the safety and feasibility of Treg-based therapy, and provided promising results on the ability of the treatment to taper immunosuppression and to prevent organ rejection at 1-year post-transplantation. Despite these results, several issues remain to be addressed. First, it is still to be defined the long-term safety profile related to Treg cell plasticity. Infused Tregs have indeed the potential to be destabilized in strong inflammatory conditions *in vivo,* adopting pathogenic T cell phenotype and functions, thereby possibly mediating graft rejection. Moreover, it is still an open question the overall long-lasting impact of Tregs on hampering immunity against infections and malignancies (55). Some of these questions will be addressed in ongoing phase II/III clinical trials.

Despite the promising clinical outcomes, cell isolation, manufacturing, dosing, specificity, and Treg tracking after infusion has been, so far, difficult. Moreover, *ex vivo* donor-specific Tregs or engineered Tregs compared to polyclonal expanded Tregs seems to be better; however, more investigation is needed to confirm the preliminary results. From clinical standpoint, one concern regarding the transgenic TCR is the mispairing with the endogenous TCR that can cause off-target effects. Moreover, engineered Tregs may have the risk of insertional mutagenesis due to viral transduction. These can be overcome by the development of CRISPR/Cas9 technology, which will further optimize the cell product (56, 57). Future potential application might be the combination of different engineering approaches to generate a more powerful (e.g., IL-10 or FOXP3) and allo-specific (e.g., CAR or TCR) cell product.

MRC-Based Therapy in Organ Transplantation

Myeloid cells are involved in mediating immune responses after organ transplantation. Donor DCs migrate from the graft to lymph nodes and activate alloreactive T cells, which then migrate back to the graft to mediate rejection. Moreover, tissue-resident macrophages by secreting pro-inflammatory mediators sustain graft rejection contributing to alloreactive T cell expansion. In the tolerated graft the anti-inflammatory microenvironment allows the differentiation of MRCs that in turn promote Treg expansion or the conversion of allo-specific T cells into Tregs (58, 59). These evidences together with the development of protocols to differentiate MRCs *in vitro* prompted investigators to apply MRCs as cell-based therapy to promote tolerance in the contest of SOT (**Figure 1**).

A protocol to generate human regulatory macrophages (Mregs) that suppress alloreactive T cell responses *in vitro* has been established (60). Mregs convert allogeneic $CD4^+$ T cells into IL-10-producing $TIGIT^+FOXP3^+$ Tregs *in vitro* and in a Mreg-treated kidney transplant recipient *in vivo* (61). After optimization of the Mreg manufacturing (62), the medicinal products Mreg_UKR has been tested to minimize immunosuppression after kidney transplantation (NCT02085629; ONEmreg12 trial), showing that Mregs, pre-operatively administered to transplant recipients, limited the number of infection-related adverse events and allowed tapering immunosuppression (15).

DC manipulation through exposure to anti-inflammatory and immunosuppressive agents have been shown to promote the differentiation of tolerogenic DC (tolDC) with the ability to modulate T cell responses and to promote Treg differentiation (63). The seminal study that led to the use of tolDC as cell therapy to prevent graft rejection showed that adoptive transfer of donor-derived tolDC prolonged heart graft survival in mice (64). After this work, several reports in pre-clinical models confirmed the ability of donor-derived tolDC alone or in

combination with costimulatory blockade, or cyclophosphamide, to prevent allograft rejection (59). These results were confirmed in non-human primates (65–67). More recently, it has been shown that administration of autologous tolDC, named ATDCs, generated in the presence of low-dose GM-CSF, prevented graft rejection in pre-clinical models and in non-human primates (68). ATDCs, through the generation of a lactate-rich environment, dysregulate the aerobic glycolysis of T cells, which suppress T cell proliferation, and promote Treg expansion (69). These data paved the clinical testing of TolDC-based therapy (NCT03726307; NCT0164265, and NTC0225055). Donor-derived DCreg generated with Vitamin D3 and IL-10 (70) administered 1 week prior to transplantation prolonged renal allograft survival and attenuates anti-donor $CD8^+$ memory T cell responses (71), and ATDCs infused in living kidney donor transplanted patients, demonstrated the ability to lower immunosuppression (15).

Comparison of clinical-grade tolDC generated with vitamin D3, IL-10, dexamethasone, TGFβ, or rapamycin demonstrated that all tolDC have a stable phenotype, but IL-10-modulated DC reproducibly induced suppressor Tregs (72). We and others developed IL-10-modulated DC (31, 73–75), and comparative analysis of DC-10, IL-10-modulated DC generated *in vitro* through the exposure of monocytes to IL-10 during DC differentiation (31), and IL-10-DC, monocyte-derived DC exposed to IL-10 during the last 2 days of DC differentiation (73), demonstrated that both cell types inhibited primary allogeneic T cell responses, but DC-10 were more effective in promoting allo-specific Tr1 cells *in vitro* (Gregori S. et al., personal communication). More recently, an efficient protocol to generate IL-10-producing human DC (DC^{IL-10}) through the transduction of monocytes with a LV encoding for IL-10 has been established (76). DC^{IL-10} secrete supra-physiological levels of IL-10, are stable upon exposure to pro-inflammatory signals, recapitulate the tolerogenic ability of DC-10, and inhibited allogeneic T cell responses *in vivo* (76).

MRC-Based Therapy Conclusions and Future Perspectives

MRC-based therapy represents an emerging approach on the context of SOT to taper general immunosuppression and to promote transplantation tolerance (77). Thus far, single administration of MRCs have been applied to transplanted patients; however, based on the assumption that tolDC promote tolerance via multiple mechanisms of immunomodulation, including the generation of a tolerogenic microenvironment that leads to a self-sustained tolerogenic process (78), possible multiple rounds of MRC administration may be more effective in dampening allogeneic T cell responses and in promoting allo-specific Tregs.

Despite the different methods to generate MRCs and the different models used, the common features converge in low expression of costimulatory and MHC molecules, maturation resistance, high expression of immunomodulatory molecules, modulation of T-cell responses and induction of regulatory cells. However, definition of shared markers and pathways by MRCs will help the comparison of the products and of their effects. Efforts to define guidelines, named minimum information, for MRCs (MITAP) have been recently reported, allowing some comparison between different cell products (79). Finally, in comparison with Tregs, MRCs have a limited lifespan upon *in vivo* delivery, overall lowering the risk of promoting adverse responses and long-term immunosuppression.

OVERALL CONCLUSIONS

Tregs and MCRs have been tested in clinical trials, overall demonstrating the safety and feasibility of the approach but the efficacy must be further investigated. Several hurdles have been encountered by investigators in performing such clinical testing using these advance medicinal products (ATMPs) [reviewed in Trzonkowski et al. (80) Ten Brinke et al. (81)]. Some of the burden include the difficulties in implementing GMP-compliant protocols to manufacture cell products, the cumbersome legislation for running trials, and the regulatory and ethical approvals, which vary among the countries. Despite the results obtained thus far, a number of important issued remains to be defined such as the dose and schedule of cell infusion/s, the identification of the appropriate immunosuppressive regimen, and the best suited cells for given diseases. It cannot be indeed excluded that specific regulatory cell can be suitable for one particular approach or another. Another key aspect in the field of regulatory cell-based therapy is the identification of effective and informative assays to monitor efficacy and signs of unwanted activation of adverse immune responses. Results from ongoing trials focusing on precise immune-monitoring will provide the identification of biomarker of efficacy and will offer important tools for optimizing regulatory cell-based therapy to prevent organ transplant rejection and promoting long-term tolerance. In this regard, initiatives similar to that undertaken by "the ONE study" for comparing regulatory cell products in the same setting and immuno-monitoring, are highly recommended. Moreover the European Union Cooperation in Science and Technology (COST) Action BM1305, "Action to Focus and Accelerate Cell-based Tolerance inducing Therapies-A FACTT," (A-FACTT) or Action BM1404, "European Network of Investigators Triggering Exploratory Research on Myeloid Regulatory Cells (Mye-EUNITER) by gathering expertise and investigators in the specific field of regulatory cell-based therapy enabled the creation of consensus on standard of common protocols and harmonizing guidelines for the analysis of cell products and clinical monitoring of immune responses after therapy. More recently, the INsTRuCT consortium, an Innovative Training Network (ITN) funded by the European Union H2020 Programme (https://www.instruct-h2020.eu/) established a network of European scientists, from academic and industry, designed to foster the pharmaceutical development of novel MRC-based therapies, by training the new generation of researchers in the field.

In conclusion, several efforts have been taken to advance regulatory cell-based therapy in the field of SOT and a number of additional investigations are necessary for rendering

this approach routinely applicable to transplant recipients. The required patient specificity, thus far, hampered the wide application of cell-based strategies, since high level of expertise, time and money are needed. The use of third-party (unrelated to the donor or recipient) cells to generate an "off-the-shelf" cell product is a promising endpoint. The ongoing efforts will shed light on the development of innovative and effective strategies applicable to SOT, which will allow long term survival of the graft, preventing rejection.

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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REVIEW
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Immunomodulatory Effects of Histone Deacetylation Inhibitors in Graft-vs.-Host Disease After Allogeneic Stem Cell Transplantation

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Histone deacetylase inhibitors are currently the most studied drugs because of their beneficial effects on inflammatory response. Emerging data from numerous basic studies and clinical trials have shown that histone deacetylase inhibitors can suppress immune-mediated diseases, such as graft-vs.-host disease (GVHD), while retaining beneficial graft-vs.-leukemia (GVL) effects. These drugs prevent and/or treat GVHD by modifying gene expression and inhibiting the production of proinflammatory cytokines, regulating the function of alloreactive T cells, and upregulating the function and number of regulatory T cells. Some of these drugs may become new immunotherapies for GVHD and other immune diseases.

Keywords: allo-reactive T cells, allogeneic hematopoietic stem cell transplantation, epigenetic regulation, graft-vs.-host disease, histone deacetylase inhibitors

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INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is one of the most effective therapies for hematological malignancies. Although the overall effect of allo-HSCT has improved with the improvement in conditioning regimens, effective control of infection, HLA matching technology, and donor selection, the incidence of graft-vs.-host disease (GVHD) is still 30–60%, with a mortality rate of 30–50% (1). GVHD is the main cause of death after transplantation (2), which limits the success of allo-HSCT. Acute GVHD (aGVHD) has been reported to mainly involve the skin, liver, and gastrointestinal tract of patients within 100 days after transplantation. Chronic GVHD is usually diagnosed after day 100 and mainly manifests as autoimmune symptoms, including dry syndrome, scleroderma and obliterative bronchitis. The pathogenesis of GVHD has been confirmed to involve an alloreactive immune response mediated by the activation of donor T lymphocytes (3).

The combination of a calcineurin inhibitor (CNI; i.e., tacrolimus or cyclosporine) plus methotrexate (MTX) and/or mycophenolate mofetil (MMF) is a standard GVHD prophylaxis regimen used with posttransplantation cyclophosphamide (PTCy) in most haploidentical donor transplant (HIDT) protocols (4–6). Alternative immunosuppressive drug combinations may further help reduce the risk of treatment failure. The incorporation of proteasome inhibitors into GVHD-prevention regimens represents one such strategy, which has generated significant interest (7, 8). However, the incidence of aGVHD is still high and CNIs can also inhibit graft-vs.-leukemia (GVL) effect, thereby increasing relapse rate. In addition, currently, methylprednisolone

is the first-line treatment for GVHD. However, the probability of complete remission of patients treated with methylprednisolone is <50% (9), and the long-term use of steroids may lead to steroid dependency and steroid-related adverse events, such as infection. Therefore, there is an urgent need to develop a new, safe, and effective strategy for prophylaxis and treatment of GVHD in the field of allo-HSCT.

Histone deacetylase (HDAC) inhibitors (HDACis) are currently used as anticancer drugs. Their effects in immune-mediated diseases have been studied. For instance, butyrate (pan-HDACi) has been reported to inhibit inflammatory response in a murine model of GVHD (10). This paper reviews advances in research on the application of HDACis for GVHD, and discusses their profound implications in immune cells involved in GVHD.

OVERVIEW OF HISTONE DEACETYLATION INHIBITORS

Histones, as structural proteins, are an important component of chromatin. According to the "histone code hypothesis," specific residues of histone tails exposed to the chromatin surface can be covalently modified, such as through lysine acetylation, to form "histone codes" and then trigger downstream events (11). Histone acetylation level is a result of the interaction between histone acetyltransferases (HATs) and HDACs. These two groups can acetylate or deacetylate histones (mainly H3 and H4) or some specific lysine residues of certain proteins, thereby altering the chromatin structure and ultimately affecting gene expression.

HDACs can be divided into four categories, of which classical classes I, II, and IV have sequence similarity, and their enzyme activities are dependent on Zn^{+}. Class I HDACs (HDAC1, 2, 3, and 8) are mainly located in the nucleus, class II HDACs (HDAC4, 5, 6, 7, 9, and 10) often shuttle between the nucleus and cytoplasm, and class IV HDAC (only HDAC11) is mainly located in the nucleus. Class III HDACs are Sir2-related enzymes (SIRT), which are deacetylases that depend on nicotinamide adenine dinucleotide, and this class has seven members, SIRT1–7, which are located in various organelles based on their functions (12, 13).

HDACis can inhibit the activity of specific histone deacetylases and upregulate the acetylation level of histones in specific cells as well as other specific non-histone molecules, thus regulating cell growth, differentiation, and immune response. HDACis have different effects on different cells. Their mechanisms include influencing DNA damage, DNA repair, and glycometabolism; altering gene expression; influencing cell growth; and inducing apoptosis, mitosis abnormalities, active oxygen redox, antiangiogenesis, antitumour metastasis, and autophagy of tumors (**Figure 1**). HDACis can be divided into six categories based on their chemical structure, including hydroxamic acids, short-chain fatty acids, cyclopeptides, electrophilic ketones, benzoamides, and other compounds. At present, four kinds of HDACis have been approved as anti-tumor drugs by the Food and Drug Administration (FDA). Among them, suberoylanilide hydroxamic acid (SAHA) and romidepsin can be used to treat T-cell lymphoma, panobinostat can be used to treat multiple melanomas, and belinostat to treat peripheral T-cell lymphoma (14). Notably, several HDACis have been used in clinical trials for prophylaxis or treatment of GVHD and show dramatic effects, such as a reduction in proinflammatory cytokine secretion and improvement of clinical symptoms (15) (**Table 1**).

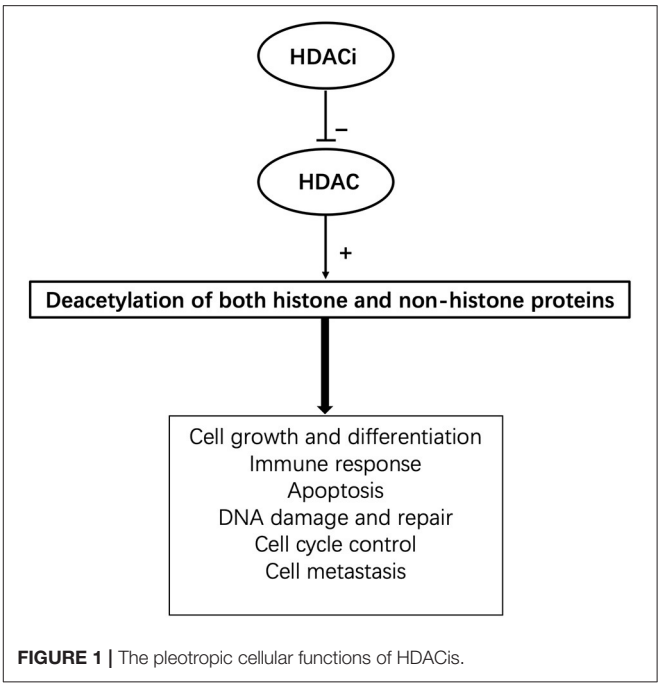

FIGURE 1 | The pleotropic cellular functions of HDACis.

Hydroxamic Acids

Hydroxamic acids chelate with metal atoms and bind reversibly with the zinc ions required for HDAC catalytic activity. This type of HDACi, including trichostatin A (TSA), vorinostat (SAHA), panobinostat (PANO), and belinostat, inhibits HDAC activities through competitive binding with the active site. TSA is a natural pan-HDACi that can inhibit class I and II HDACs. It has been used in diverse studies, including *in vitro* and *in vivo* experiments on various cancer strains and immune diseases. TSA reduces the expression of interleukin (IL)-12, interferon γ (IFN-γ), and IL-6 at both the mRNA and protein levels by promoting the acetylation of histones H3 and H4, and ultimately reduces renal disease in lupus mice (20). TSA has also been reported to regulate the expression of various costimulatory/adhesion molecules (such as CD28 and CD154) to alter T-cell function (21). However, the toxicity of TSA limits its clinical application (22). SAHA is a synthetic analog of TSA, but it has significantly

Abbreviations: HDAC, histone deacetylase; GVHD, graft-vs.-host disease; Allo-HSCT, allogeneic hematopoietic stem cell transplantation; HDACi, HDAC inhibitors; HATs, histone acetyltransferases; SIRT, Sir2-related enzymes; NAD, niacinamide adenine dinucleotide; SAHA, suberoylanilide hydroxamic acid; PANO, panobinostat; TSA, trichostatin A; IFN-γ, interferon γ; SCFAs, short-chain fatty acids; IECs, intestinal epithelial cells; MHC, major histocompatibility complex; VPA, valproic acid; Treg, regulatory T; ITP, idiopathic thrombocytopenic purpura; APCs, antigen-presenting cells; NK, natural killer; CD4+ Tconvs, CD4+ conventional T cells; IFIT1, tetratripeptide repeats 1.

TABLE 1 | Summary of the specificities and clinical trials of HDAC inhibitors in GVHD.

Name	Classification	HDAC specificity	Clinical research stage in GVHD	For GVHD
SAHA	Hydroxamic acids SCFAs	Pan-HDACi	I/II (16, 17)	Prophylaxis
Panobinostat		Pan-HDACi	I/II[1],[2]	Treatment
Butyrate	SCFAs	Pan-HDACi	II (18)[3]	Prophylaxis
Romidepsin	Cyclic peptides	Class I and II HDACs (mainly HDAC1 and 2)	I[4]	Prophylaxis
Chidamide	Benzamides	Pan-HDACi	Not reported yet	
Nicotinamide	SIRT inhibitors	Class III HDACs	I/II (19)	Prophylaxis

lower toxicity. Therefore, SAHA is more widely used than TSA in experimental investigations and clinical applications. SAHA has been shown to prevent GVHD after bone marrow transplantation in mice in an indoleamine-2,3-dioxygenase-dependent manner (23). Another study has also shown that the prevention of GVHD by SAHA is related to the regulation of the inflammatory cytokine environment and the inhibition of signal transducer and activator of transcription 1 (STAT1) (24). The feasibility of SAHA (100 mg, twice a day) combined with tacrolimus and MTX for GVHD prophylaxis after allo-HSCT was evaluated in a prospective phase I/II clinical trial (NCT00810602). Fifty patients diagnosed with high-risk hematological malignant diseases were enrolled in this trial. All the patients had an available 8/8 or 7/8 HLA-matched related donor and underwent reduced-intensity conditioning. The cumulative incidence of grade II–IV acute GVHD by day 100 was 22% (95% confidence interval [CI] 13–36%). The most common non-hematological adverse events included electrolyte disturbances ($n = 15$), hyperglycemia ($n = 11$), infections ($n = 6$), mucositis ($n = 4$), and increased activity of liver enzymes ($n = 3$) (16).

In addition, in a single-center prospective phase II clinical trial, a novel regimen, consisting of SAHA and standard prophylatic drugs, was evaluated after unrelated-donors HSCT. The results showed that the addition of SAHA reduced the incidence of grade II–IV aGVHD on day 100 from 48 to 28% or lower, assuming a type I error of 5%, and enhanced the acetylation of histone H3 in peripheral blood mononuclear cells while reducing IL-6 secretion (median, 4.2 vs. 7.6 pg/mL; $P = 0.028$) (NCT01790568) (17).

PANO has been approved by the FDA as a third-line treatment for multiple myeloma (25). Bug et al.[1] reported a phase I/II clinical trial of oral maintenance therapy using PANO for patients with myelodysplastic syndrome or acute myeloid leukemia who underwent allo-HSCT. Furthermore, in a phase I/II clinical study (NCT01111526), PANO was used in combination with glucocorticoids for the treatment of GVHD[2]. All participants in this study took PANO at a maximum tolerated dose (5 mg) three times a week for a month. At 36 days after study initiation, complete responses were observed in 12 patients (75%), partial responses were observed in 3 (19%), and progressive disease in 1 (6%). These trials demonstrated that PANO is safe to use after allo-HSCT; moreover, it can control GVHD and additionally function in targeting minimal residual lesions.

Short-Chain Fatty Acids (SCFAs)

SCFAs cannot bind to Zn^{2+} in the active center of HDAC; therefore, their inhibitory effect on HDAC is weaker than that of hydroxamic acids. SCFAs are the products of bacterial degradation of unabsorbed starch and non-starch polysaccharides (e.g., fibers). They are important anions in the colon's cavity and affect the morphology and function of colon epithelial cells (26). Some studies have shown that SCFAs can be absorbed by the intestinal epithelial cells (IECs), from where they enter the circulatory system through the liver, and ultimately affect cardiovascular function and inflammatory response (27, 28). Acetate, propionate, and butyrate are the main components of SCFAs. Among these, butyrate is the most important HDACi; it inhibits class I and II HDACs and has been shown to inhibit inflammatory responses in various inflammatory models. Cleophas et al. (29) found that butyrate can inhibit the expression of proinflammatory cytokines (IL-1β, IL-6, IL-8, and IFN-γ) in gouty arthritis and has strong anti-inflammatory effects. Furthermore, it has been found that fecal butyrate levels are decreased in patients after allo-HSCT. In a clinical study, stool samples from patients were obtained at baseline (before conditioning regimen), and on day 0 (day of allo-HSCT), day 7 post transplant, and day 14 post transplant. The results showed that the level of butyrate was significantly lower on day 14 than at the baseline, which was collected before allo-HSCT ($P = 0.0039$) (18). In 2016, to verify the effectiveness of butyrate in GVHD prophylaxis, a prospective phase II clinical trial (NCT02763033) was initiated to determine whether resistant starch can reduce the incidence of aGVHD[3]. In their ongoing study, the investigators speculated that the short-term administration of resistant starch increases intestinal butyric acid levels, thereby reducing the incidence of GVHD. In addition, in a previous study by Mathewson et al., (10) butyrate was effective in the treatment of GVHD in a mice model. The reduced butyrate in IECs after allo-HSCT resulted in decreased histone acetylation, whereas butyrate restoration improved the intestinal epithelium junction, re-established the intestinal flora structure, decreased IECs apoptosis, influenced IECs to present major histocompatibility complex (MHC) class II antigens.

Cyclic Peptides

Cyclic peptides are the most complex class of HDACis. They inhibit the enzyme activity of class I and II HDACs by interacting with Zn^{2+} at HDAC's active sites. Cyclic peptide HDACis can be classified as sulfur-containing and sulfur-free inhibitors. FR235222 is a sulfur-free cyclotetrapeptide inhibitor. It was first isolated from the fermentation broth of Cladosporium

[1]ClinicalTrials, n.d., ClinicalTrials.gov. NCT01451268.
[2]ClinicalTrials, n.d., ClinicalTrials.gov. NCT01111526.
[3]ClinicalTrials, n.d., ClinicalTrials.gov. NCT02763033.

(*Acremonium* sp. No. 27082) by Mori et al. (30). They found that FR235222 has a strong immunosuppressive ability, which effectively inhibited the proliferation of T cells and delayed hypersensitivity in mice and adjuvant-induced arthritis in rats. AS1387392 is an analog of FR235222, but it has better pharmacokinetic characteristics and is an orally bioavailable HDACi. Therefore, AS1387392 can be used as a new and effective immunosuppressant (31). Romidepsin is a sulfur-containing peptide HDACi, with a unique ring structure. It has been approved by the FDA for the treatment of cutaneous T-cell lymphoma (32). In addition, recent studies have found that romidepsin can inhibit the activation of STAT1 and STAT3 by inducing suppressor of cytokine signaling 1 expression, and it can suppress the expression of proinflammatory cytokines (e.g., IL-1β) induced by sodium urate crystals (33). In an ongoing phase I clinical trial (NCT02512497), romidepsin was administered in combination with fludarabine and busulfan before and after allo-HSCT to verify whether it helps in controlling leukemia or lymphoma and evaluate the safety of this combination[4].

Benzamides

Entinostat (MS-275) is a typical synthetic benzamide HDACi that selectively inhibits class I HDAC enzyme activity. Saito et al. (34) first discovered that MS-275 has a pronounced antitumour activity in mice. In addition to this activity, MS-275 has been used as an effective anti-inflammatory agent in recent studies and has been verified to be effective in some inflammatory models, such as experimental autoimmune encephalomyelitis and rheumatoid arthritis (35, 36). Lin et al. (35) found that MS-275 can effectively improve collagen-induced arthritis in animal models of rheumatoid arthritis; considerably reduce claw swelling, bone erosion, and absorption; and reduce serum IL-6 and IL-1β levels. Moreover, another study found that MS-275 can effectively inhibits inflammatory response in experimental autoimmune neuritis (EAN) in rats by inhibiting inflammatory T cells, macrophages, and proinflammatory cytokines, and inducing anti-inflammatory immune cells and molecules. This indicates that MS-275 may be an effective candidate drug for treating autoimmune neuropathy (36).

Chidamide is an orally absorbed benzamide pan-HDACi that was independently developed in China. It can stimulate the expression of Foxp3, a key transcription factor of regulatory T (Treg) cells, in patients with idiopathic thrombocytopenic purpura (ITP) and in ITP model mice. Moreover, it upregulates the expression of intracellular cytotoxic T-lymphocyte-associated protein 4 in Treg cells, induces Treg cell expansion, and restores immune tolerance (37). However, it is still worth exploring further whether benzamides HDACi can effectively prevent GVHD.

Sirtuin (SIRT) Inhibitors

Sirtuin inhibitors include nicotinamide, which inhibits all class III HDACs, and specific SIRT1/2 inhibitors, such as sirtinol, cambinol, and EX-527. Nicotinamide can inhibit proliferation and induce the apoptosis of chronic lymphoblastic leukemia cells by activating the p53/miR-34a/SIRT1 network (38). In a phase I/II clinical trial, it was confirmed that nicotinamide can be used to expand umbilical cord blood cells *in vitro*, and the median recovery times of neutrophils and platelets were shortened by 9.5 days (95% CI, 7–12 days) and 12 days (95% CI, 3–16.5 days), respectively, after umbilical cord blood transplantation, which significantly improved the safety of cord blood transplantation and reduced the incidence of GVHD (19). Anusara et al. (39) found that SIRT-1 knockout in mice enhanced p53 acetylation in T cells and promoted Treg stability. Furthermore, selective inhibition of SIRT1 by EX-527 significantly alleviated GVHD, improved survival of the mice, and preserved the GVL effect mediated by donor T cells.

EFFECTS OF HDACIs ON GVHD

The pathological mechanism of GVHD has now mostly been clarified and can be divided into three stages. In the first stage, tissue damage is caused by conditioning chemotherapy or infection, which activates "dangerous signal pathways" and leads to the secretion of proinflammatory cytokines (such as IL-1 and TNF-α). The second stage is the activation and amplification of effector T cells, in which antigen-presenting cells (APCs) and proinflammatory cytokines jointly activate donor T cells and cause their proliferation and the secretion of proinflammatory cytokines. The third stage is the immune effect stage, in which the activated donor T cells and abundant proinflammatory cytokines lead to tissue damage of the host, and this organ injury can further activate T cells. The following section summarizes the possible mechanism of action of HDACis in the treatment of GVHD in terms of occurrence and development stages of GVHD (**Figure 2**).

HDACis Inhibit the Production of Inflammatory Mediators

Reducing the secretion of proinflammatory cytokines, thereby downregulating immune response, is an effective strategy for the prophylaxis and treatment of GVHD. ITF2357 has been reported to decrease the expression of various mRNAs induced by IL-1β, including those of cytokines (IL-6 and IL-8), chemokines (CXCL2, CXCL5, CXCL6, and CXCL10), matrix-degrading enzymes (MMP1 and ADAMTS1), and other inflammatory mediators, and promote the degradation of IL-6, IL-8, PTGS2, and CXCL2 mRNAs (40). SAHA, which has been approved by FDA for the treatment of T-cell lymphoma, has antitumor effects at micromolar concentrations, whereas a nanomolar SAHA concentration can reduce the secretion of inflammatory cytokines, such as IFN-γ, TNF-α, IL-1, and IL-12. SAHA can downregulate the mRNA levels of TNF-α and IFN-γ by enhancing the acetylation level of histone H3 and inhibit the secretion of inflammatory cytokines (41, 42). Moreover, butyrate can enhance the acetylation of TNF-α and IL-6 promoters and block the binding of RNA polymerase II with TNF-α and IL-6 gene promoters. In other words, transcription initiation is inhibited, and the expression of TNF-α and IL-6 is reduced (43).

[4]ClinicalTrials, n.d., ClinicalTrials.gov. NCT02512497.

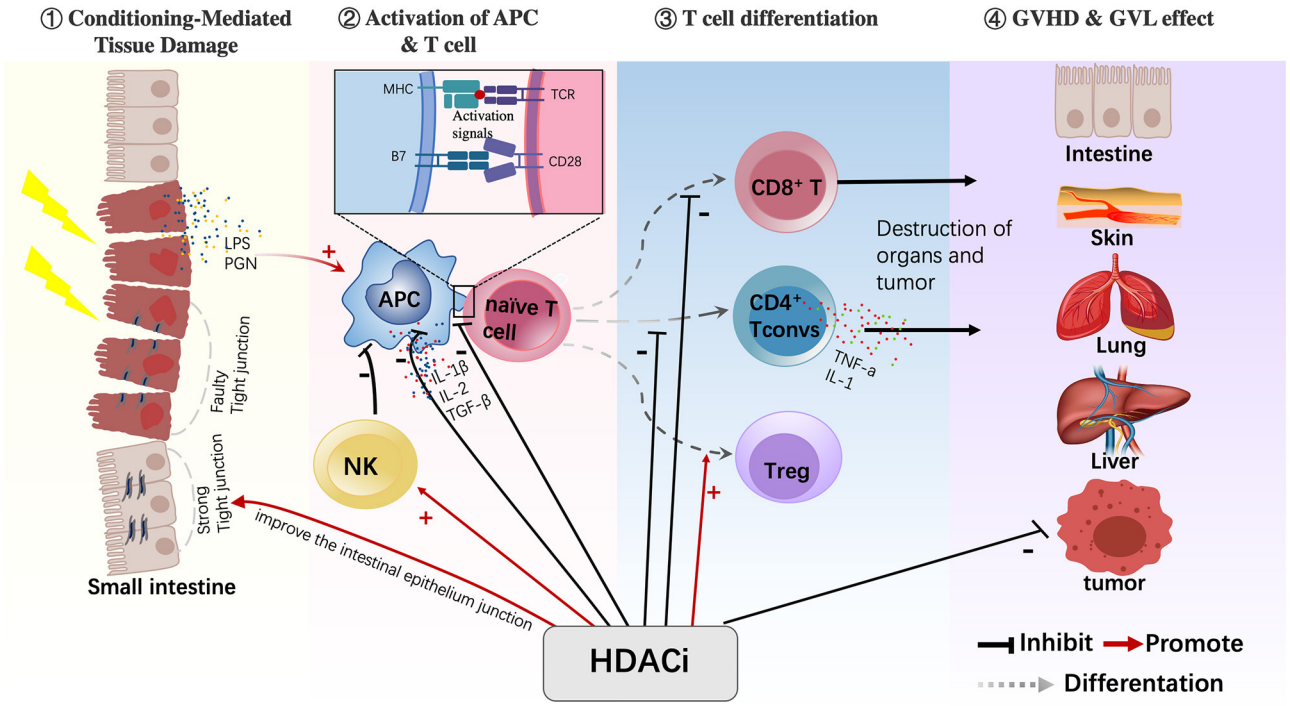

FIGURE 2 | HDACis have various immunomodulatory effects on different cells. HDACis play an important role in regulating the maturation of APCs, reducing their antigen-presenting capacity and inhibiting the production of proinflammatory cytokines. HDACis also promote the conversion of naive T cells into Tregs and increase their function. In addition, HDACis activate NK cells and inhibit $CD4^+$ Tconv cells and $CD8^+$T cells. Moreover, HDACis can improve the intestinal epithelium junction during the GVHD process (HDACi, histone deacetylase inhibitor; APC, antigen-presenting cell; Treg, regulatory T cell; NK, natural killer cell; $CD4^+$ Tconv, $CD4^+$ conventional T cells).

HDACis Regulate the Function of APCs

Dendritic cells (DCs) are the most effective type of APCs; they play an important role in the pathological process of GVHD. On the one hand, DCs can activate donor T cells by presenting host antigens; on the other hand, they can secrete numerous pro-inflammatory cytokines to further aggravate tissue damage. Pretreatment with TSA reduces the antigen-presenting activity of lipopolysaccharide (LPS)-induced DCs in a dose-dependent manner. TSA plays a role in regulating the maturation of DC cells; thus, pretreatment of DCs with TSA before LPS stimulation reduces the expression of maturation markers to the same level as that of immature DCs. In addition, TSA reduces the production of IL-2 in mature DCs stimulated by LPS (44). Furthermore, TSA can reduce the levels of proinflammatory cytokines (IL-1β, IL-12, and TGF-β) secreted by DCs (45).

HDACis and Regulatory T Cells

Sakaguchi et al. (46) first discovered that a small group of CD4+ T cells, named Tregs, expresses high levels of CD25 and that the removal of Tregs leads to autoimmune diseases. Tregs play a key role in maintaining peripheral immune tolerance by preventing autoimmunity and chronic inflammation (46). Subsequently, Hori S et al. demonstrated that Foxp3 is specifically expressed in Tregs and is a key regulator of cell development and function (47). In recent years, preclinical studies have shown that adoptive retransfusion of Tregs can inhibit GVHD and prevent or delay allograft rejection (48).

The acetylation of lysine in Foxp3 is necessary to maintain Treg function. Foxp3 acetylation promotes its binding with the IL-2 promoter and subsequently inhibits endogenous IL-2 production. HDAC can inhibit *FOXP3* gene transcription to some extent, whereas HDACis can enhance the homeostasis mediated by Treg proliferation. Therefore, HDACis are considered to be effective for increasing the number and inhibitory function of Tregs (49). Choi et al. (50) analyzed the immune response of patients receiving vorinostat for GVHD prevention after HSCT. Their results showed increases in the number of Tregs, methylation level of the Treg-specific demethylated region, and CD45RA and CD31 expression on the surface of Tregs, as well as enhanced inhibitory function.

Effect of HDACis on Natural Killer (NK) Cell Function

Donor NK cells can reduce the occurrence of GVHD by eliminating host APCs and secreting IL-10 in the early stage of transplantation, and they can directly eliminate recipient tumor cells. Delayed expansion of NK cells, especially immature NK cells, is associated with an increased aGVHD incidence and severity. Compared with patients without GVHD, patients with GVHD showed a significant decrease in the number of

NK cells in peripheral blood (51). Entinostat enhanced NK cell function through epigenetic upregulation of the IFIT1-STING-STAT4 pathway. In that study, the researchers found that entinostat significantly increased the expression of NKG2D, an essential NK cell-activating receptor. Furthermore, the killing function of NK cells was also enhanced. In terms of its mechanism, entinostat increases the accessibility of the chromatin in the promoter region of interferon-induced protein with tetratripeptide repeats 1 (IFIT1), thus upregulating the mRNA and protein expression levels of IFIT1 and enhancing the IFIT1–STING–STAT4 pathways mediated by IFIT1 (52). However, further studies investigating whether HDACi also promotes the killing function of NK cells by regulating acetylation, thereby eliminating recipient APCs and inhibiting GVHD, are warranted.

Effect of HDACis on Helper T Cells

As mentioned above, donor-derived T cells are the key cell subsets in the development of GVHD, whereas GVL also requires allogeneic T cells. Th1, Th17, and Th2 subpopulations contribute to GVHD, but they mediate GVHD to different degrees of severity (Th1 and Th17 mediating more severe GVHD) and different distributions of GVHD in target tissues (conversion to Th1 or Th17 cells is related to intestinal GVHD, whereas conversion to Th2 cells is related to lung GVHD) (53). Long et al. (54) demonstrated that valproic acid (VPA) can reduce the incidence and lethality of GVHD after allo-HSCT in mice, which is related to the downregulation of Akt phosphorylation and thus, inhibition of Th1 and Th17. In addition, TSA can inhibit inflammation by increasing the number of Th2 cells and enhancing their ability to secrete IL-4 (55).

DISCUSSION

This paper reviews the classification of HDACi and their direct or indirect effects on immune cells involved in GVHD. HDACi are an important class of anti-tumor drugs that have been used to treat a variety of tumors. Moreover, an increasing number of basic research and clinical trials have shown that HDACi have a strong anti-inflammatory effect and can negatively regulate GVHD while retaining beneficial GVL effects. Therefore, HDACi may become new immunotherapeutic options for prophylaxis and treatment of GVHD or other immune diseases.

AUTHOR CONTRIBUTIONS

Under the supervision of HH and YZ, the manuscript was written by XX and XL. All authors contributed to the article and approved the submitted version.

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Apoptotic Donor Cells in Transplantation

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Despite significant advances in prevention and treatment of transplant rejection with immunosuppressive medications, we continue to face challenges of long-term graft survival, detrimental medication side effects to both the recipient and transplanted organ together with risks for opportunistic infections. Transplantation tolerance has so far only been achieved through hematopoietic chimerism, which carries with it a serious and life-threatening risk of graft versus host disease, along with variability in persistence of chimerism and uncertainty of sustained tolerance. More recently, numerous *in vitro* and *in vivo* studies have explored the therapeutic potential of silent clearance of apoptotic cells which have been well known to aid in maintaining peripheral tolerance to self. Apoptotic cells from a donor not only have the ability of down regulating the immune response, but also are a way of providing donor antigens to recipient antigen-presenting-cells that can then promote donor-specific peripheral tolerance. Herein, we review both laboratory and clinical evidence that support the utility of apoptotic cell-based therapies in prevention and treatment of graft *versus* host disease and transplant rejection along with induction of donor-specific tolerance in solid organ transplantation. We have highlighted the potential limitations and challenges of this apoptotic donor cell-based therapy together with ongoing advancements and attempts made to overcome them.

Keywords: apoptosis, tolerance, transplantation, EDCI-SP, cell-based therapies

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INTRODUCTION

The use of immunosuppressive medications for transplantation has significantly decreased the incidence of acute allograft rejection, however they have had limited to no impact on chronic rejection and overall long-term graft survival (1). On the contrary, this pharmacological immunosuppression has side effects that include infections, malignancies, metabolic disease together with drug toxicities to the allograft itself. These detrimental side effects and non-specific immunosuppression can be potentially eliminated through donor-specific tolerance induction. Thus far in humans, one strategy that has been employed with encouraging results in solid organ transplantation is the use of combined kidney and hematopoietic stem cell transfers (CKHCT). This results in a state known as mixed chimerism, wherein both donor and recipient hematopoietic stem cells coexist and tolerance is achieved primarily through the central tolerance mechanism of intra-thymic deletion of donor-reactive T cells. This strategy has been successful in Human Leukocyte Antigen (2) identical transplants with the use of total lymphoid irradiation and T cell depletion for conditioning. However, in HLA-mismatched donor-recipient pairs, more aggressive conditioning

was required together with administration of a higher number of donor T cells that significantly increased the risk of the life-threatening complication of graft versus host disease (3–5). In the realm of non-chimeric approaches, immunoregulatory cell-based therapies have recently come into clinical trial space as well, with the most frequently used cells being regulatory T cells (T_{regs}), tolerogenic antigen-presenting-cells (APC) such as dendritic cells (DC) and regulatory macrophages, and lastly, myeloid-derived suppressor cells (MDSCs) (6–8). These cells have been used in treatment of graft-versus-host-disease (GVHD), rejection in hematopoietic stem cell transplant (HSCT) as well as tolerance induction in solid organ transplantation. However, the major challenges and hurdles of this approach include cumbersome manufacturing processes of these cells, selection of optimal timing and dose, conferring antigen specificity, and lastly, their *in vivo* instability.

Many of the aforementioned challenges encountered with the mixed chimerism approach and immunoregulatory cell therapy can be overcome with the use of apoptotic cells which can effectively deliver donor antigen while also creating an immunosuppressive milieu that promotes donor specific tolerance. Not only has this potential been utilized for tolerance induction and treatment of rejection in solid organ transplant, in HSCT it has also shown efficacy in reverting GVHD (9).

MECHANISMS

Apoptosis is essential to the maintenance of self-tolerance, thus mutations in apoptosis regulating genes such as Fas and Fas ligand (FasL) in humans as well as in mouse models have been implicated in autoimmune diseases (10, 11). Specifically, inability to effective clear dying cells can result in persistence of cellular debris which may lead to systemic autoimmunity such as systemic lupus erythematosus (12–14). Apoptotic cells attract and recruit macrophages to dying cells through "find-me" signals and facilitate engulfment through "eat-me" signals in a process known as efferocytosis (15). Efferocytosis involves four steps: recruitment, recognition, tethering and signaling and engulfment. At the onset of apoptosis, recruitment is carried out through "find-me" signals produced by apoptotic cells. These are sensed by phagocytes which are then recruited to the site of apoptosis. The second step, involves the interaction of binding ligands ("eat-me" signals) on the surface of apoptotic cells and their receptors on the surface of macrophages. As a consequence, the cytoskeletal rearrangement within the phagocyte occurs by a Rac1-mediated signaling pathway (16). The final step of engulfment follows this and internalization of apoptotic particles and their decomposition takes place within phagocytes.

One such "find me" signal is lysophosphatidylcholine, a lipid mediator that is produced and released from apoptotic cells and by interacting with the G2 accumulation receptor, it recruits macrophages (17). This is a G-protein-coupled receptor expressed in macrophages, dendritic cells, neutrophils, mast cells, T lymphocytes and B lymphocytes that is involved in regulating cell cycle, proliferation, and immunity. Its further functions are not known well, however it's interaction with lysophosphatidylcholine possibly results in the production of chemoattractants such as monocyte chemotactic protein-1 (MCP-1), IL-8 and chemokine ligand 5 (CCL5) for the recruitment of monocytes, neutrophils and lymphocytes. Another "find me" signal is sphingosine-1-phosphate that acts on macrophages to increase erythropoietin (EPO) expression, subsequently activating the peroxisome proliferator-activated receptor-γ (18). This enhances the expression of numerous phagocyte receptors like MerTK, MFGE8, Gas6, and CD36, all of which play a role in promoting phagocytosis.

Cells express phosphatidylserine (PtdSer) on their surface when undergoing apoptosis, which then acts as an "eat-me" signal (19, 20). Using Annexin I as a bridging molecule, PtdSer interacts with the TAM family (21) of receptors to promote phagocytosis. This TAM family are tyrosine kinases receptors for Gas6 and protein S which bind PtdSer and antagonize inflammatory cytokine production by STAT-1-dependent induction of suppressor of cytokine signaling (SOCS) proteins 1 and 3 (22, 23). Furthermore, apoptotic cell-mediated activation of Mer inhibits lipopolysaccharide (LPS) driven PI3K/AKT-dependent NF-κB activation (24). As NF-κB signaling results in production of numerous inflammatory cytokines, targeting of MerTK and possibly other TAM receptors therefore has the potential for inhibiting inflammatory cytokine production. Interestingly, the precipitation of a severe autoimmune phenotype in mice deficient in TAM receptor expression suggests that they may play a role in induction of suppressive macrophages (25). Therefore as briefly outlined above, unlike necrosis, not only does apoptosis not elicit an inflammatory response, it has immunomodulatory effects that are exerted through leukocytes such as APCs, regulatory cells and soluble factors as described further and illustrated in **Figure 1**.

Soluble Factors

Apoptotic cells themselves release soluble mediators in their local milieu such as IL-10, TGF-β, and annexin A1 which exert immunosuppressive effects (26–28). In addition to that, macrophages that interact with apoptotic cells also downregulate immune response through release of IL-10, TGF-β and PGE_2 together with a reduction in inflammatory cytokines such as IL-1α, IL-1β, IL-6, IL-12p70 and TNF-α (29–31). The downstream effects of these cytokines include but are not limited to the prevention of differentiation of T helper type 1 (Th1) and repression of MHC-II and costimulatory molecule expression on APCs. This deters further antigen presentation and T cell activation.

The release of TGFβ *in vitro* has been demonstrated to be carried out by recipient macrophages ingesting apoptotic cells but not during any other type of phagocytosis (32). This production is due mainly due to the ligation of PtdSers exposed on apoptotic cells to their receptor expressed on macrophages (32, 33). TGFβ induces T_{regs} (identified by expression of $CD4^+CD25^+CD45RB^{low}$ $CD62L^{high}$ intracellular CTLA-4^{high} and high forkhead-box transcription factor p3 (Foxp3) mRNA) in both peripheral blood and spleen in murine bone marrow transplantation model receiving

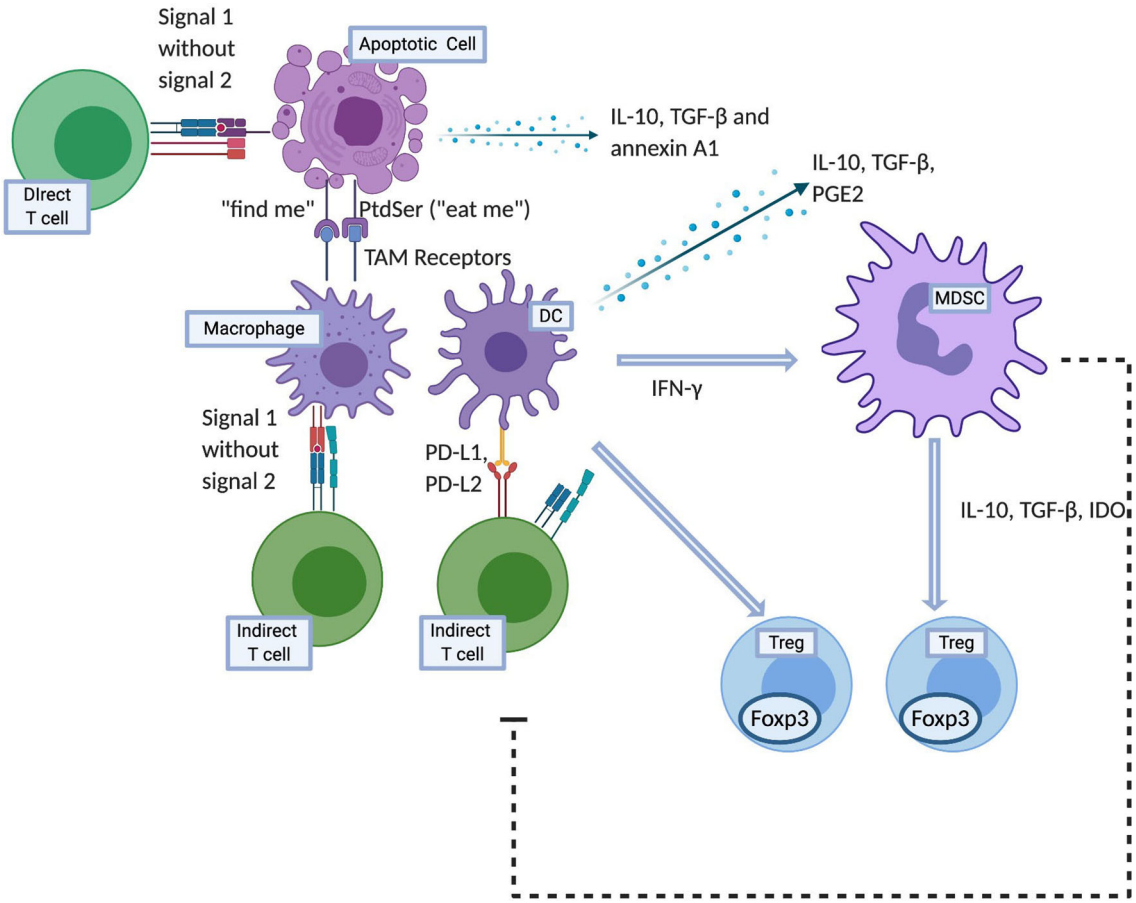

FIGURE 1 | Mechanisms of apoptotic cell induced tolerance. Created with BioRender.com.

apoptotic cell infusions (34). This effect on T_{regs} was not seen with TGF-β neutralization. This process is functionally relevant as well, wherein depletion of these T cells results in an augmented allogenic response.

IL-10 specifically is an anti-inflammatory cytokine that plays a role in tolerance induction and suppression of DC maturation (35). However, conclusive evidence linking apoptotic cell-induced suppression of adaptive immune responses exerted through IL-10 is lacking. This suggests that the mechanistic expanse of the immune responses to apoptotic cells likely extends beyond solely cytokine-mediated effects. Verbovetski et al. also outlined a role of complements in this process by demonstrating that uptake of iC3b-opsonized apoptotic cells resulted in upregulation of the expression of CCR7 on immature DCs, rendering these cells capable of migrating in response to CCR7 ligands to secondary lymphoid organs to initiate or maintain T cell peripheral tolerance (36).

Control of APC Functions

Investigations of the effect of apoptotic cells on APCs have shown that ingestion of apoptotic cells by immature DCs leads to their resistance to maturation and activation, therefore inhibition of MHC Class II, CD40 and CD80/86 (37, 38). This in turn can decrease their ability to stimulate T cells despite intact apoptotic cell-derived antigen presentation. Effector T helper 17 (Th17) cells are suppressed while T_{regs} are induced through ingestion of apoptotic cells by DCs and the subsequent DC-T cell interaction in the presence of altered co-stimulatory and coinhibitory signals (39). Antigen coupled apoptotic cells induce T cell tolerance *via* IL-10 production and upregulation of PD-1 expression on APCs (40). PD-L1 on APCs then binds to CD80 expressed on T cells with a greater affinity than CD28 binding, and negatively regulates T cell activation (41).

One could hypothesize that macrophages contribute significantly to the tolerogenic response given that they induce T_{regs} (42). Supporting that hypothesis, various studies show the essential nature of macrophages in settings of tumor and autoimmune disease-related tolerogenic responses to apoptotic cells (43, 44).

Beyond macrophages and DCs, another distinct cell population that has been shown to play a role in apoptotic cell related immunosuppressive effect are monocytic-like ($CD11b^{+}Ly6C^{high}$) and granulocytic-like ($CD11b^{+}Gr1^{high}$) MDSCs (45). In cardiac allograft model, these cells exert their immunosuppressive effect by trafficking to the allograft where they inhibit local CD8 T cell

accumulation and potentially induce and recruit T_{regs}. Both populations have been shown to suppress T cell proliferation *in vitro* through antigen-dependent as well as antigen-independent methods *via* a variety of effector mechanisms, including nitric oxide (46), arginase, and reactive oxygen species (47–50). Furthermore, they promote T_{reg} induction through production of IL-10, TGF-β and indoleamine 2, 3-dioxygenase (51, 52). Most evidence suggests that MDSC subsets require IFN-γ, both for their induction and their effector function (53–56). Consequently, neutralization of IFN-γ completely abolishes the suppressive capacity of this population (57). For phagocytosis of apoptotic cells in the spleen, macrophages, T and natural killer (NK) cells are the potential sources of IFN-γ (58).

Another distinct APC population of interest is plasmacytoid dendritic cells (pDC). They have been not been demonstrated to be directly affected by apoptotic cells. However, the soluble factors released by macrophages upon interaction of with apoptotic cells can induce pDC activation, manifesting as an increased expression of CD86 and IFN-α (59). These pDCs can then stimulate T_{reg} generation through TGF-β dependent mechanisms. In cardiac allograft transplantation, alloantigen-presenting pDCs home to the lymph nodes in tolerogenic conditions, where they mediate alloantigen-specific T_{reg} cell development and prolong graft survival (60). Apoptotic cells can also drive activated pDCs to adopt a regulatory phenotype, capable of inducing IL-10-secreting T cells (61).

Regulatory Cells

APCs are pivotal in priming T cell responses, but also in the induction of $Foxp3^+$ T_{regs}. This has been demonstrated after intravenous apoptotic cell infusions, local apoptotic death of epithelial cells and it occurs in a TGF-β dependent environment (62). Interestingly, the induced T_{regs} are likely antigen specific as was demonstrated in a murine arthritis model (63). The precise mechanisms that induce naïve T cell differentiation to T_{regs} requires further investigation however it's distinctly clear that they play a vital role in maintenance of tolerance.

Apoptotic cells also activate splenic B cells to assume a regulatory phenotype which further induces CD4+ T cells to secrete IL-10. In a mouse collagen induced arthritis model, apoptotic cell therapy delayed the clinical onset and protected mice from severe joint inflammation and bone destruction through this mechanism where inhibition of IL-10 *in vivo* reversed the beneficial effects of apoptotic cells. These regulatory B cells (B_{regs}) cells also produce IL-10 themselves and their passive transfer provides significant protection from arthritis to the mice (64).

APOPTOTIC CELL THERAPIES IN SOLID ORGAN AND TISSUE TRANSPLANTATION

As outlined above, apoptotic cells have the potential to be utilized in the field of transplantation due to their immunomodulatory potential and being a source of allo-antigens that can be captured and presented by APCs in an immunoquiescent environment. Intravenous infusion of apoptotic cells is the most commonly employed method of delivery. The use of donor derived apoptotic cells efficiently combines the delivery of apoptotic cells and donor antigens. However, provision of apoptotic signals and donor antigens can also be dissociated. For example, as outlined in the various studies described in the later part of this review, major histocompatibility complex (MHC) match between the apoptotic cells and the donor does not appear to be essential to induce tolerance in the recipient, as the delivery of any source of apoptotic cells (syngeneic, allogeneic, and xenogeneic) can induced recipient tolerance to the antigens co-delivered with the apoptotic cells. Therefore, while the source of apoptotic cells can be variable, the tolerance induced in this manner carries antigen-specificity that is established by the specific antigens provided at the time of apoptotic cell infusions (for example: apoptotic cells of donor origin; apoptotic cells infused with donor bone marrow cells; apoptotic cells infused to treat rejection or GVHD when donor cells are already present in the recipient). Once infused, these cells accumulate initially in the periphery of the splenic follicles within the marginal zone DCs and macrophages. Not only are apoptotic cells processed by recipient APCs to downregulate the indirect pathway T cells *via* negative co-stimulatory molecules, they can also directly interact with the direct pathway T cells and anergize these T cells by providing signal 1 without signal 2 (**Figure 1**) (65, 66).

Several *in vitro* methods can be utilized to induce apoptosis of cells. These include radiation strategies such as γ-radiation (65) or UV-B irradiation (66–68); and chemical treatments such as ethylene carbodiimide (ECDI) (69–72) or paraformaldehyde (73). An important consideration during the process of inducing apoptosis is to ensure early stage of cell apoptosis by the process, as late stages of apoptosis can in fact lead to immune activation due to loss of plasma membrane integrity, and subsequent release of intracellular contents and engagement of damage-associated molecular patterns (DAMPs) (74, 75). To determine the spectrum of stages from apoptosis to necrosis that the cells are in, one method is to quantify their surface annexin V and propidium iodine PI (PI) expression, wherein annexin V positivity marks apoptosis and PI positivity marks necrosis (72). The other important consideration is the timing of apoptotic cell infusion, most studies have achieved maximum benefit when infusions are administered 7 days prior to transplantation. This is likely to because it gives ample time for the processing of apoptotic cells by splenic APCs and subsequent induction of the aforementioned regulatory cell populations.

One of the effective methods that we have extensive experiences with and utilize to deliver donor apoptotic cells is through chemical treatment of donor splenocytes with ECDI (ECDI-SP) (71, 72, 76–78). ECDI is a hygroscopic, water-soluble chemical peptide cross-linker that acts by activating free carboxyl groups, catalyzing the formation of covalent peptide bonds between the active carboxyl group and primary amines (79, 80). The advantages of ECDI-treated cells are that they demonstrate better viability when maintained at 4°C, but within hours of *in vivo* administration they undergo rapid apoptosis (81). Cell based therapies such as donor specific transfusion (DST) carry a significantly higher risk of recipient

sensitization, especially in those with pre-existing alloimmunity, while ECDI-SP might possibly confer therapeutic benefit in that scenario (82). In transplantation, ECDI treated cells have been used in non-human primates; while in autoimmune diseases, autoantigen-coupled syngeneic leukocytes have been developed for a phase I clinical trial for multiple sclerosis and have demonstrated the safety of this approach in this study (83).

Preclinical data from different groups has shown in murine models of cardiac transplantation that prolonged vascular allograft survival can be achieved through intravenous infusion of apoptotic donor splenocytes prior to transplantation. Sun et al. utilized UV or γ irradiation to induce apoptosis in splenocytes from donor strain rats, followed by confirming the apoptotic stage by using annexin V and PI staining (65). Apoptotic donor splenocytes were subsequently injected at a dose of 5 x 10^7 per recipient a week prior to transplantation. This treatment alone resulted in a significant prolongation of graft survival from a median survival time of 7 days in untreated controls to 53 days in the treatment group. Histological analysis also revealed reduced leukocyte infiltration in the allograft in the treated recipients. Furthermore, the authors demonstrated that *in vivo* blockade of phagocytic activity prevented graft protection by this treatment. Another group led by Wang et al. independently tested the utility and mechanism of donor apoptotic cell infusions in a fully mismatched aortic allograft murine model (67). They established that donor apoptotic cell infusions downregulated indirect anti-donor response and improved chronic allograft vasculopathy (CAV). Through directly targeting DCs with allo-antigens, the anti-donor indirect T and B cell responses in allograft recipients were ameliorated. In liver transplantation in rats, donor apoptotic splenic lymphocytes have been shown to promote liver graft acceptance and increase peripheral T_{regs} as well (84, 85). Furthermore, in liver transplant rejection, administration of tolerogenic DCs with apoptotic lymphocytes alleviated the rejection while inducing immune tolerance (86).

Donor apoptotic cell infusions in islet transplantation in mice, have shown to prolong islet survival through T_{reg} induction and tolerogenic DCs (87, 88). Beyond murine studies, in non-human primates using donor apoptotic cell infusions have also shown promising results in allogeneic islet transplantation. An earlier study in non-human primates by Lei et al. showed prolonged islet allograft survival in monkeys infused with ECDI-SP on the day of transplantation; however, the effect was not sustained and the duration of graft survival following discontinuation of immunosuppression was 48 to 133 days, although the infusion of ECDI-SP was associated with significant $CD4^+CD25^+Foxp3^+$ generation and expansion (89). Singh et al. used peri-transplant apoptotic donor leukocyte infusions, 7 days prior to transplant and 1 day after, along with short-term immunotherapy consisting of antagonistic anti-CD40 antibody, rapamycin, soluble tumor necrosis factor receptor, and anti-interleukin 6 receptor antibody for tolerance induction for intra-portal allogeneic islet transplantation in rhesus macaques (90). All of the five rhesus macaques showed operational tolerance to their islet allografts and demonstrated intact islets on histopathology of the liver at necropsy when the end point was reached. This strategy was successful in inducing long-term (≥1 year) tolerance of islet allografts in five of five non-sensitized, MHC class I-disparate, and one MHC class II DRB allele-matched rhesus macaques. Compared to monkeys that did not receive peri-transplant ECDI-SP infusions, the administration of ECDI-SP was associated with suppression of anti-donor CD4+ and CD8+ T effector memory (TEM) cell expansion within the circulating and liver mononuclear cells (LMNCs) and mesenteric lymph node (LNs). Additionally, a higher percentage of circulating natural suppressor and T_{reg} cells were present in the ECDI-SP-treated cohort. Notably, another cohort of fully MHC mismatched donor recipient pair did not show similar induction operational tolerance, or an increase in regulatory cell types or suppression of TEM responses. This could suggest that in this non-human primate study one-DRB-matched ECDI-SP infusion possibly provided a shared MHC II necessary for T_{reg} activation and/or expansion. Both studies demonstrate the overall safety of ECDI-treated leukocyte infusions, therefore providing a strong foundation for clinical translation of this approach (90).

To date, the only clinical trial utilizing a modified cell infusion for induction of transplant tolerance in solid organ transplant is a phase I trial of mitomycin-treated donor mononuclear cell infusions in ten kidney transplant recipients (91). The primary outcome of demonstrating safety of the infusions was achieved with the infusions being well tolerated without side effects. Importantly, none of the patients developed *de novo* donor specific antibodies (DSAs) or experienced any rejection episodes. The infusions were administered to three different subgroups of patients, in increment doses and at different time points with respect to their day of transplantation (group A: 1.5 x 10^6 per kg body weight (BW) on day −2; group B: 1.5 x 10^8 per kg BW on day −2 and group C: 1.5 x 10^8 per kg BW on day −7). Interestingly, subsequent testing showed suppression of donor-stimulated recipient leukocyte proliferation, whereas response to third party stimulation was intact. The best results were observed with the higher dose given at the early (day −7) time point. The presence of a strong $CD19^+CD24^{hi}CD38^{hi}$ B_{reg} induction together with IL-10 production and evidence of an immune tolerance signature similar to that seen in immune tolerance network studies (92) suggest that donor apoptotic cell infusions may promote donor-specific tolerance. This can be compared to the aforementioned similar IL-10 producing B_{reg} induction seen mice autoimmune disease model with apoptotic cell infusion treatment (64, 93).

Interestingly, the authors noted that infections caused a transient disappearance of donor-specific hypo-responsiveness as demonstrated by *in vitro* donor-stimulated recipient lymphocyte proliferation. This trial thus successfully demonstrated the safety and possible efficacy of donor apoptotic cells in inducing donor-specific hypo-responsiveness for solid organ transplantation.

APOPTOTIC CELL THERAPIES IN BONE MARROW TRANSPLANTATION

MHC disparity between donor and recipient remains a challenge to HSCT. Presence of T cells of donor origin in the graft faciliates

bone marrow engraftment and prevents disease relapse, however it can increase the risk of GVHD. Similarly, recipient T cells that are not eliminated during conditioning impairs bone marrow engraftment and increases the risk of disease relapse and graft failure. This constitutes a unique challenge with using T cell depletion strategies in bone marrow transplatantion(BMT) that result in T cell depletion of donor and recipient origins, therefore underscores the need for alternatives to global T cell depletion strategies in BMT.

In the last decade, due to their immunomodulatory effect, apoptotic cell therapies have entered clinical translation and been tested as a prophylactic therapy for acute GVHD in HLA-matched myeloablative allogenic BMT (94) (2). Notably, a phase I/IIa clinical trial enrolled 13 patients with hematological malignancies, and infused them with incremental doses of donor mononuclear apoptotic cells (ApoCell) on day −1 followed by BMT with a myeloablative conditioning regimen on day 0. Overall, six of the patients who received the higher dose of ApoCell showed 0% incidence of grade II to IV GVHD, and the remaining seven patients showed a lower incidence of GVHD compared to published data of historical controls not receiving ApoCell infusions. Notably, ApoCell infusions had no effect on the time to engraftment, chimerism, or incidence of infections among the treated subjects. These observations support the needs for larger trials with even higher doses and possibly more frequent dosing of ApoCells in BMT as a GVHD prophylaxis (94).

Bittencourt et al. evaluated the effect of administration of irradiated apoptotic leukocytes from either donor or non-donor sources in murine model of mismatched BMT to determine whether the source of the apoptotic cells had an effect on the outcome (68). The addition of apoptotic cells resulted in a significant increase in the number of engrafted mice, along with a higher percentage of donor type cells in the mice that received apoptotic splenocytes. Interestingly, this effect was indiscriminate of whether the injected apoptotic leukocytes were from third party or syngenic hosts, or even from xenogeneic hosts such as human blood mononuclear cells, suggesting that the MHC molecules of the apototic cells do not need to match to either the donor or the recipient for this approach to be effective. This study thus demonstrated that apoptotic cells could have a utility in overcoming MHC barriers in BMT through possibly cross-tolerizing anti-donor recipient T cells, and therefore may also be used to reduce the intensity of conditioning regimens (68). Donor and third party apoptotic cell infusions have shown to lower the incidence of donor allo-immunization with only one out of forty-four mice developed DSA (95). This finding is in agreement with the reported poor immunogenicity of apoptotic cells compared with identical viable or non-replicating cells.

ROLE OF APOPTOSIS IN EXTRACORPOREAL PHOTOPHERESIS

Extracorporeal photopheresis (ECP) refers the process of UV-A radiation of autologous mononuclear cells obtained *via* leukapheresis, followed by photosensitization with by 8-methoxypsoralen (8-MOP) and infusion back to the patient. ECP was initially used to treat patients with cutaneous T-cell lymphoma (CTCL), but its indications for use have now extended to other conditions such as GVHD (96), scleroderma (97), and solid organ transplantation (98–100). In a standard ECP treatment, usually only 10% of total blood circulating mononuclear cells are obtained and exposed to 8-MOP, and the susceptibility to ECP-induced apoptosis varies from cell type to cell type (101). The exact mechanisms of the therapeutic effect of ECP still remains to be elucidated, but in CTCL it has been described that the ingestion of apoptotic cells by APCs results in production of anti-tumor cells targeting malignant lymphoid cells (102). This explains its beneficial effect in CTCL, however its utility in GVHD is likely to be due to a wider scope of less well-defined immunomodulatory effects.

Gorgun et al. demonstrated a shift in the cytokine profile toward a Th2 response in patients who underwent ECP for GVHD treatment (103). Specifically, they demonstrated an increase of IL-4, IL-10 and TGF-β and a concurrent decrease of IL-12, IL-1, interferon-γ, and TNF-α. Furthermore, leukocyte proliferation assays using DCs from patients undergoing ECP showed decreased proliferation of antigen-stimulated autologous and allogeneic T cells. Circulating T_{regs} with ECP therapy suppressed proliferation of allogenic effector T cells and their IFN-γ secretion (104). The above described T cell responses have prompted its use together with conventional pharmacotherapy for the treatment of GVHD as well as acute rejection of cardiac allografts in humans (101, 105–110).

LIMITATIONS

Prior Sensitization

Transplant recipients with memory cells as a result of previous sensitizing events can be challenging to transplant as they mount a rapid and aggressive immune response compared to their non-sensitized counterparts, thereby increasing the risk for immediate graft loss (111–113). The presence of donor specific antibodies (DSAs) can also lead to an accelerated rejection through complement activation, resulting in endothelial damage in solid organ transplantation (113). Burns et al., demonstrated in a sensitized murine cardiac transplant model that memory B cells override the tolerogenic effect of donor-specific transfusions (DST) combined with co-stimulation blockade by anti-CD154. Furthermore, they also facilitate the priming of alloreactive T cells and thus, in the presence of DSAs, result in accelerated graft loss (82, 114). A similar concern may also exist for apoptotic donor cell infusions in the presence of DSAs.

On the other hand, when DSAs are at low or negligible levels, we have demonstrated in a sensitized murine islet transplant model that infusions of donor ECDI-SP together with transient anti-CD154 and rapamycin are effective in early inhibition of alloreactive T and B memory cells, therefore protect islet allograft function. Analysis of donor-specific T memory cells in these

recipients treated with this combination therapy showed almost a complete absence in the islet allograft as well as in draining lymph nodes. Memory B cells also met a similar fate in that in recipients treated with this combination therapy, their numbers in draining lymph nodes were also significantly suppressed. These findings correlated with superior islet allograft survival in these previously sensitized recipients. Thus, the use of donor EDCI-SP also shows promises for transplantation of sensitized recipients (115).

Infection and Tolerance

Opportunistic infections and latent viral activation, such as CMV, pose a considerable challenge in transplantation overall. In the context of tolerance, many of the aforementioned authors have described both in murine models, non-human primates and phase I clinical trials, that infections can negatively impact tolerance induction. Such infections have also been demonstrated to be deleterious to the stability of donor-specific tolerance, thereby effecting long-term host alloreactivity and graft survival (116).

Of the common pathogens, cytomegalovirus (CMV) is a highly prevalent virus that causes a symptomatic infection that has been noted as an independent risk factor for the development of acute rejection (117). Our lab has demonstrated in a murine islet transplant model that acute murine-CMV(MCMV) infection alters MDSC differentiation, promoting maturation of immature myeloid cells to become inflammatory monocytes which subsequently prime alloreactive CD8 T cells that prevent the induction of tolerance (78). In mice where MCMV infection was introduced days after donor ECDI-SP infusions, it not only led to the disruption of tolerance otherwise induced by donor ECDI-SP infusions, but also resulted in accelerated rejection of a subsequent same-donor islet transplant as a consequence of anti-donor memory T cell response (118).

Other pathogens that have been studied include the gram-positive intracellular bacteria Listeria monocytogenes (Lm). Wang et al. demonstrated that a sublethal dose of Lm in a tolerized cardiac transplant mouse model resulted in rejection of the cardiac allograft in nearly 40% of the recipients, while an additional 30% showed a slowing of the heartbeat and an enlargement of the allograft with histological evidence of increasing lymphocytic infiltration (119). Furthermore, through analysis of the gene signature of tolerized *versus* rejecting mice, they noted that only partial restoration of the tolerized gene signature had occurred at day 30 post Lm injection. Notably, with resolution of infection, intra-graft T_{reg} percentage returned to the pre-infection level. This suggests that partial, but not complete, return of tolerance occurred with resolution of the infection. In this model, Wang et al. further noted that the disruptive effect of Lm was prevented by IFNαR gene deficiency in their cardiac and skin transplantation recipients. Conversely, administration of IFN-β even without Lm infection, shortened skin allograft survival. Supporting this finding, Young et al. showed that Lm infection induced a transient increase in circulating IL-6 and IFN-β and with recovery from the infection, these cytokines returned to baseline (120). These findings suggest a role of type-1 interferon in tolerance disruption in setting of a Lm infection.

The data outlined above emphasizes the need for therapies that maintain tolerance or restore complete tolerance in the setting of inadvertent microbial infections. The potential targets whose roles need to be further elucidated in this process include type-1 interferon, IL-6, and other inflammatory cytokines.

The other facet relating donor-specific tolerance to risk of opportunistic infections is the potential of tolerance to minimize allograft inflammation and eliminate chronic immunosuppression, both of which may contribute to prevention of latent viral (e.g., CMV) reactivation, especially from the transplanted allograft. However, with the tolerance approach *via* bone marrow chimerism, aggressive conditioning regimens needed for BMT may in fact promote CMV reactivation, thus impairing bone marrow engraftment, and/or promoting subsequent loss of chimerism and tolerance (121, 122). These concerns again underscore that the alternative approach using apoptotic cell-based might be a more attractive option, taking into consideration of potential opportunistic infections particularly latent CMV reactivation.

SUMMARY AND FUTURE DIRECTIONS

The profound immunoregulatory effects of donor apoptotic cells have been harnessed thus far in several murine and non-human primate experimental models where they have shown promising efficacy for transplant tolerance induction. Furthermore, recent early phase I/II clinical trials in both solid organ transplant and BMT have demonstrated the safety of this approach. As highlighted above, the major challenges with the use of apoptotic cell infusion include limitations in sensitized recipients and the loss of tolerance in setting of opportunistic microbial infections. Other potential obstacles include controlling for the early stage of apoptosis and the limited practicality of using donor apoptotic cells in diseased donor transplantation.

One pragmatic approach that can overcome logistical obstacles is the use of acellular carriers for solubilized donor antigens. This would obviate the need for procurement of a large number of donor cells, a particular logistical challenge in case of deceased donor transplantation. It can also make storage easier and ensure consistent quality in the manufacturing process. One such acellular carrier is polylactide-co-glycoside (PLG)-based nanoparticles. PLG nanoparticles can be coupled with membrane donor antigens, and in combination with a low dose rapamycin, have been shown to inhibit anti-donor response and prolong allograft survival as well as to prevent GVHD (123, 124). Furthermore, geometric modifications of PLG particles can modify cellular signaling networks and program them to alter subsequent immune cell activation therefore be utilized to create an immunoquiescent environment. Once such modification involves the presentation of phosphatidylserine which typically is expressed on the surface of apoptotic cells and may interact with phagocytic APC receptors. The subsequent signaling of this interaction likely through TGF-β production leads to activation of alloreactive T cells while promoting expansion of T_{regs} (125). Altogether, these data highlight the enormous potential of bioengineering the full

immunomodulatory signaling program of apoptotic cells onto acellular carriers for the induction of transplant tolerance.

Promisingly, the future holds exciting potential for apoptotic cell therapy with its recent translation into clinical trials. However, a great deal remains to be learned of the underlying mechanisms together with methods to overcome its limitations when aiming for a more widespread clinical application.

AUTHOR CONTRIBUTIONS

IH and XL reviewed the literature relevant to the research topic. IH wrote the manuscript under consultation with XL. XL contributed to the critical revision of the article and the final approval of the version to be published. All authors contributed to the article and approved the submitted version.

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REVIEW
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A Large-Scale Bank of Organ Donor Bone Marrow and Matched Mesenchymal Stem Cells for Promoting Immunomodulation and Transplant Tolerance

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Induction of immune tolerance for solid organ and vascular composite allografts is the Holy Grail for transplantation medicine. This would obviate the need for life-long immunosuppression which is associated with serious adverse outcomes, such as infections, cancers, and renal failure. Currently the most promising means of tolerance induction is through establishing a mixed chimeric state by transplantation of donor hematopoietic stem cells; however, with the exception of living donor renal transplantation, the mixed chimerism approach has not achieved durable immune tolerance on a large scale in preclinical or clinical trials with other solid organs or vascular composite allotransplants (VCA). Ossium Health has established a bank of cryopreserved bone marrow (BM), termed "hematopoietic progenitor cell (HPC), Marrow," recovered from deceased organ donor vertebral bodies. This new source for hematopoietic cell transplant will be a valuable resource for treating hematological malignancies as well as for inducing transplant tolerance. In addition, we have discovered and developed a large source of mesenchymal stem (stromal) cells (MSC) tightly associated with the vertebral body bone fragment byproduct of the HPC, Marrow recovery process. Thus, these vertebral bone adherent MSC (vBA-MSC) are matched to the banked BM obtained from each donor, as opposed to third-party MSC, which enhances safety and potentially efficacy. Isolation and characterization of vBA-MSC from over 30 donors has demonstrated that the cells are no different than traditional BM-MSC; however, their abundance is >1,000-fold higher than obtainable from living donor BM aspirates. Based on our own unpublished data as well as reports published by others, MSC facilitate chimerism, especially at limiting hematopoietic stem and progenitor cell (HSPC) numbers and increase safety by controlling and/or preventing graft-vs.-host-disease (GvHD). Thus, vBA-MSC have the potential to facilitate mixed chimerism, promote complementary peripheral immunomodulatory functions and increase safety of BM infusions. Both HPC, Marrow and vBA-MSC have potential use in current VCA and solid organ transplant (SOT)

tolerance clinical protocols that are amenable to "delayed tolerance." Current trials with HPC, Marrow are planned with subsequent phases to include vBA-MSC for tolerance of both VCA and SOT.

Keywords: immune tolerance, chimerism, bone marrow, vascular composite allograft, regulatory T cells, solid organ transplant, mesenchymal stem cells, hematopoietic stem cell

INTRODUCTION

SOT has become standard of care over the last half century, resulting in not only a significant life extension but also an enhancement of quality of life (QOL) for end-stage organ failure patients (1, 2). More recently, VCA has become a life changing procedure for patients with severe deformities due to traumatic injury or congenital defects (3). While short-term outcome of transplant recipients using refined conventional immunosuppressive protocols have steadily improved, long-term outcome for the vast majority of patients has not changed over the last decades of experience with transplantation; chronic rejection nearly inevitably leads to organ loss and, depending on the transplanted organ, also to patient death unless a retransplantation is performed (4). Ten years after transplantation only roughly 50% of all heart, liver and kidney and 30% of lung and intestinal grafts are still functioning[1].

The continuing negative impact of chronic rejection, combined with the severe adverse effects of conventional immunosuppressive regimens, has spurred intense research into new and safer strategies to prevent allograft rejection. While chronic allograft failure is associated with more frequent hospitalization, higher morbidity and increased health care costs, chronic immunosuppression (IS) is linked to side effects that range from malignancy, infection, toxicities (kidney, central nervous system, hematopoietic system) to cardiovascular and metabolic diseases (5–15). Medication related adverse effects, amount of pill-intake, combined with high costs of immunosuppressive drugs, translate to high rates of patient non-compliance/non-adherence. In kidney transplantation, approximately one third of all patients lose their graft due to non-adherence making it one of the leading causes of allograft loss (16–18). Ultimately, the most desirable outcome and often referred to as the "Holy Grail" of transplantation is the establishment of transplant tolerance as this would abrogate the need for chronic IS, thereby transforming organ transplantation from a chronic treatment to a permanent cure (19). Tolerance in the setting of organ and tissue transplantation not only leads to improved QOL, it also eliminates drug-related side effects, mitigates the impact of adherence and compliance, substantially lowers health care cost, extends organ half-life, and thereby addresses the ongoing critical issue of organ shortages (10, 12, 13, 20–26).

Besides tolerance regimens, various alternative strategies to inhibit rejection are in development to replace or reduce the need for current mainstay IS drugs. These regimens seek to shift the balance of lymphocytes in favor of regulatory T (Treg) cells over effector/memory T (Tem) cells, as opposed to pan-T cell inactivation with calcineurin or mTOR inhibition (27, 28). Of particular note is the increasing number of exploratory cell-based immunoregulatory and tolerizing therapies (29–37). One such immunomodulatory protocol that is already in clinical use utilizes unmodified deceased donor-derived BM cell infusion following HLA-mismatched VCA using a Campath-based induction regimen (38). Even though, only extremely low levels of mixed chimerism were induced, the co-infusion of BM cells after VCA allowed for a substantial reduction of maintenance immunosuppression to a single-agent regimen (32). In contrast to tolerance protocols were transient or stable mixed chimerism-mediated Treg cell expansion and central deletion of donor-specific Tem cells are major drivers of tolerance [reviewed by (33, 36)], durable tolerance in the absence of stable mixed chimerism involves contribution of the graft to long-term promotion of donor-specific T cell suppression/depletion (39).

The vascularized BM component of VCA has innate immunomodulatory properties; however, this is not sufficient to fully tolerize recipients to the highly immunogenic skin component of the composite tissue (29–31, 34, 36, 40). Over 120 upper extremity and >40 facial transplants have been performed worldwide with positive outcomes, demonstrating not only the immunological feasibility but also the potential of this revolutionary life-enhancing modality to restore lost functionality to traumatic injury victims (41, 42). Because reconstructive transplantation addresses a life-changing, but not life-saving, health issue, the risks of non-myeloablative conditioning regimens required to promote mixed chimerism are not warranted. Thus, obtaining durable tolerance in the absence of auxiliary mixed chimerism is a challenge inherent to all forms of transplantation but undoubtedly greatest to VCA (43–47).

Although superficially similar in that both approaches administer hematopoietic cells, there are fundamental differences with respect to safety and mechanisms between the mixed chimerism-based approaches that are currently used in clinical trials to promote tolerance in SOT and the immunomodulatory approach in clinical use for VCA. Induction of tolerance through mixed chimerism necessitates non-myeloablative conditioning in the form of irradiation (total body, total lymphoid, or thymic irradiation), cytotoxic agents (e.g., cyclophosphamide, fludarabine) and cell-depleting agents [e.g., ATG, rituximab; (36, 48)]. Lack of conditioning prior to BM infusion in the VCA tolerance protocol limits chimerism to extremely rare transient events. While cytotoxic effects of conditioning are required to induce tolerance, this toxicity limits its use and is responsible for associated side effects (43–47). Lack of conditioning in immunomodulation not only decreases toxic side effects, but also largely prevents GvHD, which is another major safety

[1]UNOS https://unos.org/data/transplant-trends/ (accessed October 27, 2020)

concern with hematopoietic stem cell transplantation (HSCT) for SOT (49). Additional strategies to augment BM infusion-mediated immunomodulation in the absence of conditioning to promote mixed chimerism are currently being explored as described below.

Clinical Experience With BM-Derived Products for Inducing Tolerance and Immunomodulation

Currently, there are three U.S. centers (Massachusetts General Hospital (MGH), Stanford University, and Northwestern University) that are investigating clinical protocols for inducing SOT tolerance (50). The protocols have been reviewed in detail elsewhere (36, 46, 47, 51). Each of these protocols uses a whole or fractionated BM-derived cell transplant to induce stable or transient mixed chimerism. In order to induce tolerance, each of these protocols relies on non-myeloablative conditioning to prepare the BM niche for the engraftment of donor-derived stem cells (52). Current successes in clinical trials using these protocols to induce tolerance of SOT through mixed chimerism have been achieved exclusively in the setting of living donor kidney transplantation (39). Most of the current protocols use a preconditioning regime which is implemented days before the transplant; only one regime exists that starts concomitantly with the transplantation. Due to logistic constraints the procedures are presently limited to elective living donor procedures. However, >80% of all transplant recipients receive grafts from deceased donors. Hence, establishing tolerance protocols for deceased donor organ transplantation would greatly expand the number of patients who could potentially benefit from this life-saving procedure.

VCA grafts are invariably from deceased donors, which are also a source of high quality BM obtained from the donor vertebrae that can be cryopreserved for subsequent infusion (32, 53, 54). In the absence of recipient conditioning, the goal of BM infusion following VCA is to augment chimerism inherent to the composite graft. The protocol used at Johns Hopkins to induce immunomodulation in VCA recipients employs cryopreserved BM that is infused 2 weeks after transplantation. The full complement of mechanisms involved in augmentation is not known but at least partially involves supplementation with regulatory cell types and may additively involve alloreactive clonal T cell exhaustion and deletion (55).

Toward Developing Clinical Delayed Tolerance Protocols

The achievement of immunomodulation with BM infusion that clinically translates into significantly reduced need for IS in VCA demonstrates that (1) harvesting and cryostorage of deceased donor BM is feasible, (2) cryopreserved deceased donor BM can be safely infused, and (3) delaying infusion of previously cryopreserved deceased donor BM over a significant period following VCA still achieves desirable biological effects. This suggests that delayed tolerance with deceased donor SOT may be possible.

Feasibility of delayed BMT for tolerance in SOT in fact has been demonstrated in rodent and non-human primate models of solid organ and vascularized composite allotransplantation (for details see **Table 1**). These new protocols paved the way for the introduction of the term "delayed tolerance" which have the distinct advantage of allowing for a recovery period to stabilize graft function and enable inflammation resulting from the surgical procedure as well as ischemia reperfusion injury upon revascularization to subside which may enhance tolerance-promoting effects of the BMT. However, the concomitant increased risk due to expansion of alloreactive Tem cells during the interim must be effectively reduced, which appears feasible in non-human primates using an anti-CD8 monoclonal antibody (67, 68). This finding opens up the potential for banking deceased donor BM for future transplantation to promote tolerance in current as well as future SOT recipients.

Ethical Considerations of Translation and Commercialization of Cell Products

Cell therapies are a rapidly growing field that have the potential to significantly impact the practice of medicine, not only in the field of transplantation but for a wide range of diseases (69). Despite their immense potential, cell therapies are significantly more complex in their mode of action and due to biological variation and differences in quality of the starting material, not as standardized as other pharmaceutical products (70). In addition, ethical concerns exist regarding cell and tissue sources and especially the use of altruistically donated cells for commercialization. Similar to the US, European regulations make it illegal to buy or sell human cells and tissues. Yet, it is accepted to compensate for reasonable costs that arise for procurement, processing and storage (71, 72). Ethical and safety concern in the early 2000s led to the implementation of regulations by the U.S. Food and Drug Administration and European Medicines Agency regarding cell- and tissue-based products and therapies (73–76). These regulations ensure strict principles of cell and tissue procurement, product development, processing, testing, distribution, and traceability to maintain quality and safety. However, full compliance with all implemented regulations result in significantly increased production costs disqualifying many products that have been produced by single institutions (77).

Development of a Genetically Diverse Bank of Deceased Organ Donor Bone Marrow

Deceased donor BM represents a large, untapped source of hematopoietic stem and progenitor cells (HSPCs). As has also been demonstrated over the last few decades with cryopreservation of cord blood, it is well-established that BM remains biologically active following long-term cryopreservation (78–81). The larger volumes of HSPCs that can be recovered from a deceased donor compared to aspiration from living donors allow for multiple HSCT procedures or repeat infusions in cases of graft failure. The recovered BM can be precisely packaged, tested for quality, and cryopreserved for subsequent on-demand use. The cryopreserved units can be stored indefinitely (82), with

TABLE 1 | Overview on delayed tolerance protocols in small and large animal models of solid organ and vascularized composite allotransplantation.

Author	Organ	Year	Time delay	Conditioning regime	Citation
NON-HUMAN PRIMATE					
Yamada et al.	Kidney	2012	4 months	TBI, TI, Atgam, anti-CD154mAb, anti-CD8mAb	(56)
Lee et al.	Kidney	2013	4 months	TBI, TI, Atgam, anti-CD154mAb, LFA3-Ig	(57)
Tonsho et al.	Lung	2015	4 months	TBI, TI, Atgam, anti-CD8mAB, anti-CD154mAb, anti-IL6RmAb	(58)
Tonsho et al.	Heart, Heart and Kidney	2016	4 months	TBI, TI, anti-thymocyte globulin, anti-CD154 mAb, anti-CD8 mAb	(59)
Huh et al.	Heart and Kidney	2017	2 and 4 months	TBI, TI, anti-thymocyte globulin, anti-CD154 mAb, anti-CD8 mAb	(60)
Hotta et al.	Kidney	2018	4 months	TBI, TI, Thymoglobulin, Belatacept	(61)
Oura et al.	Kidney and Islet	2019	4 months	TBI, TI, Atgam, Belatacept, anti-CD40mAb, LFA3-Ig	(62)
Lellouch et al.	VCA	2020	2 and 4 months	TBI, TI, Atgam, anti-CD8mAB, anti-CD154mAb, anti-IL6RmAb	(63)
MOUSE					
Guo et al.	VCA	2019	30 days	TBI, anti-Thy1.2Ab, Cyclophosphamide	(64)
RAT					
Chen et al.	Kidney, VCA	2012	2 months	TBI, anti-αβTCRmAb, anti-CD8mAb, ALS	(65)
Xie et al.	Liver	2017	4 weeks	TBI, anti-αβTCRmAb	(66)

Ig, immunoglobulin; mAb, monoclonal antibody; TBI, total body irradiation; TI, thymic irradiation; VCA, vascularized composite allotransplantation.

the advantage over living donor registries of having essentially no attrition.

Efforts are currently underway in collaboration with the national Organ Procurement Organization (OPO) network in the U.S. to build the first bank of cryopreserved deceased donor BM. The U.S. OPO network provides an existing refined infrastructure for procuring and transporting bone tissue recovered from deceased donors. Approximately 10,000 deceased donor organs are recovered each year in the U.S., with a further 40,000 donations, yielding approximately 30,000 organs and over a million tissues recovered annually[2]. The high numbers of bones recovered through this system each year supports the inventory required to establish an integrated system of bone procurement, recovery, and transport, linked to BM processing and banking centers. It has been demonstrated that protocols can be developed and enforced to maintain a favorable ischemic environment from the point of bone procurement and recovery, through cross-country shipping, to arrival at a BM processing center (83). Through these efforts, banking of BM product (HPC, Marrow) for transplantation is currently underway.

Protocols for isolation of HPC, Marrow from deceased donor vertebral bodies were based on original work at University of Pittsburgh and optimized at Johns Hopkins University (32, 53, 54). Those protocols formed the basis for the now fully good manufacturing practice (GMP) compliant process that conforms to 21 CFR Part 1271 regulations and is tested for release in a CLIA-certified laboratory using fully validated testing procedures. Certain improvements to the process were made to increase throughput and enhance reproducibility as well as the aforementioned establishment of logistical procedures for recovery and shipment of vertebrae across large geographic regions. Donor eligibility requirements were established to reduce the risk of adventitious agent transmission (health screening and serological testing) as well as health status incompatible with functioning BM. Finally, cryopreservation conditions were optimized and stability validated to ensure functionality of each HPC, Marrow unit released for transplantation. The result is a product with high viability, high colony forming unit potential and the ability to stably engraft irradiated mice following primary and secondary transplants (manuscript in preparation).

Discovery and Clinical Development of BM Compartment Mesenchymal Stem/Stromal Cells (MSC) Not Recovered in HPC, Marrow

Our team has identified an abundant population of MSC associated with the vertebral body (VB) bone fragment byproduct of HPC, Marrow recovery. These MSC remain tightly adhered to cancellous bone fragments and can only be released by enzymatic treatment. We have determined that these vertebral bone adherent MSC (vBA-MSC) are identical to BM-MSC when cultured (84). The vBA-MSC population yields roughly 2,000x the number of viable, low passage cells from one donor compared with MSC recovered through aspiration from iliac crests of living donors. This bank of vBA-MSC matched to solid organ and VCA donors is a unique resource that overcomes limitations of using third-party MSC by reducing the risk of introducing additional alloantigens and, thus, lowering the risk of sensitization and alloimmune activation (85, 86). Furthermore, the abundance of vBA-MSC allows for generating hundreds of billions of low passage (i.e., P2) cells, allowing multiple infusions (**Figure 1**).

The potent immunoregulatory properties of MSC comprise a spectrum of secreted and cell-bound molecules that modulate a wide array of innate and adaptive immune responses. The multifaceted mechanisms of MSC immunomodulation have been detailed in numerous reviews and, therefore, will only be briefly introduced here. The MSC secretome includes both freely soluble factors as well as those encapsulated by extracellular vesicles. Mechanisms include metabolic inhibition (e.g., indoleamine-pyrrole 2,3 dioxygenase; IDO), immunomodulatory cytokines

[2]unos.org/data/transplant-trends/ (accessed October 27, 2020).

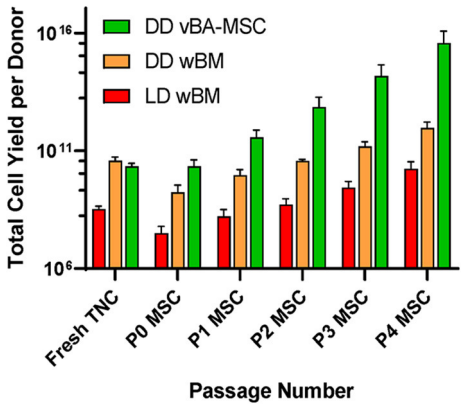

FIGURE 1 | Comparison of total numbers of low passage MSC obtained from living and diseased donor BM. Sources were deceased donor vBA-MSC (DD vBA-MSC) and deceased donor whole BM (DD wBM) and live donor aspirated whole BM (LD wBM). Yields were calculated based on pilot manufacturing runs with either 3 (wBM sources) or 7 (vBA-MSC) donors for each. Averages ± SD shown.

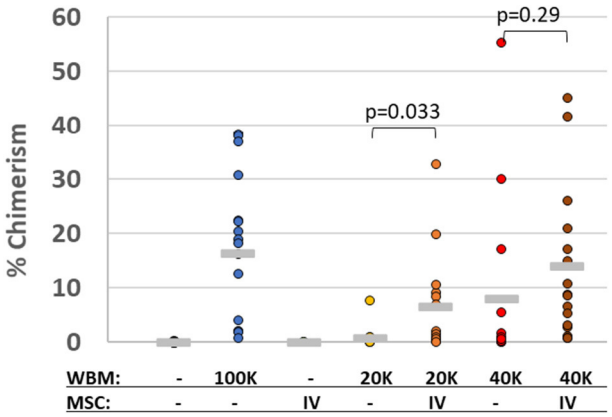

FIGURE 2 | Human MSC promote chimerism of limiting doses of congenic murine bone marrow cells. Lethally gamma-irradiated (875 cGy) C57Bl/6 (CD45.2+) mice were 24 h later with either saline vehicle or one of three doses of whole bone marrow (WBM) isolated from congenic BoyJ (CD45.1+) mice. The WBM doses were either 2×10^4, 4×10^4 or 1×10^5 total nucleated cells (TNC). At 24 and 72 h after irradiation, some groups of mice were also injected with human vBA-MSC (passage 2) at a dose of 1×10^6. Bone marrow was collected from mice surviving 30 days and analyzed for the level of chimerism by flow cytometric analysis with antibodies specific for CD45.1 and CD45.2 surface proteins. The percentage of CD45.1+ chimerism for individual mice in each group is indicated as well as the average per group (horizontal gray line). *P*-values were determined by Student's *t*-Test.

(e.g., transforming growth factor-β; TGF-β), and checkpoint inhibitors (e.g., programmed death ligand 1; PD-L1). These myriad factors inhibit T cell activation and proliferation, as well as enhance proliferation of regulatory cells (85–88).

Preclinical studies have demonstrated the therapeutic potential of MSC for inducing operational tolerance of SOT and VCA (89–102), providing proof-of-principle for clinical testing in the transplant setting (100, 103–108). The effect of MSC infusion, including in humans and non-human primates, is to skew the T cell population in favor of Treg over Tem cells (97, 99, 109). Clinical studies of MSC-induced immune tolerance of mismatched kidney transplants have demonstrated safety and efficacy (103–108). In one small study of two kidney transplant patients treated with minimal conditioning and MSC found that levels of CD8+ Tem cells decreased without a decrease in overall T cells (103, 106). Teff cells also demonstrated hyporesponsiveness to alloantigen (110–112). A larger controlled study found significantly higher levels of Tregs at 30 days in the MSC treated cohort compared to the control group (109). Thus, MSC beneficially modulate the ratio of Treg/Tem cells to prevent rejection.

In addition to potentially facilitating graft survival through ameliorating alloreactivity, MSC have demonstrated considerable potential for suppressing GvHD which could be an unintended consequence of SCT to induce tolerance (33, 46, 111, 113–126). In fact, based on a wealth of clinical data, MSC are approved in some countries for the treatment of steroid refractory GvHD and there are strong indications that the cells could be used for prophylaxis (121, 127–131). This potent immunomodulatory function of MSC could mitigate the risk of immune tolerance protocols that promote development of GvHD. However, evidence suggests that this function of the cells is dependent on minimal passaging of the cells, with over-expanded cells losing the ability to modulate acute GvHD (132, 133). The large depot of donor-matched vBA-MSCs facilitates minimal expansion to achieve doses required for treatment in humans (**Figure 1**).

Another mechanism to minimize the risk of GvHD is titrating down the HSPC graft to a minimal efficacious dose, which correspondingly reduces the donor T cell load. MSCs have been reported to facilitate and enhance engraftment of allogeneic HSPC clinically, even after initial graft failure and rejection of conventional stem-cell grafts (134). Preclinical studies suggest that MSCs enhance mixed chimerism when co-infused with HSPC (135) by migrating to the BM stroma to help establish a favorable micro-environment within the hematopoietic niche (136). This appears to minimize the number of HSPC required for transplantation (137, 138). We have confirmed these findings with vBA-MSC in irradiated non-immunocompromised mice treated with limiting dilutions of congenic whole BM with and without co-infusion, followed by a second dose at 48 h, of human vBA-MSC (**Figure 2**).

Besides GvHD, engraftment syndrome (ES) that occurs in 7–90% of cases during neutrophil recovery after autologous and allogeneic HSCT poses a potential limitation (139). It is associated with fever, pulmonary vascular leak, rash, and organ dysfunction and has also been described in combined HLA-haploidentical BM and kidney transplant recipients. In the described cases, ES manifested not only with fever and fluid retentions but also with a marked acute kidney injury (140), prompting speculations on an increased susceptibility with freshly transplanted kidney grafts, especially in combination with CNI treatment (141). Even though the exact pathophysiology is unclear, ES is thought to be mediated by endothelial cell injury, activated leukocytes, and proinflammatory cytokines. The inflammatory nature of ES is underlined by the good

response to treatment with corticosteroids (141, 142). As vBA-MSCs have strong anti-inflammatory, antioxidative, and immunomodulatory properties, co-administration could potentially mitigate the risk or severity of ES after HSCT (143–145). Thus, the combination of promoting BM chimerism and the immunomodulatory functions of MSC suggest that their use as an adjuvant to BM transplants will safely enhance induction of immune tolerance.

Potential for Incorporating Deceased Donor BM and vBA-MSC Into Current Tolerance Protocols

Each of the current protocols for inducing tolerance in VCA and SOT lend themselves to deceased donor BM augmentation and BMT, respectively, with modification to accommodate donor availability. Inclusion of vBA-MSC either prophylactically or to treat GvHD is possible. Following BM isolation and quality control testing, HPC, Marrow would be cryogenically preserved until shipping under the same conditions for infusion into the patient 14 days following surgery, as described previously (32). Simultaneously, vBA-MSC could be prepared from the bone fragments and expanded before cryopreservation and shipping with HPC, Marrow.

In regard to SOT, the MGH delayed tolerance protocol appears to be the most easily adaptable to HPC, Marrow, providing that encouraging results in NHP and early clinical trials in humans are repeated in future larger clinical trials (56–58, 61, 62, 68, 146–148). Transplantation at 4 months following SOT would allow more than enough time to prepare, qualify and store HPC, Marrow as well as expanded vBA-MSC. It is well-established that cryopreservation preserves cellular function for decades so long as proper controls are implemented to prevent transient warming events (82).

The Stanford protocol, which relies on an infusion of a mixture of isolated mobilized peripheral blood-derived CD34+ and T cells could in theory be adapted to using HPC, Marrow for selection of these cells (49, 149). The amount of HPC, Marrow typically recovered from a full donor contains hundreds of millions of CD34+ cell (53, 54, 83). We have adapted CD34 selection methods to develop a GMP process that has yielded an average of 125×10^6 $CD34^+$ cells from HPC, Marrow recovered from three donors. Importantly, these methods can be used on either freshly isolated or previously cryopreserved HPC, Marrow; thus, providing flexibility in cell production. The Stanford protocol infuses cryopreserved selected cells at 11 days after kidney transplant which would provide sufficient time to prepare HPC, Marrow as well as over a billion very early passage GMP vBA-MSC (**Figure 1**). Given that MSC are commonly dosed at 1×10^6/kg, this would provide more than adequate vBA-MSC for co-infusion as well as any additional dosing if further expansion was not feasible. A company, Medeor, has been established to demonstrate commercial potential of the Stanford protocol and, according to their website[3], a delayed tolerance protocol for living donor kidney transplantation is in development. As of yet, efficacy has not been established using this protocol with deceased donor kidney transplants.

The Northwestern tolerance protocol for kidney transplantation differs by the use of full body non-myeloablative conditioning with the goal of promoting full chimerism rather than transient (i.e., MGH protocol) or durable (i.e., Stanford protocol) mixed chimerism (150–154). The protocol uses an engineered cell source, termed facilitating cells (FC), derived from kidney donor mobilized blood collected at least 2 weeks prior to transplant combined with HSPC to promote chimerism (155, 156). Providing the protocol is amenable to a delayed tolerance approach, deceased donor HPC, Marrow could offer a distinct advantage for manufacture of FC given the high abundance of BM cells available and the enhanced time provided for manufacture and testing. To this end, we have demonstrated that HPC, Marrow is amenable to manipulation using a CliniMACS system (Miltenyi Biotec). As GvHD appears to be a concern with this protocol, infusion of low passage vBA-MSC could be advantageous.

Limitations of Current Tolerance Protocols

Despite clinical realization of tolerance and preclinical evidence supporting the feasibility of delayed tolerance protocols as outlined above, tolerance induction is still limited to a few highly specialized centers (47). Widespread adoption is currently hindered by the risks associated with complex recipient conditioning regimes which have a variety of toxic side effects. The most promising strategy of tolerance induction thus far is the mixed chimerism approach, however, tolerance induction efficacy is still limited. HSCT is also associated with a risk of GvHD, which has been observed in protocols aiming for durable chimerism in a small number of patients (36). To overcome these hurdles, a concerted effort of clinicians, scientists, stakeholders (e.g., insurance companies and hospitals), and funding agencies is crucial. In recent years, transplant tolerance has regained attention and a variety of new agents have been identified that have the potential to make induction regimens significantly less toxic, reduce associated risks of GvHD, and increase efficacy. The realization of a deceased bone marrow bank, as outlined in this review, is another step in the process of making transplant tolerance a clinical reality for a larger number of patients.

Conclusions and Future Perspectives

Future broad success with BM and MSC induction of tolerance and potent immunoregulation will have profound effects on transplant patients. Achieving immune tolerance, in particular, will alleviate the burden of life-long IS and associated morbidity, avoid chronic rejection, and significantly improve overall outcomes. Furthermore, it will overcome current compliance and adherence-based limitations that negatively impact graft survival due to subsequent subliminal rejection and the development of donor-specific antibodies. Tolerance and immunoregulation will be especially impactful for patients by increasing accessibility to transplantation and through positively shifting the risk:benefit ratio by reducing associated long-term risk.

[3]https://www.medeortx.com/our-pipeline.php/ (accessed October 27, 2020).

In view of these potential opportunities and substantial benefits, the establishment of a bone marrow bank for delayed tolerance protocols marks a crucial step in making this resource available for present as well as future transplant patients. The complementary treatment with vBA-MSC could further increase safety with the added potential of enhanced efficacy. Furthermore, the ability of vBA-MSC to promote HSPC BM engraftment would allow lowering of HPC, Marrow doses which effectively extends the number of patients who receive organs from a single donor that are able to benefit from this procedure.

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REVIEW
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Adoptive Transfer of Regulatory Immune Cells in Organ Transplantation

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Chronic graft rejection remains a significant barrier to solid organ transplantation as a treatment for end-organ failure. Patients receiving organ transplants typically require systemic immunosuppression in the form of pharmacological immunosuppressants for the duration of their lives, leaving these patients vulnerable to opportunistic infections, malignancies, and other use-restricting side-effects. In recent years, a substantial amount of research has focused on the use of cell-based therapies for the induction of graft tolerance. Inducing or adoptively transferring regulatory cell types, including regulatory T cells, myeloid-derived suppressor cells, and IL-10 secreting B cells, has the potential to produce graft-specific tolerance in transplant recipients. Significant progress has been made in the optimization of these cell-based therapeutic strategies as our understanding of their underlying mechanisms increases and new immunoengineering technologies become more widely available. Still, many questions remain to be answered regarding optimal cell types to use, appropriate dosage and timing, and adjuvant therapies. In this review, we summarize what is known about the cellular mechanisms that underly the current cell-based therapies being developed for the prevention of allograft rejection, the different strategies being explored to optimize these therapies, and all of the completed and ongoing clinical trials involving these therapies.

Keywords: transplantation, solid organ transplant, regulatory T cells, myeloid derived suppressive cells, chimeric antigen receptor, immunoengineering, graft rejection, IL-10-producing B cells Bregs

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INTRODUCTION

At present, solid organ transplantation remains the only curative treatment for patients with end-stage organ disease. Organ transplantation has evolved over the past 60 years to become the predominant treatment option for end-organ failure, as advancements in immunosuppressive therapies have led to significantly reduced rates of acute organ rejection with improvement in 1-year graft survival (1). However, long-term survival of grafts and the prevention of chronic rejection has remained a

Abbreviations: AMR, Antibody-mediated rejection; APC(s), Antigen presenting cell(s); B10 Cell, IL-10 secreting B cell; BCR, B cell receptor; CAR, Chimeric antigen receptor; EAE, Experimental autoimmune encephalomyelitis; iNOS, Inducible nitric oxide synthase; iTreg, Induced Treg; M-MDSC(s), Monocytic MDSC(s); MDSC(s), Myeloid derived suppressor cell(s); NK Cell, Natural killer cell; PMN-MDSC(s), Polymorphonuclear (granulocytic) MDSC(s); TCR, T cell receptor; Tr1 Cell, T regulatory type 1 cell; Treg(s), Regulatory T cell(s).

significant hurdle in the success of solid organ transplant therapy. While the long-term survival rate of grafts has not seen significant improvement, the burden of lifelong immunosuppressive regimens contributes to the morbidity and mortality transplant recipients (1). The most commonly used maintenance immunosuppressive drugs used in solid organ transplant include steroids, calcineurin inhibitors (CNIs), antiproliferative agents (i.e. mycophenolate mofetil) and drugs that inhibit the mammalian target of rapamycin (mTOR). All of these drugs have drug-specific side-effects that can lead to nonadherence, as well as common use-restricting toxicities such as nephrotoxicity, increased cardiovascular risk, and systemic overimmunosuppression that can result in opportunistic infections as well as some malignancies (2). Given the significant side-effect burden of current immunosuppressive therapies and a persistent rate of chronic graft rejection, there is a need for minimization strategies that reduce (or eliminate) the amount of immunosuppressive drugs required for graft survival, with the ultimate goal being immunologic tolerance (i.e. stable graft tolerance in the absence of any systemic immunosuppression).

In solid organ transplantation, graft rejection occurs by two main pathways: the direct pathway and the indirect pathway. It is generally believed that the direct mechanism of T cell activation predominates early in graft rejection as there is an abundance of APCs present in the graft (i.e. donor passenger leukocytes), but that progressive depletion of the donor passenger leukocytes over time ultimately leads to a predominance of the indirect mechanism of T cell activation (3–7). Thus, it is the indirect pathway that ultimately persists leading to chronic graft rejection by priming effector T cells to induce cellular rejection, while also promoting a delayed-type hypersensitivity reaction that drives antibody-mediated rejection (AMR) and the inflammatory response of the innate immune system (5–10). Given this knowledge, it is logical to pursue adoptive cell-based therapies that have indirect allo-specificity to combat the progression of chronic allograft rejection and promote immune tolerance.

Much of the research involving the induction of graft tolerance has focused on cell-based therapies that use regulatory cell types belonging to both the innate and adaptive immune systems. Of particular interest have been regulatory T cells (Tregs), which were identified in a landmark study in 1995 showing a subpopulation of $CD4^+$ T cells that expressed the IL-2 receptor (CD25) and were responsible for preventing the development of autoimmune disease (11). Other regulatory cell types have been identified, including myeloid-derived suppressor cells (MDSCs), immunosuppressive IL-10 secreting B cells (B10), tolerogenic dendritic cells (DCs), and natural killer cells (NKs). Each of these cell types act by distinct and sometimes synergistic methods, with varying degrees of promise for clinical utility in the setting of solid organ transplant. A number of studies have focused on either expanding these cell types *in vivo* in transplant recipients, while others have developed protocols for expanding regulatory cell types *ex vivo* and adoptively transferring them into transplant recipients (12–20). The bulk of the published research thus far has focused on Tregs, MDSCs, and B10 as the most promising candidates, and each of these potential therapeutic strategies are reviewed here.

REGULATORY CELL TYPES AND HOW THEY DEVELOP

Regulatory T Cells

One of the most heavily investigated types of regulatory cells are a subset of CD4+ cells that primarily act to promote tolerance of both self and non–self-antigens, commonly known as Tregs. Naturally occurring Tregs are produced either in the thymus (central Tregs) or can be induced in the periphery (iTregs). While there is some heterogeneity in the markers expressed by specific subsets of Tregs, in both humans and mice they can generally be identified by co-expression of CD4 and CD25, as well as Foxp3 which serves as the "master regulator" for Treg development (21, 22). In their initial 1995 paper identifying the $CD4^+CD25^+$ Treg population, Sakaguchi et al. also showed that CD25 knockout mice exhibited heightened immune response to allogeneic skin transplantation, which could be normalized by reconstitution with $CD4^+CD25^+$ cells, collectively showing that $CD4^+CD25^+$ T cells (Tregs) are important for the maintenance of self-tolerance as well as tolerance to some non–self-antigens (11). The majority of human Tregs that maintain self-tolerance develop in the thymus, and their development is dependent on the strength and duration of T cell receptor (TCR) signaling, based on interaction with MHC-self peptides, as well as a combination of cytokines including IL-2, IL-15, and TGF-β (23–25). Of critical importance to Treg development in both humans and mice is selective demethylation of an element within the Foxp3 locus known as the Treg-specific demethylated region (TSDR) (26, 27). Studies in both humans and mice have demonstrated that epigenetic imprinting within this region is initiated during early stages of thymic Treg development, resulting in long-term stability of Foxp3 expression and commitment to the Treg lineage (24, 28). Fontenot et al. showed in a murine model that Foxp3 expression is required for both the development and suppressor function of Tregs, as Foxp3 knockout mice developed lethal autoimmune disease, and ectopic expression of Foxp3 was able to confer suppressor function to $CD4^+CD25^-$ T cells (29). Of note, Jeffrey Bluestone and colleagues showed in 2006 that CD127 (IL-7Rα) serves as an additional marker to differentiate highly suppressive human Tregs, as CD127 expression inversely correlates with suppressive capability (30). Nadig et al. built upon this finding by showing in 2010 that *ex vivo* expanded Tregs sorted based on low expression of CD127 ($CD127^{lo}$) provide a more potent therapy compared to conventional Tregs in a humanized mouse system modeling transplant arteriosclerosis (12).

While Tregs that maintain self-tolerance primarily develop in the thymus, another population of $CD4^+Foxp3^-$ T cells in the periphery can be stimulated to become $CD4^+Foxp3^+$ Tregs primarily in response to non–self-antigens, termed induced Tregs (iTregs) (31). Using a murine model, Kretschmer et al. demonstrated that repeated, small antigen doses with suboptimal

dendritic cell activation, along with the addition of TGF-β, resulted in increased conversion of these cell types (31). Multiple studies have investigated the signaling required for the induction of Tregs in the periphery, collectively showing that $CD4^+CD25^-$ cells coming from the thymus can be induced to become antigen-specific $CD4^+CD25^+Foxp3^+$ iTregs by a combination of TCR signaling along with TGF-β and IL-2 signaling (32–34). In addition to promoting the differentiation of iTregs in the periphery, IL-2 also functions to inhibit the development of Th17 cells, thereby constraining the production of IL-17 and providing additional tolerogenic function (35). Using a murine model, Gottschalk et al. further elucidated the specific strength and duration of TCR stimulation that is required to induce Tregs in the periphery, and they found that low dose of a strong agonist in the setting of suboptimal co-stimulation provided the maximum stimulation for induction of $Foxp3^+$ Tregs *in vivo* (33). This suggests that recognition of antigens by TCRs to which the organism has chronic exposure to leads to the differentiation of iTregs, resulting in tolerance.

There is a subpopulation $CD4^+CD25^-$ iTregs in humans known as T regulatory type 1 (Tr1) cells characterized by their ability to produce predominantly IL-10 and TGF-β and to transiently upregulate Foxp3 expression to induce tolerance (36). These cells are of special interest to the application of transplant therapy as they were first described in patients who developed tolerance after HLA-mismatched fetal liver hematopoietic stem cell transplantation and preliminary clinical trials have shown safety and efficacy of the use of these cells in human patients (36). While Tr1 cells are not as well-characterized as Tregs, it has been suggested that Tr1 cells can be differentiated in both humans and mice based on co-expression of CD49b and LAG-3 (37). The phenotypic markers that delineate Tregs and Tr1 cells in both mice and humans are summarized in **Table 1**.

Myeloid-Derived Suppressor Cells

MDSCs were first identified by tumor biologists studying how the tumor microenvironment facilitates tumor evasion from the hosts anti-tumor immune response (38–40). These cells, which were initially defined as $CD11b^+Gr\text{-}1^+$ in mice, displayed robust immunosuppressive capabilities against the tumor-specific T cell response, creating an environment that allowed the tumors to grow unopposed (38–41). MDSCs have since been identified in a number of inflammatory settings in both human and mouse models, including infection, sepsis, trauma, auto-immunity, and transplant rejection (42–47). Given their immunosuppressive function, MDSCs have garnered particular interest in the field of transplant immunology as potential therapeutic tools to prevent graft rejection.

MDSCs can be subclassified into two main categories: monocytic MDSCs (M-MDSCs) and polymorphonuclear MDSCs (PMN-MDSCs, also referred to as granulocytic MDSCs), named for their phenotypic and morphologic similarities to monocytes and polymorphonuclear cells, respectively (48). The relative number of M-MDSCs and PMN-MDSCs has been shown to vary depending on cancer type and inflammation setting and can potentially be used to predict risk of graft versus host disease (49, 50). The importance of the ratio between M-MDSCs and PMN-MDSCs in the setting of organ transplantation has yet to be fully elucidated; however, limited data suggests that it is the monocytic subtype that predominates in mediating transplant tolerance (46, 51). Scalea et al. review some of the generally accepted surface markers of MDSCs, which vary between humans and mice (52). Human M-MDSCs can be characterized by dual expression of CD11b and CD14, as well as $HLA\text{-}DR^{low/-}$ and lack of CD15 (52). These cells can be distinguished from mature human monocytes which share CD11b and CD14 expression but are HLA-DR– (48, 52). Human PMN-MDSCs, on the other hand, can be characterized by expression of CD11b and CD15 with no CD14 expression (48, 52). In humans, these cells have traditionally been distinguished from non-MDSC PMNs by density gradient centrifugation, but more recent studies have shown that LOX-1 expression may serve as a reliable marker to separate MDSC PMNs from non-MDSC PMNs *via* flow cytometry (48, 53). It has also been suggested that cytosolic calcium binding protein S100A9 expression can be used to further distinguish M-MDSCs from PMN-MDSCs *via* flow cytometry (54).

In mice, MDSCs are classically characterized by dual expression of CD11b and Gr1 (the myeloid lineage marker composed of Ly6C and Ly6G) (52, 55). Like human MDSCs, mouse MDSCs can be sub-classified as either M-MDSCs and PMN-MDSCs based on relative expression of Ly6C versus Ly6G (52, 55). M-MDSCs are characterized by high expression of Ly6C and lack of Ly6G ($CD11b^+Ly6G^-Ly6C^{high}$), while PMN-MDSCs are characterized by expression of Ly6G and low levels of Ly6C ($CD11b^+Ly6G^+Ly6C^{lo}$) (52, 55). Mouse M-MDSCs can further be distinguished from PMN-MDSCs based on the expression of CD49d on M-MDSCs (52).

MDSCs can be induced from hematopoietic stem cells under a variety of inflammatory conditions, as mentioned above. Normally, hematopoietic stem cells differentiate into common myeloid precursor cells (CMPs), which then further differentiate into immature myeloid cells (IMCs). In the absence of pathological inflammatory conditions, IMCs can migrate to secondary lymphoid organs and differentiate into mature macrophages, dendritic cells, or neutrophils (56). However, under the influence of mediators of chronic inflammation, these IMCs can develop into immunosuppressive MDSCs, which correlates with downregulation of interferon regulatory

TABLE 1 | Phenotypic characterization of regulatory cells in mice and humans.

Cell	Mouse	Human
Tregs	$CD4^+$ $CD25^+$ $Foxp3^+$	$CD4^+$ $CD25^+$ $Foxp3^+$
Tr1	$CD4^+$ $CD49b^+$ $LAG\text{-}3^+$ $IL\text{-}10^+$ $CD25^-$ $Foxp3^-$	$CD4^+$ $CD49b^+$ $LAG\text{-}3^+$ $IL\text{-}10^+$ $CD25^-$ $Foxp3^-$
M-MDSCs	$CD11b^+$ $Gr\text{-}1^+$ $Ly6G^-$ $Ly6C^{hi}$ $CD49d^+$	$CD11b^+$ $CD14^+$ $HLA\text{-}DR^{lo/-}$ $CD15^-$ $S100A9^{hi}$
PMN-MDSCs	$CD11b^+Gr\text{-}1^+$ $Ly6G^+Ly6C^{lo}$ $CD49d^-$	$CD11b^+$ $CD14^-$ $HLA\text{-}DR^{lo/-}$ $CD15^+$ $LOX\text{-}1^+$
B10	$CD1d^{hi}$ $CD5^+$ $CD19^{hi}$ $TIM\text{-}1^+$	$CD1d^{hi}$ $CD5^+$ $CD19^{hi}$ $CD24^{hi}$ $CD27^+$

factor-8 (IRF-8) *via* a STAT3 transcription factor-dependent mechanism (56, 57). The main driver of MDSC expansion is G-CSF/GM-CSF, along with other pro-inflammatory mediators such as IL-2, IL-6, TGF-β, LPS, TNFα, IFN-gamma, and CXCL-1/2 (41, 48, 52, 58–60). In a study conducted by Marigo et al., the authors report that G-CSF, GM-CSF, and IL-6 could be used to rapidly generate functional MDSCs from human bone marrow precursor cells (58). However, they found that different combinations of these cytokines resulted in MDSCs with varying levels of tolerogenic activity, with MDSCs induced by a combination of GM-CSF$^+$IL-6 possessing the highest tolerogenic activity (58). Interestingly, it has been shown that after MDSCs differentiate from precursor cells in the bone marrow, they can be maintained by activated T cells (61). IL-10 secreted from activated T cells promotes STAT3 phosphorylation on MDSCs, which subsequently leads to B7-H1 expression, a key molecule mediating MDSCs development and suppressor function (61). The phenotypic markers that delineate M-MDSCs and PMN-MDSCs in both mice and humans are summarized in **Table 1**.

Regulatory B Cells (B10)

B cells classically play a central role in the adaptive immune response, most significantly as a component of humoral immunity; however, initial evidence that there exists a subset of B cells capable of down-regulating T cell-mediated inflammatory response came from studies with experimental autoimmune encephalomyelitis (EAE) in mice, showing that recovery from the Th1-driven autoimmune condition was dependent on B cells capable of producing IL-10 (62). In these studies, mice with selective IL-10 deficiency in the B cell compartment (but not the T cell compartment) exhibited a persistent type 1 autoimmune condition (62). In a similar murine model, lack of B cells resulted in delayed induction of Tregs in the CNS (63). Further investigation to elucidate the role of IL-10-producing B cells, termed "B10" cells, has shown that a phenotypically distinct CD1d^{hi}CD5$^+$CD19^{hi} B cell subset exists as a rare population of cells (1%–2% of all splenic B cells and 7%–8% of peritoneal B cells) that can be significantly expanded in the setting of T cell-mediated inflammation (64). Normally, B10 cells predominantly localize to the spleen and peritoneal cavity and are absent from the lymph nodes and peripheral blood (64, 65). Using a contact hypersensitivity (CHS) model in mice, Yanaba et al. showed that B10 cells exit the spleen and enter circulation and upregulate their IL-10 expression during the CHS response to downregulate the T cell response (64).

B10 cell development and maturation requires antigen receptor diversity, as transgenic mice with a fixed B cell receptor (BCR) exhibit 90% reduction levels of B10 cells (66). Further, both innate and adaptive signals can promote the expansion and maturation of B10 cells from B10 progenitor cells, most significantly by LPS and CD40L, respectively (65, 66). B10 development and activation appears to be T cell and pathogen-independent (65, 66). Of note, other regulatory B cells have been identified, including CD5$^+$ B-1a cells, CD1d$^+$ marginal zone B cells, and transitional-2-marginal zone precursor B cells (65). However, the bulk of regulatory B cell research focusses on the IL-10-competent CD1d^{hi}CD5$^+$CD19^{hi} subset (B10s) because these are responsible for the majority of B cell-derived IL-10 secretion and appear to be the most potent regulators of the T cell-mediated immune response in mice (65). Interestingly, TIM-1 (also known as Hepatitis A virus cellular receptor 1), a co-stimulatory molecule that regulates the immune response, has been identified as unique identifier of IL-10 producing regulatory B cells in mice (67). In a model of islet cell allograft transplant, TIM-1$^+$ B cells were found to be highly enriched for IL-10 and IL-4 expression, and the subset of B cells expressing TIM-1 was significantly expanded (from 5%–8% up to 10%–15%) after allograft transplantation (67). These findings suggest that TIM-1 could be used as a unique marker to identify IL-10 competent regulatory B cells within other established subsets, such as the CD1d^{hi}CD5$^+$CD19^{hi} subset. In humans, cell surface markers CD24 and CD27 have been identified as additional identifiers of the B10 population (68). The phenotypic markers that delineate B10 cells in both mice and humans are summarized in **Table 1**.

HOW THEY EXERT THEIR TOLEROGENIC EFFECTS

Tregs

Tregs have the ability to suppress the differentiation of naïve T cells into mature effector T cells, as well as suppress the functions of differentiated effector T cells and other players of the both the innate and adaptive immune systems, including B cells, macrophages, NK cells, and dendritic cells (21, 69). These tolerogenic effects are mediated through both cell surface molecules present on Tregs and soluble factors secreted by Tregs (**Figure 1**). One of the cell surface molecules that appears to play a central role in their immunosuppressive capabilities is CD25, a subunit of the IL-2 receptor (IL-2R), which is an important component of Treg differentiation and survival, as mentioned above. In addition to maintaining Treg homeostasis, the high levels and high affinity of IL-2R expression on Tregs results in IL-2 deprivation-mediated apoptosis of effector T cells, as IL-2 is also critical for the maintenance and survival of CD4$^+$ and CD8$^+$ effector T cells *in vitro* (69–72). However, *in vivo* studies have shown that IL-2 is actually not required for the maintenance of effector T cells, and that Tregs are able to exert their immunosuppressive effects even in mice that lack IL-2R on effector T cells (69). Another important contact-dependent mechanism by which Tregs suppress effector T cells *via* surface molecules involves an interaction between CTLA-4 on Tregs and CD80/86 on effector T cells (73–75). In addition to the direct interaction between Tregs and effector T cells, CTLA-4 on Tregs also interacts with CD80/86 present on the surface of dendritic antigen presenting cells (APCs) (76, 77). In this mechanism, engagement of CD80/86 ligands by CTLA-4, tolerogenic dendritic cells upregulate tryptophan metabolism *via* an indoleamine 2,3-dioxygenase (IDO)-dependent pathway, thereby inhibiting T cell proliferation (69, 76–78). Similarly, Tregs express PD-1, which has been shown to play an important role in suppressing autoreactive B cells in mice *via* interaction with PD-L1 expressed on B cells (79). CD39 and CD73

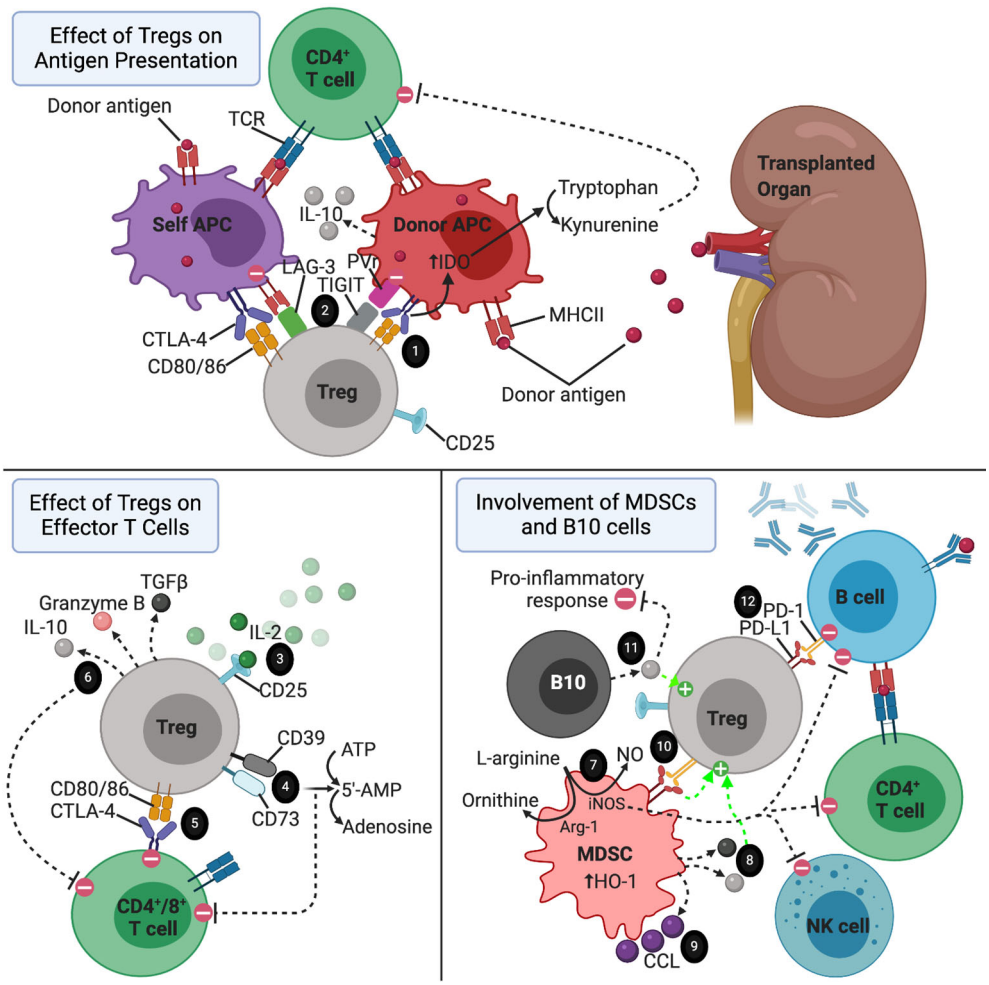

FIGURE 1 | The immune environment surrounding a transplanted organ. 1) CTLA-4-CD80/86 interaction between Tregs and APCs resulting in increased tryptophan metabolism by APCs *via* IDO-dependent pathway. 2) LAG-3 and TIGIT on Tregs directing APCs towards a more tolerogenic phenotype. 3) Treg consumption of IL-2. 4) CD39 and CD73 acting as ectonucleotidases to break down ATP and 5'AMP to adenosine. 5) Tregs suppressing effector T cells *via* CTLA-4-CD80/86 interaction. 6) Tregs secreting anti-inflammatory cytokines to reduce the pro-inflammatory response, induce apoptosis of effector T cells, and promote the expansion of regulatory cell types. 7) MDSCs suppressing effector T cell, B cell, and NK cell proliferation *via* consumption of L-arginine in an iNOS dependent pathway. This mechanism is enhanced but upregulation of Arg-1 and HO-1 by MDSCs. 8) IL-10 and TGF-b secreted by MDSCs promoting the activation of Tregs. 9) CCL5 secreted by MDSCs establishing a graft-to-periphery gradient to recruit Tregs. 10) MDSCs promoting the suppressive function of Tregs *via* interaction between PD-L1 and PD-1. 11) IL-10 secreted by B10 cells promoting expansion of Tregs and exerting a broad array of anti-inflammatory effects. 12) Tregs inducing apoptosis of autoreactive B cells *via* interaction of PD-1 expressed on Tregs with PD-L1 on B cells.

are two additional surface molecules on Tregs that suppress effector T cells by acting as ectonucleotides to convert ATP and 5′-adenosine monophosphate (5'-AMP), generated by pro-inflammatory cells like neutrophils, into adenosine, an anti-inflammatory molecule (80–82). Both human and mouse Tregs can also be induced to express high levels of the surface molecules, lymphocyte activation gene-3 (LAG-3) and TIGIT, which both exert their immunosuppressive effects primarily by interacting with APCs (83, 84). LAG-3 binds MHC II on DCs to suppress their antigen presenting capabilities, while TIGIT binds to poliovirus receptor on DCs to modulate their differentiation towards a more tolerogenic phenotype with enhanced IL-10 production (83, 84).

In addition to the surface molecules mentioned above, Tregs also secrete several soluble factors to exert contact-independent immunosuppressive functions. In a cytolytic mechanism of immunosuppression, Tregs secrete granzyme B to induce apoptosis of effector T cells and APCs (72, 85–87). This mechanism has been shown to be of particular importance in the maintenance of transplant tolerance (85). Tregs also secrete TGF-β and IL-10. As mentioned earlier, Tr1 cells are a subset of inducible Tregs that appear to be the main contributors of Treg-derived IL-10 production (36, 88, 89). The secreted IL-10 exerts broad immunosuppressive activity by downregulating MHC II and costimulatory molecules, suppressing the immunostimulatory capacity of APCs, and inhibiting the production of various pro-

inflammatory cytokines by macrophages and DCs, overall resulting in reduced proliferation and activity of effector T cells (90–92). TGF-β secreted by Tregs appears to predominantly affect the cytolytic function $CD8^+$ T cells while sparing $CD4^+$ effector T cells (93–95). Given the importance of TGF-β signaling in the induction and activation of regulatory cell types, including Tregs and MDSCs, the TGF-β secreted by Tregs may also promote tolerance by enhancing these regulatory cell populations (23, 25, 32, 52).

MDSCs

MDSCs, like Tregs, exert their immunosuppressive effects *via* a variety of both contact-mediated and soluble factor-mediated mechanisms (**Figure 1**). The primary targets of these mechanisms are effector T cells and NK cells (45, 96). One of the main mechanisms by which MDSCs act, especially in the setting of transplant tolerance, involves production of NO by inducible nitric oxide synthase (iNOS) (44, 45, 51, 97–100). This iNOS-dependent mechanism has a profound regulatory impact on effector T cells, B cells, and NK cells by suppressing the differentiation, proliferation, and various functions of these effector cell types (101). iNOS also suppresses T cell proliferation by consumption of L-arginine, an important substrate for T cell proliferation and the precursor substrate used by iNOS to produce NO (102). This mechanism is enhanced by arginase-1 (Arg-1), another enzyme that is upregulated by MDSCs which cleaves L-arginine to form ornithine and urea (97). MDSCs also upregulate hemoxigenase-1 (HO-1), and in a skin allograft transplant model using mice, MDSC-mediated T cell suppression and prolongation of graft survival was dependent on HO-1 expression (103).

MDSCs also have substantial interactions with Tregs, enhancing their migration, proliferation, and function (14, 41, 46, 51, 104–108). One of the main mechanisms involves an interaction between B7-H1 (PD-L1) on MDSCs and PD-1 expressed on Tregs (51, 108). In a murine model of islet cell transplantation, B7-H1 knockout mice were unable to exert their immunosuppressive capabilities or induce Tregs (108). Additionally, the presence of IFN-γ stimulates MDSCs to secrete IL-10 and TGF-β, thereby activating Tregs (41, 109). MDSCs also appear to play an interesting role in the setting of organ transplantation by establishing a graft-to-periphery gradient of CCL5 chemokine, which directs migration of Tregs from secondary lymphoid organs to the site of the graft in rat models of heart and kidney transplantation (106). Given these findings that MDSCs and Tregs act synergistically, it is reasonable to suggest that adoptive transfer of both MDSCs and Tregs together may provide a greater beneficial effect for achieving transplant tolerance than either one alone. To support this, in a model using MHC class II disparate allogeneic donor skin transplantation, mice receiving administrations of either G-CSF to induce MDSCs or IL-2 to induce Tregs resulted in prolonged survival of the graft, and the combination of both treatments resulted in even better survival of the graft (14). Interestingly, this same study showed that the induced MDSCs were more potent at suppressing T cell responses compared to naive MDSCs (14).

B10 Cells

As their name implies, B10 cells predominantly exert their tolerogenic effects by producing and secreting the anti-inflammatory cytokine IL-10 in an antigen-specific manner (**Figure 1**). As mentioned above the in the setting of Tr1 cells, IL-10 suppresses the Th1 response, inhibits the antigen-present capabilities of APCs, and reduces the production and secretion of pro-inflammatory cytokines by macrophages and activated macrophages (65, 90, 91, 110). B10 cells have been shown to play a critical role in regulating the immune response in multiple models of autoimmunity in mice, including contact hypersensitivity, EAE, and collagen-induced arthritis (CIA) (62, 111, 112). In these models, adoptive transfer of CD40 mAb-stimulated B cells reversed the autoimmune pathologies, while transfer of $IL\text{-}10^{-/-}$ B cells had no effect, confirming the critical role of IL-10 production by B10 cells (62, 113).

Like MDSCs, B10 cells also promote tolerance by inducing the expansion of Tregs (67, 114, 115). One study demonstrated that human alloantigen-specific Foxp3-expressing Tregs can be generated in high frequencies by co-culturing $CD4^+CD25^-$ precursor T cells with CD40L-stimulated regulatory B cells (116). In a study investigating the role of B10 cells in the induction of oral tolerance, Sun et al. demonstrated that tolerance to a repeatedly administered antigen could be induced in mice in a Treg-dependent manner by transferring naïve T cells as long as IL-10-producing B cells were also present (117). Expansion of the antigen-specific Treg population, and therefore induction of tolerance, was absent in B cell-depleted mice, while co-transfer of B cells and naïve T cells into B cell-depleted mice restored the Treg population and resulted in tolerance (117). Like MDSCs mentioned above, these results suggest that co-transfer of both B10 and Tregs (or all three: B10, Treg, and MDSCs) in transplant patients could provide a synergistic therapeutic effect in the reduction of transplant rejection.

THERAPEUTIC POTENTIAL OF REGULATORY CELL TYPES IN TRANSPLANT MODELS

Tregs

Tregs have been extensively implicated as therapeutic options in a variety of organ transplant models, including skin, heart, kidney, islet cell, and lung (**Table 2**). The specific therapeutic strategy (*ex vivo* expansion versus *in vivo* induction, adjunctive immunosuppression, and specific subset of Tregs utilized) varies between studies, and it is likely that the optimal strategy may depend on the specific organ being transplanted. In a pivotal study published in Nature Medicine in 2010, Nadig et al. showed that *ex vivo* expanded $CD127^{lo}$ Tregs could be adoptively transferred to inhibit the development of transplant arteriosclerosis (TA) in a clinically relevant chimeric humanized mouse system (12). This marked the first time that human Tregs were used to prevent TA in human arteries, which is the hallmark of chronic allograft dysfunction (12). In another recent study, Ratnasothy et al. demonstrated that exogenous administration

TABLE 2 | Animal transplant models utilizing regulatory cells.

Cell	Organ	Species	Cell Origin (recipient/donor/3rd party)	Adjunctive Therapy	Mean Survival Time of Graft: Treatment vs. Control (Days)	Reference (Examples)
Tregs	Skin	Mouse; humanized mouse	Donor; recipient	IL-2 (118); IL-33 (119)	40 vs. 12 (14); 29 vs. 13 (118); >30 vs. 12 (119); 76 vs. 10 (120)	(14, 118–120)
	Heart	Mouse	Donor; recipient	IL-33 (15)	29 vs. 9 (15); >100 vs. 7 (16); 91 vs. 67 (126); >150 vs. 59 (146)	(15, 16, 126)
	Kidney	Nonhuman primate	Donor	Sirolimus (122)	416 vs. 22 (121); 48.5 vs. 22 (122)	(121, 122)
	Islet cell	Mouse; humanized mouse	3rd party; recipient	Rapamycin + anti-CD8 (146)	32 vs. 17 (17); >60 vs. 15 (108)	(17, 108, 146)
	Lung	Humanized mouse	3rd party;	N/A	Intimal thickening: 0.4% vs. 39.9% (127)	(127)
Tr1	Islet cell	Mouse	3rd party	N/A	>100 vs. 25 (128)	(128)
CAR-Tregs	Skin	Mouse	3rd party	N/A	>40 vs. 37 (129); 14 vs. 8 (131)	(129–131)
MDSCs	Cornea	Mouse	Recipient; 3rd party	Glucocorticoids (135)	22.71 vs. 15.65 (43); 28.3 vs. 15.73 (136)	(43, 135, 136, 147)
	Skin	Mouse	Recipient; 3rd party	G-CSF (14); IL-33 (119)	40 days vs. 16 days (14); 13.9 vs. 8.8 (43); 40 vs. 28 (59); >100 vs. 40 (99); >100 vs. 29 (109); >30 vs. 12 (119); 15 vs. 11 (132); 45 vs. 23.5 (133); 54.8 vs. 12.7 (148)	(14, 43, 59, 99, 103) (109, 119, 132, 133, 148)
	Heart	Mouse	Recipient; 3rd party; donor	Rapamycin (105); anti-CD40L mAb (51); IL-33 (15)	29 vs. 9 (15); 67 vs. 7 (105); 58 vs. 10 (134)	(15, 51, 105, 134, 149)
	Islet cell	Mouse	Recipient; 3rd party	N/A	>60 vs. 15 (100); >60 vs. 15 (104); >60 vs. 15 (108)	(58, 100, 104, 108)
B10	Islet cell	Mouse	3rd party	Anti-TIM-1-mAb (67)	>100 vs. 15 (67)	(67)

of IL-2 lead to the preferential expansion of adoptively transferred donor-specific Tregs (specific for the MHC class I molecule K^d), but not polyclonal Tregs, producing a synergistic effect that resulted in prolonged skin graft survival (from a mean of 13 days without treatment to 29 days with Tregs + IL-2) (118). In multiple other models of skin allograft in mice, Tregs were induced *in vivo* using exogenous administration of either interleukin-2 complex (IL-2C) or interleukin-33 (IL-33), resulting in prolonged survival of the skin grafts in the absence of immunosuppressive drug therapy (14, 118–120). In multiple models of kidney transplantation using non-human primates, adoptive transfer of *ex vivo* expanded donor-specific Tregs has been shown to prolong graft survival and prevent acute rejection (121, 122). Observational data has also suggested the potential efficacy of adoptive Treg therapy in human kidney transplant patients. In a retrospective study of human living donor kidney transplant recipients, flow cytometry analysis revealed significant increase in frequency of activated Tregs in the first 3 months after transplantation (123). Additionally, operationally tolerant kidney transplant patients have a higher frequency of more potent memory Tregs compared to patients with stable graft function or with chronic graft rejection, a trend which is also observed in operationally tolerant liver transplant recipients (124, 125).

Tregs have also been induced *in vivo* or adoptively transferred to prevent chronic rejection of heart transplants in mice (15, 16, 126). Takasato et al. demonstrated that donor-specific Tregs expanded *via* the indirect pathway were most effective in prolonging cardiac allograft survival (16). Interestingly, in the study conducted by Ma *et al*, low dose of the commonly used immunosuppressive drug sirolimus appeared to have a synergistic effect with Tregs promoting their expansion and homing to secondary lymphoid organs in the setting of heart transplantation (122).

In a humanized mouse model studying the role of Tregs in lung transplantation, adoptive transfer of allogenic human peripheral blood mononuclear cells enriched for Tregs resulted in significantly reduced transplant arteriosclerosis and intimal thickening (127). Finally, multiple studies have demonstrated the efficacy of using adoptively transferred human Tregs or inducing Tregs using adoptively transferred MDSCs to delay islet cell allograft rejection (17, 108). While minimal studies have utilized adoptive transfer of Tr1 cells in delaying graft rejection, the adoptive transfer of donor-specific (but not polyclonal) Tr1 cells has been shown to be efficacious in preventing islet cell allograft rejection (128).

Using chimeric antigen receptor (CAR) technology, multiple groups have developed Tregs expressing HLA-A2-specific CARs that have more potent immunosuppressive capabilities compared to polyclonal Tregs in the setting of humanized mouse models with HLA-$A2^+$ skin xenografts, resulting in prevention of skin graft rejection (6, 129–131). Utilization of this technology overcomes several barriers associated with the use of natural Tregs. Namely, that the induction and expansion of antigen-specific Tregs involves a technically challenging protocol requiring repeated stimulation with the antigen of interest, which may not be feasible in the setting of clinical

transplantation (129). These groups working with CAR technology have developed short transduction protocols that circumvent the need for extensive *in vitro* expansion (129). Additionally, these donor-specific CAR Tregs appear to be more specific and more potent than natural Tregs (129–131).

MDSCs

The therapeutic role of adoptively transferred MDSCs has been extensively demonstrated in mouse models of skin transplantation (**Table 2**). Multiple groups have published protocols for inducing and activating MDSCs *in vitro* to be adoptively transferred into skin transplant recipients, including induction with LPS, TNF-α, human inhibitory receptor immunoglobulin-like transcript 2 (ILT-2), IFN-γ, or recombinant G-CSF, GM-CSF, or IL-6 (58, 59, 99, 103, 109, 132, 133). MDSCs induced *in vivo* with administration of G-CSF or IL-33 have also been shown to promote graft tolerance in skin transplanted mice (14, 119). Drujont et al. found that a single injection of LPS-activated MDSCs on the day of skin transplantation resulted in significant increase in survival of the graft, while repeated weekly injections resulted in even greater graft survival, suggesting that the full therapeutic potential of adoptive transfer of MDSCs may depend on repeated injections of activated MDSCs (59).

In heart allograft transplantation, both induced and adoptively transferred MDSCs have been successfully used to prolong graft survival in animal models. Garcia et al. demonstrated that donor MDSCs can be adoptively transferred and induced in the recipient by treatment with anti-CD40L mAb, resulting in MDSCs that migrate into the transplanted organ to prevent the initiation of the adoptive immune response and enhance the development of Tregs (51). Similarly to the skin transplant models described above, IL-33 has been used to induced *in vivo* expansion of MDSCs and Tregs to promote cardiac allograft survival in mice (15). Bryant et al. demonstrated that apoptotic donor splenocytes could be treated with the chemical cross-linker ethylcarbodiimide (ECDI) and preemptively infused into cardiac allograft recipient mice to induce MDSCs, resulting in long-term allograft survival (134).

He et al. found that sepsis-induced MDSCs could be harvested and adoptively transferred into mice immediately following corneal and combined corneal-skin transplantation, resulting in substantial expansion of MDSCs in the recipients bone marrow and in the corneal graft and increasing corneal graft survival from a mean of 15.65 days to 22.71 days (43). Glucocorticoids are known to induce expansion of MDSCs *in vitro*, and it has been shown that both systemic administration of glucocorticoids and adoptive transfer of glucocorticoid-induced MDSCs following corneal transplantation results in enhanced proliferation and mobilization of MDSCs, inducing immune tolerance (135). He et al. compared the tolerogenic capacities of inflammation-induced MDSCs versus tumor-induced MDSCs in the setting of corneal transplantation (136). In terms of reducing neovascularization and prolonging graft survival in the absence of immunosuppressive drugs, they found that inflammation-induced MDSCs were comparable to tumor-induced MDSCs when adoptively transferred to transplant recipients by retroorbital injection (136).

There is also extensive evidence to support the use of MDSCs to promote the survival of islet cell allografts. Marigo et al. demonstrated that MDSCs generated by treating bone marrow precursor cells with a combination of GM-CSF and IL-6 could be adoptively transferred to islet cell transplant recipients with four weekly injections immediately following transplantation (58). These MDSC's inhibited the priming of $CD8^+$ T cells and their adoptive transfer resulted in long term survival of allogenic islet cell transplant, with 75% of mice remaining euglycemic 200 days post-transplantation (58). MDSCs can also be generated *ex vivo* by co-culturing bone marrow precursor cells with GM-CSF, dendritic cells, and hepatic stellate cells (100). These MDSCs can be adoptively transferred to promote islet cell allograft survival in a manner that is dependent on iNOS expression and also results in the expansion and accumulation of antigen-specific Tregs in lymphoid organs close to the grafts when MDSCs are co-transplanted (100, 108).

In humans, MDSCs have been implicated as important regulators of tolerance in kidney and lung transplantation (46, 107, 137, 138). $CD14^+$ M-MDSCs expand in renal transplant patients following transplantation, and these MDSCs are highly efficient in suppressing the proliferation of $CD4^+$ T cells in mixed leukocyte reactions and are also capable of expanding Tregs *in vitro*. Additionally, there is a linear relationship between these MDSCs post-transplantation and circulating levels of Tregs (46, 138). In a study involving 50 patients with biopsy-proven acute T cell-mediated rejection (ATCMR), Meng et al. found that higher circulating levels of MDSCs post-transplantation correlated positively with allograft function and survival (107). *In vitro*, the MDSCs isolated from these patients were capable of expanding Tregs and inhibiting production of IL-17 (107). In a study investigating the role of MDSCS in human lung transplantation, it was found that circulating MDSCs are increased in stable lung transplant recipients versus non-transplant controls, and that patients with chronic lung allograft dysfunction (CLAD) had lower levels of MDSCs compared to stable recipients (137). These findings in humans, combined with the successful use of adoptive MDSC transfer in animal models described above, suggest that adoptive transfer of MDSCs could prolong organ allograft survival and promote graft tolerance in humans.

B10

While studies involving the therapeutic use of B10 in transplantation are limited compared to Tregs and MDSCs, there is evidence implicating them in promoting tolerance in kidney, heart, skin, and islet cell transplantation (**Table 2**) (67, 139–144). In a mouse model of islet cell transplantation, anti-TIM-1 antibody was used to expand $TIM\text{-}1^+$ B10 cells *in vivo* to significantly prolong islet cell allograft survival (67). Adoptively transferred $TIM\text{-}1^+$ B10 cells exhibited potent tolerogenic activity in an antigen-specific fashion to prolong islet cell allograft survival while also enhancing the frequency of Tregs in the recipient (67). In multiple human and animal models of transplantation, including kidney, heart, skin, and islet cell, depletion of B cells during the period shortly following the transplant procedure when tolerance is being induced results in an enhanced T-cell response and accelerates graft rejection (140–143).

In human kidney transplant recipients, patients who achieve operational tolerance exhibit elevated levels of regulatory B cells compared to stable patients still requiring immunosuppression

or patients with chronic rejection (139, 145). While there is a general paucity of studies directly investigating the adoptive transfer of B10 cells to promote tolerance, all of the above evidence suggests that B10 cells play an important role in inducing transplant tolerance and should be pursued as a potential therapeutic option alongside Tregs and MDSCs.

CLINICAL TRIALS INVOLVING ADOPTIVE TRANSFER OF REGULATORY CELL TYPES

In recent years, a number of clinical trials have been initiated to study the use of adoptive cell therapy in organ transplantation. Thus far, these studies have focused on the use of Tregs, with a paucity of trials investigating MDSCs or B10 cells. Kidney and liver have been the main organs involved in these trials. A summary of all completed and ongoing clinical trails involving adoptive transfer of regulatory cell types in the setting of organ transplantation is presented in **Table 3**.

Kidney

A number of key phase I trials have been initiated investigating the use of regulatory cell types in kidney transplant recipients, including the TASK trial, the TRACT trial, and the ONE study (18, 150, 151). The TASK trial (NCT02711826) was conducted by researchers at UCSF to investigate the safety and feasibility of autologous polyclonal expanded Tregs in three patients with biopsy-proven subclinical graft inflammation at 6 months post-transplant (150). The group found no infusion reactions or serious adverse therapy-related events. The isolated Tregs received two rounds of stimulation with anti-CD3 and anti-CD28 beads and IL-2, along with deuterated glucose to label and track the cells (150). While the patients were maintained on an immunosuppressive regiment of tacrolimus, mycophenolate mofetil, and prednisone, the infused Tregs demonstrated persistence and stability comparable to non-immunosuppressed subjects infused with the same dose of Tregs (150). These results have set the stage for future trials testing the efficacy of polyclonal and antigen-specific Tregs in the setting of subclinical inflammation in renal transplants (150).

In the TRACT trial (NCT02145325), a group from Northwestern University performed a dose-escalation trial in living donor renal transplant recipients, with three dosing tiers (0.5, 1, and 5×10 (9) cells) and three recipients per dose (18). The infused Tregs exhibited high purity (>98% $CD4^+CD25^+$) with high stability of the Foxp3 promoter. *In vivo*, the infused Tregs resulted in sustained, elevated levels of circulating Tregs. Like the TASK trial, this trial reported no adverse events related to the therapy up to 2 years post-transplant when the results were published, providing the necessary safety data move the trial into phase II efficacy studies (18).

The ONE study involved seven single-arm trials conducted at eight different institutions in across five countries, investigating the use of cell-based protocols to reduce general immunosuppression in living-donor renal transplant recipients (151). The cell-based protocols utilized in the various trials included two polyclonal Treg products (NCT02371434, NCT02129881) and two donor-antigen reactive Treg products (NCT02244801, NCT02091232), as well as one tolerogenic dendritic cell and one regulatory macrophage cell product (151). The two polyclonal Treg products, pTreg-1 (NCT02371434) and pTreg-2 (NCT02129881), were isolated and expanded using protocols published by Fraser et al. and Landwehr-Kenzel *et al*, respectively (19, 152). One of the donor-specific Treg products utilized conditions of costimulatory blockade (NCT02091232) while the other product was generated by stimulating recipient PBMCs with donor B cells that had been activated by human CD40L expressed on K562 cells (NCT02244801) (20, 153). All Treg products were delivered as a single intravenous infusion within 10 days following the day of the transplant procedure, and all patients were routinely monitored for the primary endpoint of biopsy-confirmed acute rejection (BCAR) within 60 weeks following transplantation. Combined data across all of the cell-based therapy groups revealed no safety concerns compared to the standard immunosuppressive treatment group, and the cell-based groups experienced lower infection rates. Additionally, rates of BCAR were comparable between the standard immunosuppressive group and the cell-based therapy group (12% vs. 16%), overall suggesting that adoptive transfer of Tregs could be a useful therapeutic tool for preventing rejection in renal transplant patients while reducing the burden of immunosuppression.

The STEADFAST study (EUCTR2019-001730-34-NL), a recently initiated phase I/IIa trial, has been initiated in the U.K. and the Netherlands to evaluate the safety and tolerability of an autologous HLA-A2-specific Treg therapy (TX200-TR101 product) in living donor renal transplant recipients. This will be the first clinical trial investigating the use of a CAR-Treg therapy in the prevention of transplant rejection in humans. As such, the results of this study are highly anticipated.

Liver

In 2016, Todo et al. published a pilot study on the use of adoptive transfer of donor-specific Tregs in 10 living donor liver transplant patients (UMIN-000015789) (154). Donor alloantigen-specific Tregs were generated *in vitro* by coculturing recipient lymphocytes with irradiated donor cells along with anti-CD80/86 mAbs for 2 weeks. These Tregs demonstrated donor-specific inhibition in a mixed lymphocyte reaction and were infused in all 10 patients without any significant adverse events. After transplantation and infusion with Tregs, patients underwent splenectomy and were subsequently weaned off of traditional immunosuppression of mycophenolate mofetil and tacrolimus starting at 6 months until complete cessation at 18 months. The ultimate goal of stable graft function with complete discontinuation of immunosuppression after 18 months was achieved in seven out of the 10 patients, while the other three patients developed mild rejection during the weaning period and were continued on low dose immunosuppression. Of note, these three patients all had autoimmune liver disease.

Several ongoing studies are also utilizing donor alloantigen-specific Tregs in the setting of liver transplantation, including the LITTMUS trial (NCT03577431 and NCT03654040), the ARTEMIS

TABLE 3 | Completed and ongoing clinical trials involving adoptive transfer of regulatory cell types.

Study	Phase	Condition	Intervention	Dosage	Status	Outcome
NCT02145325	1	Living donor renal transplant	Autologous polyclonal expanded Tregs	0.5, 1, 5 × 10^9 cells	Complete	No adverse events related to therapy
NCT02129881	1/2	Living donor renal transplant	Autologous polyclonal expanded Tregs	1–10 × 10^6 cells/kg	Complete	No adverse events related to therapy
NCT02371434	1/2	Living donor renal transplant	Autologous polyclonal expanded Tregs	0.5, 1, 2.5–3 × 10^6 cells/kg	Complete	No adverse events related to therapy
NCT02244801	1	Living donor renal transplant	Autologous donor-alloantigen-specific Tregs	3, 9 × 10^6 cells	Complete	No adverse events related to therapy
NCT02091232	1	Living donor renal transplant	Autologous donor-alloantigen-specific Tregs, cocultured with belatacept	N/A	Active	N/A
NCT02088931	1	Renal transplant	Autologous polyclonal expanded Tregs	320 × 10^6	Complete	No adverse events related to therapy
NCT02711826	1/2	Renal transplant	Autologous polyclonal expanded Tregs	550 × 10^6	Ongoing, recruiting	N/A
ISRCTN-11038572	2b	Living donor renal transplant	Autologous polyclonal expanded Tregs (TR001 cell product)	5–10 × 10^6 cells/kg	Ongoing, recruiting (4/2020: recruiting suspended due to COVID-19)	N/A
NCT03867617	1/2	Living donor renal transplant	Autologous Tregs + donor bone marrow + Tocilizumab	N/A	Ongoing, recruiting (2019)	N/A
NCT03943238	1	Living donor renal transplant	Autologous expanded Tregs + donor HSC's	25 × 10^6 cells/kg	Ongoing, recruiting (2020)	N/A
NCT01446484	1/2	Living donor renal transplant in children	Autologous polyclonal expanded Tregs	2 × 10^8 cells	Unknown (2011)	N/A
NCT03284242	1	Renal transplant in patients on Everolimus	Autologous polyclonal expanded Tregs	N/A	Ongoing, recruiting (2020)	N/A
EUCTR2019-001730-34-NL	1/2a	Living donor renal transplant	Autologous Antigen-Specific CAR-Tregs (TX200-TR101 cell product)	N/A	Ongoing, recruiting (2020)	N/A
UMIN-000015789	1/2	Living donor liver transplant	Autologous donor-alloantigen-specific Tregs	3.39 × 10^6 cells/kg	Complete	No adverse events related to therapy. 7/10 patients achieved complete cessation of immunosuppression.
NCT02166177	1/2	Liver transplant	Autologous polyclonal expanded Tregs (TR002 cell product)	1, 4.5 × 10^6 cells/kg	Complete	No adverse events related to therapy
NCT02188719	1	Liver transplant	Autologous donor-alloantigen-specific Tregs	50, 200, 800 ×10^6 cells	Terminated	Difficulties in manufacturing the cell product
NCT02474199	1/2	Living donor liver transplant	Autologous donor-alloantigen-specific Tregs	400 × 10^6 cells	Complete (2020)	N/A
NCT01624077	1	Living donor liver transplant	Autologous donor-alloantigen-specific Tregs	1 × 10^6 cells/kg	Unknown	N/A
NCT03577431	1/2	Liver transplant	Autologous donor-alloantigen-specific Tregs with costimulatory blockade	2.5–500 × 10^6 cells	Ongoing, recruiting (2019)	N/A
NCT03654040	1/2	Liver transplant	Autologous donor-alloantigen-specific Tregs	100–500 ×10^6 cells	Withdrawn	Investigational product manufacturing challenges
NCT03444064	1	Islet cell transplant	Autologous expanded polyclonal Tregs	400–1600 × 10^6 cells	Ongoing, recruiting (2020)	N/A
NCT03162237	N/A	Porcine islet cell xenotransplant	Autologous polyclonal Tregs	2 × 10^6 cells/kg	Complete (2020)	N/A

trial (NCT02474199), and the deLTa trial (NCT02188719). The first arm of the LITMUS trial (NCT03577431) involved the use of donor alloantigen-specific Tregs cultured and stimulated in the presence of costimulatory blockade, while the second arm of the study (NCT03654040) intended to use Tregs without costimulatory blockade but was withdrawn due to difficulty manufacturing the cell product. The deLTa trial set out to give three cohorts three different doses of donor alloantigen-specific Tregs (50, 200, and 800 ×10 (6) cells) but the study was terminated due to difficulties manufacturing the cell product. The ARTEMIS trial, which specifically investigated the use of Tregs in weaning patients off of calcineurin inhibitors (CNIs), was completed in January 2020 and results are still pending.

Trials involving liver transplant recipients have also investigated the use of polyclonal Tregs. One of these studies, known as the ThRIL trial (NCT02166177), was a phase I/IIa trial evaluating the safety, tolerability, and efficacy of polyclonal expanded Tregs and was completed in January 2018 (155). This study utilized a CliniMACS-based cell isolation protocol and expanded the Tregs using a co-culture containing anti-CD3/CD28 beads, IL-2, and rapamycin (155). Preliminary safety data from this trial was presented in abstract form at the 2017 American Transplant Congress meeting, reporting no dose-limiting toxicities in patients receiving the polyclonal Tregs (156). Results are still pending regarding the efficacy of the treatment.

Islet Cell

Two studies are currently being conducted to investigate the use of polyclonal Tregs to induce tolerance of islet cell allografts and xenografts (NCT03444064, NCT03162237) in patients with type 1 diabetes. Results of both of these studies are still pending.

DISCUSSION

The bulk of research published so far on adoptive cell therapy in the setting of solid organ transplantation has implicated regulatory cell types as potential therapeutic options for reducing the burden of systemic, lifelong immunosuppression in transplant recipients. As described in this review, each of these cell types have distinct mechanisms by which they exert their tolerogenic effects; however, there is also considerable interaction between these cell types. To date, the vast majority of animal model studies and all of the clinical trials have utilized the adoptive transfer of only a single cell type. Given the synergistic effect that these cells exert on one another, we suggest further investigation into using the adoptive transfer of multiple cell types together to induce tolerance in a single transplant recipient. This will require further research into the optimal combinations, ratios, and timing of when to transfer these cells. Additionally, thus far, clinical trials utilizing the adoptive transfer of regulatory cell types in the setting of solid organ transplantation have focused almost exclusively on Tregs. We suggest that further studies be conducted using other regulatory cell types, such as MDSCs and B10 cells, given the promising data that has been generated with their use in animal models.

Other questions that require answering before adoptive cell therapy can become a widely utilized therapeutic approach in transplantation include the optimal timing, dosing range, and dosing frequency for the different cell types and for the different organs being transplanted. This will require large-scale studies with multiple cohorts to be able to accurately compare the different strategies. Similarly, further research should be conducted to establish the most efficient and universally applicable isolation and expansion protocol for each of the different cell types, especially given that the requirement of repeated, prolonged antigen stimulation to produce antigen-specific Tregs is a limitation in many settings. This may require further pursuit of the use of CAR technology to design CAR-Tregs for clinical trials.

Immunoengineering offers promising new avenues for optimizing adoptive cell therapy. Using gene transfer technology, Tregs can be transduced with antigen-specific TCRs or CARs. While already widely used in the treatment of hematological malignancies, CAR-T cells have gone through multiple generations of optimization to increase their efficacy and limit off-target toxicities. Similar optimizations will be required for CAR-transduced Tregs before they can be widely applied to human transplant patients, including optimal co-stimulatory molecules, appropriate antigen specificity (including CARs with bi-specificity), and the possible inclusion of suicide genes to improve the safety profiles of these therapies. The high cost of these engineered cell products is also a barrier that will need to be addressed moving forward.

It should be noted that other regulatory cell types exist and have shown promise as potential therapeutic tools, including tolerogenic dendritic cells, natural killer cells, and regulatory macrophages. As the novel field of adoptive cell therapy continues to grow, these cells may emerge as important players along with Tregs, MDSCs, and B10 cells. Overall, the therapeutic potential of regulatory immune cells in the setting of solid organ transplantation is incredibly promising and will be exciting to follow as the foundational research outlined in this review is translated to the clinic.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Immune Monitoring for Advanced Cell Therapy Trials in Transplantation: Which Assays and When?

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A number of immune regulatory cellular therapies, including regulatory T cells and mesenchymal stromal cells, have emerged as novel alternative therapies for the control of transplant alloresponses. Clinical studies have demonstrated their feasibility and safety, however developing our understanding of the impact of cellular therapeutics *in vivo* requires advanced immune monitoring strategies. To accurately monitor the immune response, a combination of complementary methods is required to measure the cellular and molecular phenotype as well as the function of cells involved. In this review we focus on the current immune monitoring strategies and discuss which methods may be utilized in the future.

Keywords: immune monitoring, cell therapy, transplantation, regulatory T cell, mesenchymal stromal cell

INTRODUCTION

The long-term treatment of transplant patients with immunosuppressive drugs is associated with significant side effects including life-threatening infections, cancer development, and direct drug toxicity (1–3). A number of immune regulatory cellular therapies including regulatory T cells (Tregs) and mesenchymal stromal cells (MSCs) have emerged as novel alternative therapies for the control of transplant alloresponses (4–6), with the possibility of reducing the morbidity associated with standard immunosuppression.

To date, clinical studies of advanced cellular therapies have focused on feasibility and safety. As the goal of these cellular therapies is to modify the immune response to transplantation, detailed immune monitoring in these trials is crucial. This immune monitoring facilitates a deeper understanding of the alloresponse, while providing crucial data on treatment effectiveness as well as the potential to identify new biomarkers or therapeutic targets. The immune response is a diverse and dynamic system that interacts temporospatially at many points, and it is therefore not possible (nor relevant) to monitor a single cell type in isolation. To accurately monitor the immune response, a combination of complementary methods is required to measure the cellular and molecular phenotype as well as the function of cells involved. In this review we will discuss the current methods of immune monitoring (**Figure 1**) and how they have the potential to become standard features of clinical trials in the future.

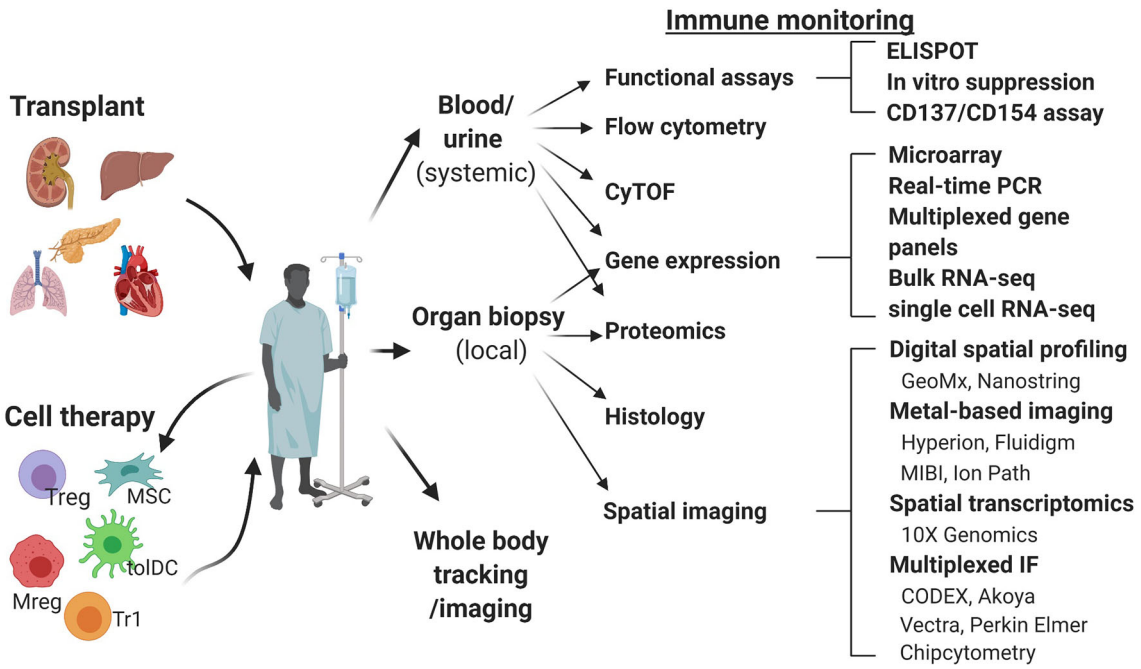

FIGURE 1 | Schematic overview of the immune monitoring methods useful in cell therapy trials in transplantation. CODEX, co-detection by indexing; CyTOF, cytometry of the time of flight; IMC, imaging mass cytometry; MIBI, multiplexed ion beam imaging; Mreg, regulatory macrophage; MSC, mesenchymal stem cell; RT-qPCR, real time quantitative polymerase chain reaction; tolDC, tolerogenic dendritic cell; Treg, regulatory T cell; Tr1, type 1 regulatory T cell.

Currently, peripheral blood is the most studied material, due to the availability of well-developed techniques of analysis and ease of repeated sampling. However, data from pre-clinical models demonstrating homing of adoptively transferred human regulatory cells to the allograft and its importance for the induction of immune tolerance highlights the need for and value of allograft tissue analysis (7, 8). We will review here the methods most commonly used for systemic immune monitoring of blood, including flow cytometry, mass cytometry, functional assays and gene expression analysis (**Figure 2**) and discuss novel techniques of tissue biopsy analysis, including gene expression analysis and spatial biology methods (**Figure 3**).

FLOW CYTOMETRY

Flow cytometry is well established as a technique for investigating the immune response. It provides rapid multi-parametric analysis of single cells in solution and is a powerful tool for immune monitoring as it can measure multiple parameters in parallel (example of data shown in **Figure 2A**) (9). Flow cytometers utilize lasers as light sources to produce both scattered and fluorescent light signals that are captured and converted into electronic signals (10). Cells are typically stained with fluorophore-conjugated antibodies or fluorescent dyes (10). This fluorescence is one of the limitations of the method, as the number of markers that can be analyzed concomitantly is limited by spectral overlap of the fluorophores used.

Whilst it is a commonly used method it is acknowledged that there can be significant variability in how samples are run and analyzed. This is of particular concern for clinical trials across different sites where it would be advantageous to be able to compare results. Efforts have been made to address this, Lee et al. published a consensus, the Minimum Information about a Flow Cytometry Experiment (MIFlowCyt) standard, detailing the minimum information that should be reported when publishing results of flow cytometry experiments (11), in order to aid both comparison and replication of results. Geissler et al. published the outcome of a TTS (The Transplant Society) symposium, where it was agreed it would be beneficial to establish consensus standard operating procedures (SOPs) for immune monitoring, the Virtual Global Transplantation Laboratory (vGTL) (12). To date, two SOPs, blood collection and PBMC isolation (13), and donor alloantigen specific IFNγ ELISpot (14) have been published. Expanding on this, Cossarizza et al. published invaluable guidelines for the use of flow cytometry in immunological studies, covering in detail the various techniques and applications of flow cytometry as well as how to analyze the results (15).

The ONE study was set up to study the immune-modulatory effect of a range of different regulatory immune cells in renal transplant patients (16). A key part of the setup was the development of a rigorous immune monitoring program, to profile the peripheral blood immune phenotype, using flow cytometry. Antibody panels were developed to profile T cell, B cell and dendritic cell (DC) subsets and their activation status (now available from Beckmann Coulter,

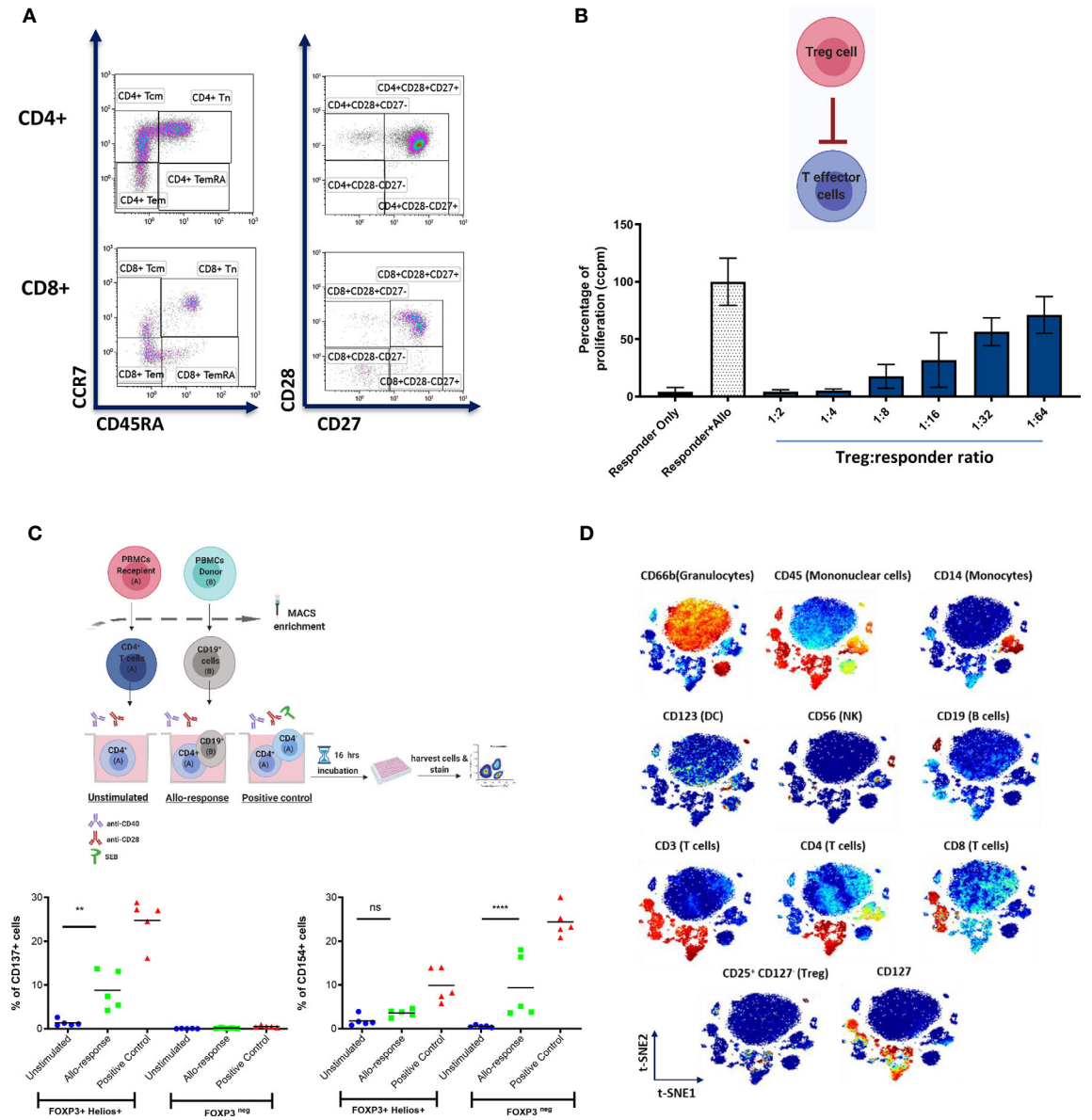

FIGURE 2 | Representative examples of systemic immune monitoring techniques. **(A)** Flow cytometry can be used to measure the frequency of various immune cell populations in peripheral blood. **(B)** In vitro suppression assays can be used to assess the suppressive potential of Tregs (or other regulatory cells). Example of 3H-thymidine incorporation readout in a test with allogeneic stimulation. **(C)** (Top) Schematic of the experimental design of CD137/CD154 assay. (Bottom) Example of CD137 (left) and CD154 (right) expression on FOXP3^{+}Helios^{+} (Tregs) and FOXP3^{neg} (conventional CD4+ T cells) cells. **(D)** Multiplexed CyTOF technology can be used for deep phenotyping analysis of leukocyte composition. An example of t-distributed Stochastic Neighbor Embedding (t-SNE) analysis of leukocyte clusters annotated based on the intensity of analyzed parameter is shown. ** = p value 0.0041, **** = p value < 0.0001, F test of variance. not significant (p >0.05).

DURAclone panels) (17). Streitz et al. reported on the results of this optimization process, including the strategy of local sample preparation using strict standard operating procedures (SOPs), followed by central analysis. They showed acceptable variability in results between multiple international sites. Using these standardized protocols provides results that can be compared between treatment groups and patients across multiple centers, vital for immune monitoring in clinical trials (17). The same panels have been incorporated by other cell therapy trials, like the TWO study (a randomized, phase II study investigating efficacy of regulatory T cells in kidney transplantation) and the Neptune study (a phase I study investigating mesenchymal stromal cells in renal transplantation).

The majority of trials looking at advanced cellular therapies in transplantation have used some form of flow cytometry analysis as part of their immune monitoring (16, 18–25). From the more basic measurement of numbers and proportions of different immune cell subsets, to following changes in the immune

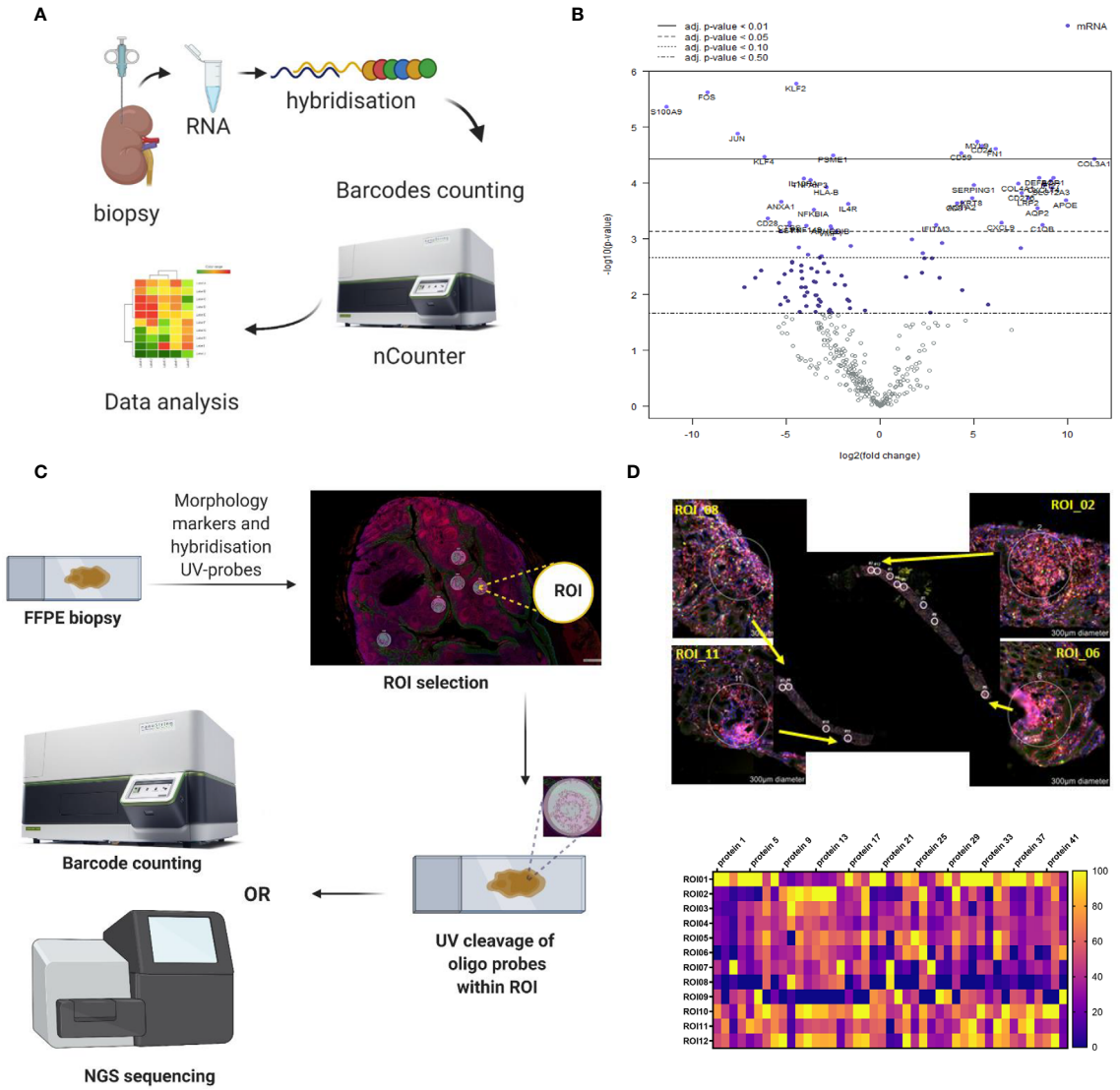

FIGURE 3 | Representative examples of the *in situ* immune monitoring techniques. **(A)** Multiplexed gene expression using the nCounter, Nanostring. Schematic of the technique. **(B)** An example volcano plot obtained using Banff - Human Organ Transplantation (B-HOT) panel on the nCounter system, kidney biopsy versus background of PBMC. **(C)** Schematic of digital spatial profiling (DSP). **(D)** Example of kidney biopsy from renal transplant recipient analyzed using DSP (GeoMx, Nanostring). Morphology staining, selected ROIs and heat map analysis are shown. Example of geometric regions of interest (ROIs) and readout with panel of antibodies. FFPE, formalin fixed paraffin embedded; ROI, region of interest.

compartment over time. Below is a brief overview of how flow cytometry has been used for immune monitoring following cellular therapy.

The ONE study

There were six different cell based medicinal products (CBMPs) trialed in the ONE study, including polyclonal Tregs (pTregs), donor alloreactive Tregs (darTregs), autologous tolerogenic dendritic cells (ATDC) and regulatory macrophages (Mregs). The immune monitoring methods detailed by Streitz et al. (17), were utilized to allow a metanalysis of the results. There was no difference in the $CD4^{+}CD25^{high}CD127^{low}$Tregs at 60 weeks in those receiving CBMPs compared to standard care (16). However, there was evidence of significant decreases in Treg specific demethylated region (TSDR) demethylation in the standard care group. Furthermore, there was an increase in $CD8^{+}T_{EMRA}$ and $CD8^{+}CD57^{+}$ chronically active T cells in the standard care group. Both the CBMP group and standard care groups had more plasmacytoid dendritic cells at 60 weeks post-transplant than healthy age/sex matched controls. Interestingly there was a normalization of marginal zone-like B cell numbers and a significant reduction in $CD14^{high}CD16^{+}$ monocytes in those who received a CBMP. Taken together this suggested that those who received CBMPs had restoration of an immune phenotype more similar to the healthy controls than those receiving standard of care (16).

Regulatory T cells

In 2014 the TRACT (Treg adoptive cell therapy) trial was commenced, a phase I trial to test safety and to trial escalating doses of autologous polyclonal Treg therapy in kidney transplantation (18). Flow cytometric analysis was the main method of immune monitoring of patients in this trial. Of note, patients had induction therapy with alemtuzumab, resulting in a significant decrease in T cells as well as B cells, NK cells and $CD14^+$ monocytes in the first month post-transplant (18). By day 90 numbers of the majority of these cells had recovered, however numbers of $CD4^+$ and $CD8^+$ T cells remained low (18). Interestingly, the authors observed an increase in Tregs that remained stable at 1 year (18).

Similarly, Todo et al. used flow cytometry as part of the immune monitoring strategy for patients who underwent liver transplantation combined with Treg therapy (19). They also noted a trend to increased Treg ($CD4^+CD25^+CTLA4^+$/ $CD4^+CD25^+Foxp3^+$) numbers post transplantation, however, they were unable to demonstrate a significant difference due to variation between recipients (19). Sánchez-Fueyo et al. used ONE study panels for immune monitoring of autologous polyclonal Treg therapy in liver transplantation. They did not find any significant changes in immune cell subsets post Treg therapy (25).

Mesenchymal Stromal Cells

The Neptune study, a phase I study of allogeneic mesenchymal stromal cells (MSCs) in kidney transplant recipients (22), utilized the same flow cytometry protocols and panels as the ONE study for immune monitoring of participants, allowing detailed monitoring of the changes in leukocyte subsets post cell therapy (22). Authors found a decrease in $CD19^+$ B cells, $CD56^+$ NK cells, $CD8^+$ T cells, $CD4^+$ T cells and Tregs post induction immunotherapy, as expected (22). The B cells and NK cells began to recover from week 25 post induction (22). Although showing signs of repopulation, T cell numbers had not returned to baseline by 12 months (22). However, $CD4^+$ T cells showed a significant increase in number after two MSC infusions (22). Later timepoint data are awaited from this trial.

Perico et al. used flow cytometry for immune monitoring of a phase I trial of autologous MSCs in renal transplantation (21). Initially they demonstrated a profound depletion of $CD8^+$ and $CD4^+$ T cells (21). The $CD8^+$ T cell numbers recovered by day 360, however $CD4^+$ T cells never regained pretransplant levels (both control and MSC group) (21). The MSC group had fewer $CD8^+CD45RO^+CD45RA^-$ memory T cells compared to the control group by day 360 (21). They also demonstrated higher numbers of $CD4^+CD25^{high}Foxp3^+CD127^-$ Tregs than the control group (21).

Peng et al. used flow cytometry to monitor immune response post MSC treatment in renal transplantation (20). No difference was seen between treatment and control groups in numbers of $CD4^+$ T cells, $CD8^+$ T cells or NK cells before or after transplant (20). However, authors did note an increased proportion of $CD27^+$ memory B cells in the MSC treated group (20). Casiraghi et al. reported a case report of tolerance following MSC infusion in liver transplant. They noted a high Treg: memory $CD8^+$ T cells ratio, compared to control. They also demonstrated expansion in naïve and transitional B cells (26).

Regulatory Dendritic Cells (DCs)

Macedo et al. used flow cytometry for immune monitoring post allogeneic DCreg infusion in living donor liver transplant patients. They reported a decrease in $T\text{-}bet^+Eomes^+CD8^+$ T cells (both central and effector memory phenotypes) following DCreg infusion (24). Conversely there was an increase in $T\text{-}bet^-Eomes^-CD8^+$T cells of naïve phenotypes with increased $PD1^+$ and $Tim3^+$ expression (24). Furthermore there was an increase in $CD4^+CD25^+Foxp3^+$Tregs, resulting in a change to the Treg : $CD8^+$ ratio and potentially a more tolerogenic profile (24).

FUNCTIONAL ASSAYS

Functional assays have an important part to play in immune monitoring. While flow cytometry is able to provide excellent data on the phenotype of cells, functional assays provide information on what role these cells may be playing. Functional assays include measurement of cell proliferation (as an indicator of activation; an example of *in vitro* suppression test with proliferation readout is shown in **Figure 2B**), measurement of chemokines or cytokines produced by cells, or their effect on other cells (for example lysis). The limitation to these studies is that the assays often require *ex vivo* manipulation of some description that may not reliably replicate what is happening *in vivo*. Below we review the functional assays that have been used to date in clinical trials of cellular therapy.

Mathew et al. used thymidine incorporation assays to test the immunocompetence of kidney transplant patients after Treg therapy (18). They looked at recipient PBMC response to a number of antigens and mitogens including PHA, ConA and CMV (18). Immediately after transplantation responses were low, likely due to induction immunosuppression, but they were shown to gradually recover in the first year post-transplant (18). Although they did not achieve pre-transplant levels it is useful to note that there were no clinical infections recorded in this time (18). Similarly, Casiraghi et al. used mixed lymphocyte reactions to demonstrate anti-donor $CD8^+$ T cell unresponsiveness following MSC transfer in liver transplant recipients, suggestive of a more tolerant profile (26).

As well as providing analysis of peripheral blood leukocyte subsets, flow cytometry may be used to look at the proliferation profile of cells. Mudrabettu et al. labeled peripheral blood mononuclear cells (PBMCs) with carboxyfluoroscein succinimidyl ester (CFSE) prior to stimulating them with anti-CD3, anti-CD28 and IL-2. The proliferation profile of $CD4^+$ T cells could then be measured using flow cytometry on the basis of CFSE staining (27). They demonstrated an initial decrease in proliferation in both control and treatment groups, likely secondary to immunosuppression. However, by day 90 after infusion the MSC treated group had a decrease in proliferation compared to control (27). Peng et al. used the same method but

found no significant differences between control and MSC-treated groups (20).

The Pleximmune™ test (Plexision Inc, USA) is another functional assay that uses flow cytometry to quantify recipient $CD8^+CD45RO^+$ memory T cells expressing CD154 after they have been cultured for 16 hours with surrogate donor PBMCs. It was used by Sánchez-Fueyo et al. to establish the donor specific alloimmune response and demonstrated hyporesponsiveness in those who had received Treg infusion (25), suggesting movement towards a more tolerogenic phenotype. It is also important to check the function of the immune cells that are being transferred to the patients. For example, $CD137^+/CD154^-$ Tregs have been shown to be reliably associated with a stable phenotype (28). Upon a short stimulation with a relevant antigen, antigen-responsive Tregs express CD137, while antigen-responsive conventional CD4 T cells express CD154 (please see an example in **Figure 2C**). The CD137/CD154 assay has been used in the ONE Study to monitor the frequency of donor-responsive Tregs and conventional CD4 T cells.

Many studies have measured cytokine and chemokine levels in patient serum. Sanchez-Fueyo et al. used LEGENDplex™ (BioLegend) to assess cytokine and chemokine (including IL-2, -5, -12, -27 and CXCL9 and 10) levels post infusion. In one patient who concurrently had a high fever these levels were raised, but in all other patients no significant changes were found. The LEGENDplex™ is a bead-based immunoassay that captures the soluble analyte between two antibodies, before then quantifying the amount using flow cytometry (25). Roemhild et al. utilized Luminex (another bead-based immunoassay) for assessment after autologous polyclonal Treg therapy in renal transplantation, as part of the ONE study (23), there was no change in either pro- or anti-inflammatory cytokines (TNFα, IFNγ, IL-1, -6, -8 or-10. Shi et al. used ELISA to measure TGFß1 and PGE2, soluble factors released by MSCs that can modulate T and B cells. Both were increased at 4 weeks following MSC infusion (29). The Neptune study measured a number of cytokines and chemokines both before and 4 hours after MSC infusion (22). Using Biorad multiplexed assays they found that TNFα and IL-10 were both decreased following the second infusion of MSCs, a result that was maintained for the rest of the study. They did not find any significant differences in IL-4 or IFNγ (22).

Perico et al. monitored the alloimmune response to donor and third party antigen by using ELISpot assays to IFNγ, granzyme B and by cell-mediated lympholysis (21). They found that patients treated with autologous MSCs had decreased anti-donor IFNγ memory T cell and anti-donor granzyme B $CD8^+$ cell responses compared to the control group (21). They also demonstrated a decreased cytolytic response of $CD8^+$ T cells (21). Similarly, ELISpot assays have been used in the ONE Study and reported by Sawitzki et al, Roemhild et al. and Harden et al. (16, 23, 30).

The Cylex Immuknow Assay is used to test the immune competence of a patient's T cells, by measuring the ATP synthesis of $CD4^+$ T cells. Todo et al. used this as part of their immune monitoring of patients who received Treg therapy after liver transplantation (19). They were able to demonstrate results in the normal range for the majority of their participants (19).

The majority of these studies also tested for the presence of donor specific antibodies (DSAs), in particular development of *de novo* DSAs after transplantation (16, 18, 19, 22–24). There was not a significant increase in patients developing dnDSAs in these preliminary trials. This was the primary method for monitoring the humoral response. As discussed in section 2 (flow cytometry) immune monitoring also frequently included panels specifically to look at the B cell subsets over time post transplantation.

It would be interesting to know how the results of this immune monitoring correlate to clinical outcomes, however the studies discussed in these sections have all been early case reports or phase I trials, therefore focused on safety data and dose optimization.

NEXT-GENERATION TECHNOLOGIES

CyTOF

Mass spectrometry with cytometry by time of flight (CyTOF) is a key technology in multiple clinical trials where deep cellular phenotyping is important (31). In traditional flow cytometry, detection of fluorochrome-conjugated antibodies is based on wavelength in which sufficiently broad emission bands are produced. In mass cytometry, fluorescent labels are replaced with heavy metal tags that produce more narrow emission bands, as detection is based on mass. This limits signal overlap of emission spectra and facilitates an increased number of parameters to be simultaneously measured. Furthermore, these metals are not commonly found in biological specimens, reducing potential background noise (e.g. from autofluorescence) (31, 32).

CyTOF has the potential to overtake flow cytometry as the method of choice for immune monitoring (example of CyTOF data is shown in **Figure 2B**). It is possible to stain intracellularly, therefore gaining functional insights and to look at antigen specificity of T cells by using metal conjugated tetramers, as well as allowing a high number of cell surface markers to be concomitantly identified (31). Furthermore, analysis is unbiased, with the potential to uncover new insights into immune cell subsets. There are some limitations, it is a slow and currently expensive method in comparison to flow cytometry and cells are not available for further studies at the end of the workflow.

Sánchez-Fueyo et al. used CyTOF to characterize the Treg compartment in patients following autologous Treg infusion. They were able to identify the expanded Tregs by comparing phenotypes of individual clusters to those examined pretransfusion. They could then follow them over time, noting by one month they had mostly disappeared. The expanded Tregs were found to be more proliferative and have increased CD25, CTLA4, CD38, GATA3, PD1 and CD274 (25).

Similarly, data from a cohort of patients enrolled in the ONE Study examined how the phenotype of Tregs changed over time post transplantation using CyTOF. Distinctive alterations were observed in clustering associated with specific post-

transplantation changes. There were significant changes in the frequency of homing markers and $CCR7^+$ Tregs (30).

Gene Expression Profiling

Within transplantation research the focus of gene expression profiling has been to identify biomarkers of rejection or tolerance. A variety of techniques, including microarray and RT-qPCR, have been used to explore the potential mechanisms involved. However, at present gene expression profiling has not widely been used as part of the immune monitoring strategy post cellular therapy.

The potential importance of gene expression profiling in immunomonitoring has been recognized by the Banff Foundation, who created a molecular diagnostics working group to assess the available literature on this topic and plan for future research. At the latest symposium in 2019 they reached a consensus on a panel of 770 genes, to cover the innate and adaptive immune response, tolerance, rejection and infection for the monitoring of transplant patients, the Banff – Human Organ Transplant (B-HOT) panel (33). In collaboration with NanoString Technologies, this has become a commercially available panel. Of note it is possible to use with formalin fixed paraffin embedded (FFPE) samples, enabling retrospective analysis of stored samples (schematic overview of the method and an example of gene expression profiling using the B-HOT panel are shown in **Figures 3A, B**).

As previously highlighted, the goal of cellular therapy is to reduce the need for harmful immunosuppression by inducing a tolerogenic state in patients. Therefore, when monitoring these patients it may be beneficial to use already developed gene signatures of tolerance as a standard for comparison, such as that described by Sagoo et al. (34). Indeed this was utilized by Hutchinson et al. to monitor patients being treated with Mreg therapy (35). They used microarray platforms to compare gene expression in their patients to those of a known tolerant cohort, finding them to be similar (35). Furthermore they found that TOAG mRNA expression (known to be decreased in acute rejection) remained high in treated patients, supporting a phenotype more often seen in healthy or tolerant patients (35).

As these biomarker profiles of tolerance or rejection become validated, it is possible to imagine how they may be used as a control or comparator group for those patients who have undergone cell therapy, to gain further insights into how these therapies are affecting the immune response.

Spatial Biology

In recent years there have been significant developments in spatial profiling techniques, making this technology Nature's 2020 Method of the Year (36). In general terms these offer the possibility of extracting spatially-resolved molecular information from tissue biopsies. Whilst bulk sequencing techniques generate detailed readouts of gene expression, they potentially miss out on small differences on a cell-per-cell basis that may be significant when taken in context of the position in which they occur. These newer techniques offer the possibility to perform more in-depth, spatially guided molecular analyses of tissue biopsies, which may be particularly relevant when trying to understand the effect of cellular therapies. We will briefly consider a few of the available techniques below.

Nanostring GeoMx DSP

The GeoMx DSP enables spatially-resolved, high-plex (10s -10,000s) digital quantitation of proteins and mRNA in tissue. It uses photo-cleavable oligo-tags to collect samples in a non-destructive way, whilst maintaining spatial information (37). Benefits include the direct, digital counting of mRNA or protein without the need for intervening enzyme steps, the ability to use archival FFPE samples, and the advantage that samples can be used for further downstream processing even after running through the GeoMx DSP workflow (schematic overview of the method and an example of kidney biopsy analyzed using GeoMx DSP are shown in **Figures 3C, D**).

10X Genomics

This uses positional molecular barcodes in the cDNA synthesis reaction with an intact tissue section, before proceeding to generating a readout *via* RNA-seq (38). It offers the same highly spatially resolved readout as the GeoMx DSP but at present is only available for use with fresh frozen samples.

Fluidigm Hyperion

This platform works in a similar fashion to the GeoMx, but instead of oligo-tags it uses metal-conjugated antibodies, followed by laser ablation and transfer of the ablated tissue to be measured by CyTOF. It can be used with FFPE samples and can look at up to 35 different antigens at one time (39).

GE Cell Dive

This may also be used with FFPE samples or tissue microarray. After staining with dye conjugated biomarker antibodies (up to four) and collecting an image, it then uses a patented dye inactivation process to allow further staining of the same sample with different antibodies (up to 60 in total). These images are stitched together for a highly multiplexed final result (40).

Codex

This platform uses immunofluorescence technology, with an iterative workflow that uses DNA tagging technology (with capture and reporters). One of the benefits of this technology is the ability to comprehensively image the whole sample and perform unbiased cell phenotyping, rather than needed to choose regions of interest at the beginning (41).

Cell Tracking

An excellent review by Tran and Thomson covers the current state of research into the tracking of adoptively transferred cells (8). This is a significant gap in our knowledge of the mechanism of action of these cellular therapies and may provide insight into how they modulate the immune response. Ashmore-Harris et al. have reviewed in detail the principles of non-invasive cell tracking, the methods available and how they may be used to develop new cellular therapeutics (42).

Hutchinson et al. pioneered the use of Mreg therapy in two kidney transplant patients (35). A proportion of the transfused Mregs were labeled with 45 Mbq oxine, allowing them to track where the cells went (using SPECT). They noted that after initially settling in the lungs they went on to travel in the circulation to liver, spleen and bone marrow. None were seen in the urinary tract, suggested good survival.

Chandran et al. monitored the fate of polyclonal Treg infusion in kidney transplant patients by labeling a proportion of transferred Tregs with deuterium and then monitoring levels over time. They found a peak in number in the first week, with the labeled Tregs still present at 30days. Numbers had fallen below the limit of detection by 3 months (43). This technique was developed by Bluestone et al. to monitor the fate of transferred polyclonal Tregs in a phase I trial in patients with type 1 diabetes (44). They also noted maximum levels of labeled Tregs at 7-14 days. Following which deuterium-Tregs decreased, to 25% of circulating Tregs at 90 days. Levels then stabilized over the next nine months. Furthermore, T effector populations did not demonstrate evidence of deuterium labeling, suggesting the transferred Tregs were stable in their identity (44).

DISCUSSION

In this paper we have reviewed the current immunomonitoring strategies used in the early and ongoing clinical trials of cellular therapy in transplantation as well as considering methods that may be of use in the future. At present the cornerstone of monitoring relies on flow cytometric analysis of peripheral blood samples to define the leukocyte subsets present. Standardized panels have been developed to enable comparison across clinical sites with good effect. These are often used in conjunction with an array of functional studies.

It is clear that the timing of sample acquisition is an important factor in any monitoring strategy. It is known that both induction and maintenance immunosuppression may have an effect on the immune cell subsets present. Indeed, a significant initial decrease in leukocytes, with slow recovery of T cell populations was demonstrated in a number of the studies discussed in this paper. This should be accounted for both when planning trials and reporting on the results. The ONE Study had a clear protocol for when samples were taken, together with the same immunosuppression, allowing for a harmonized analysis across groups.

There are a growing number of new techniques that offer the potential to explore both the phenotype and function of the immune response to cellular therapies. In particular, we are now in an era of deep spatial profiling which allows us to directly analyze transplant biopsies – the principal sites of activity - rather than surrogate tissues such as blood. These techniques offer the exciting possibility of discovering new tissue-specific treatment targets. However, the challenge will be in the analysis of data generated from these studies, which can be vast and open to misrepresentation. It will therefore be important to develop transparent standardized bioinformatic workflows to support the analysis and cross-site comparison of these data in order to fully understand their implications.

AUTHOR CONTRIBUTIONS

All authors contributed to the content design, literature searches, writing of the manuscript, and manuscript review. All authors contributed to the article and approved the submitted version.

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