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Review Article

Tregs and Other Suppressive/Regulatory/Tolerogenic Cell Therapies in Transplantation

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Abstract:

Poor long-term graft outcome remains problematic because of the inability to prevent chronic allograft rejection. Strategies based on suppression/regulation/tolerance (3 different but similarly used concepts) of the immune system often leads to other concerns.

Keywords: treg; transplantation; rejection; cell therapy

Running title: Tregs in transplantation.

Summary:

Poor long-term graft outcome remains problematic because of the inability to prevent chronic allograft rejection. Strategies based on suppression/regulation/tolerance (3 different but similarly used concepts) of the immune system often leads to other concerns. New alternatives based on facilitating the induction of alloantigen tolerance by regulatory T cells (Tregs) and other immune-suppressor cells can restore the balance between inhibitory and effector arm. This review mainly summarizes results about the use of Tregs for the control of transplant rejection, commenting also other situations and potentially similar cell therapies.

Organ transplantation is currently a successful treatment for the majority of patients with end-stage organ failure. Fortunately, improvement in transplant technology, non-invasive biomarkers, better selection of donors and recipients by Human Leukocyte Antigen (HLA) typing/compatibility and the advance of immunosuppresive agents have enabled clear progress in transplantation outcomes ameliorating the graft survival, at least in the early post-transplant stage. However, the poor long-term graft outcome remains problematic because of the inability to prevent chronic allograft rejection (CR). In fact, half of all transplanted kidneys still fail within 15 years after transplantation[1]. In this context, the current treatment of transplantation focuses on the limitation of the effector arm of immune response with nonspecific immunosuppressive drugs (ISD) that perform by inhibiting non-specific T and B cell activation pathways or by depleting lymphocytes.

The mentioned strategy based on suppression of the immune system often leads to over immunosuppression. The lack of specificity of ISD frequently diminishes patient's quality of life and gives rise to life-threatening infection episodes, malignancies, cardiovascular diseases or kidney failure causing graft loss or even death [2]. Due to the inconveniences caused in transplanted patients by this therapeutic approach, new alternatives that allow better results are being sought. In general, suppression, regulation or tolerance induction are different terms that often are interchangeably used. Although "Suppressor" cells suggest the blockage of responses, "Regulatory" should be a more flexible concept (increase or decrease functions) but just used under the meaning of suppression, and "Tolerogenic" cells are those cells that could induce specific recognition which would program no-response, the three concepts are often used as synonymous. Facilitating the induction of alloantigen tolerance by regulatory T cells (Tregs) and other immune-suppressor cells, restoring the balance between the inhibitory and the effector arm is the aim of a lot of novel strategies based on suppressive/regulatory/tolerogenic cells. Although this review mainly summarizes results about the use of Tregs as controllers of rejection in transplantation, other situations and potential similar cell therapies are also commented.

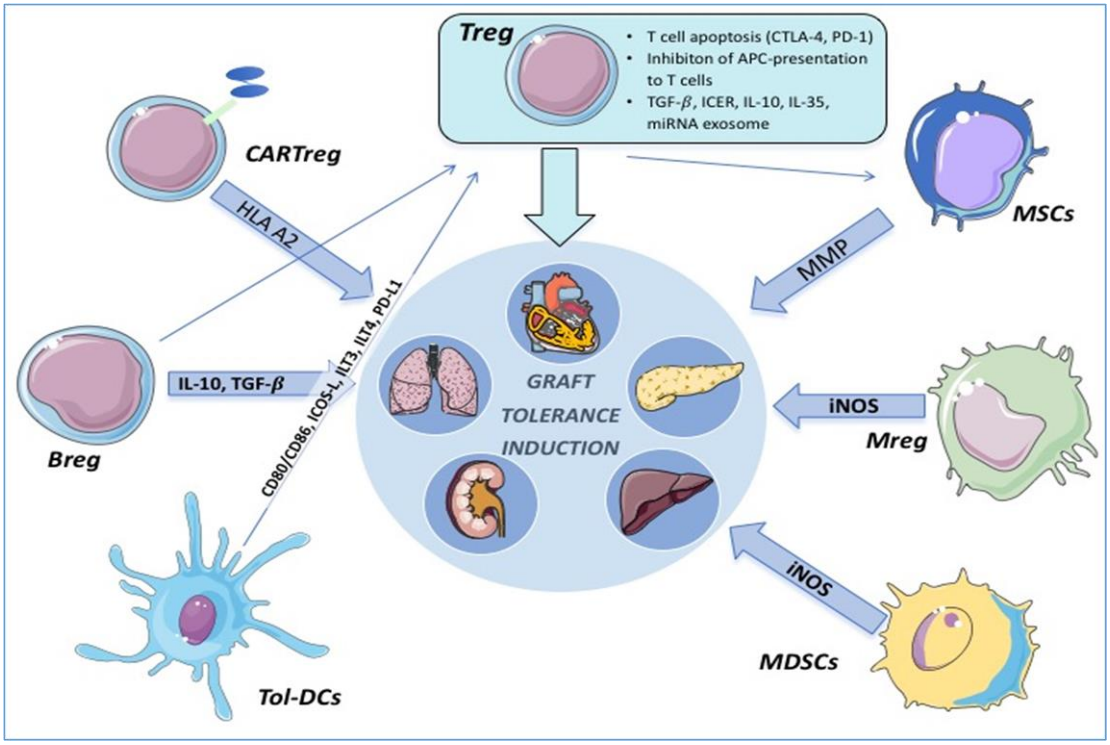

Fig 1. Mechanisms of action of suppressor/regulatory/tolerogenic cells.

Tregs: Regulatory T-cells; MSCs: Mesenchymal stromal cells; MMP: matrix metalloproteinase; Mregs: regulatory macrophages; MDSCs myeloid-derived suppressor cells; iNOs: inducible NO synthase; Tol-DCs: Tolerogenic DCs; Bregs: Regulatory B cells; CAR-Tregs: Treg cells expressing chimeric antigen receptor. Tregs induce apoptosis of alloreactive T cells via CTLA-4 and PD-1 engagement.

Besides, Tregs prevent APC's ability to activate effector T cells by CTLA-4 and LAG-3 binding. Other mechanisms such as TGF-β expression, inducible cAMP early repressor (ICER), IL-10 and miRNA exosome transference are also involved. MSCs secrete MMP types 2 and 9 facilitating the cleavage of CD25 expressed on CD4+ T cells. Both Mregs and MDSCs have immunossupressive activity in an iNOS-dependent pathway. Tol-DCs are able to induce Treg development via CD80/86, ICOS-L, ILT3, ILT4 and PD-L1 binding. Bregs can modulate immune homeostasis in an IL-10 dependent pathway or by IL-10-independent mechanisms based on IL-35 or TGF-$\boldsymbol{\beta}$. CAR-Tregs recognize specific antigens such as HLA-A2 supressing allograft rejection.

1. - General concepts about Tregs

1.1. Characterization and Ontogeny

Tregs are a subset of CD4+ T cells (comprising 1-9% of blood CD4+ T cells) whose function is to limit immune responses by maintaining self-tolerance. Tregs are traditionally classified as natural Tregs (thymus-derived), or peripheral inducible Tregs (iTregs), which are the result of natural T-cells when exposed to cytokines such as TGF-β and IL-2p[3,4]. Tregs are distinguished by the high expression of both CD4+ and CD25+ (IL-2 alpha chain Receptor) and by the transcriptional regulator Forkhead Box P3 (FOXP3)[5], which is a reliable marker specially in mouse Tregs. However, FOXP3 is also expressed in human effector T cells when activated[6] and it is required the use of other markers such as CD4+/CD25+/CD127- to characterize them. Additionally, transcription factor FOXP3 demethylation serves to preserve Treg phenotype and related epigenetic changes are now used to identify Tregs in clinical research [7].

Thymic ontogeny of Tregs starts in CD4 single-positive stage (CD4+/CD8-). Upregulation of FOXP3 and consequent differentiation of Tregs depends on a great heterogeneity of paths and cytokines ruled by environmental conditions and is strongly influenced by inflammatory cues. Antigen Presenting Cells (APCs) in thymus promote FOXP3 upregulation in these thymocytes by self-antigen-presenting in the context of self-MHC class II[8]. This event together with a satisfactory interaction with CD28 in terms of strength, duration and affinity[9] activates nuclear factor-κB (NF-κB), forkhead box protein O (FOXO) and nuclear factor of activated T cells (NFAT)[10], which is required for FOXP3 expression.

Other factors, like the presence of high concentrations of TGF-β[11], Inducible Costimulator (ICOS/ICOSL) and thymic stromal lymphopoietin are also involved[12]. Also, FOXP3 upregulation event promotes Interleukin-2 receptor alpha chain (also called CD25) surface expression allowing cytokine signalling and consequently the development of fully functional Tregs [13].

1.2. Immunosuppressive drugs and Tregs

PI3K-mTOR (mammalian target of rapamycin) signalling pathway is recognized as one of the main targets of ISD used in transplantation. mTOR is a critical signalling molecule with a crucial role in transcribing immunological cues into a specific family of T cells. Extensive studies at the molecular level of this pathway are imperative for unravelling Tregs association with immunosuppresive drugs, cancer and autoimmunity.

How mTOR regulates Treg phenotype and metabolism is not fully understood. mTOR is formed by two complexes named mTOR1(Raptor), the principal target of rapamycin (RAPA), and mTOR2 (Rictor). T cells lacking whole mTOR complex differentiate preferentially into FOXP3+ Treg rather than Th1, Th2 or Th17 effector cells [14] and expand more efficiently in the presence of IL-2 compared with normal-mTOR T cells. It has been suggested that TGF-β mediated induction of Foxp3+ regulatory cells in deficient mTOR T-cells could explain this divergence given that Tregs development is regulated by a protein named Smad3, which is more likely to be stimulated by TGF-β in mTOR-deficient Treg cells. However, mice containing Treg specific deletion of Raptor (mTOR1) lose their Treg function *in vivo* [15] and develop fatal autoimmune inflammatory state [16].

Many immunosuppressive drugs currently used base their mechanism on the mTOR pathway determining Tregs function and transplantation outcome. For instance, calcineurin inhibitors have shown a negative effect on Tregs generation and function [17] while there is substantial evidence that rapamycin favours Treg survival and function [18]. The effects of mycophenolic acid are variable [19,20] and regarding basiliximab, due to its anti-CD25 effect, may have a deleterious effect on Treg cells [21]. Nonetheless, either via mTOR or by another alternative mechanism there is a widespread observation that the percentage of circulating CD25+ CD4+ FOXP3 cells decreases after transplantation [22]. This way, the balance between immunoreactive and immunosuppressive status gets compromised concluding in the adverse events or reactions described above. That is the main reason why new approaches focusing on tolerance induction via Tregs or other promising methods such as regulatory macrophages or mixed chimerism should be considered.

2. - Tregs in transplantation

As regulatory T cells are essential for the induction and preservation of peripheral tolerance and hence for preventing graft rejection, they have been deeply studied and seriously taken into consideration as a new therapeutic tool. Data suggest that Tregs could exert a tolerant state to alloantigens *in vivo* by inducing a regulatory profile in alloreactive T cells. Before describing the therapeutic approaches by which we could take profit of Tregs, it is convenient to describe briefly the main steps where Tregs get involved suppressing allorejection to understand the multiple pathways that could be affected by manipulating these cells.

In the setting of any solid organ transplantation, donor APCs migrate to the lymph nodes and present allogeneic class I or class II MHC molecules to the recipient's CD8+ and CD4+ T cells, respectively (direct presentation). Host dendritic cells can also display and present graft alloantigens to T lymphocytes (indirect presentation) resulting in naive T cells differentiation and proliferation into effector helper T cells and cytotoxic T lymphocytes. These effector T cells migrate back into the graft and mediate cellular rejection. The usefulness of Tregs resides in their capability of regulating this rejection process in different ways.

Tregs are able to induce cytotoxic T lymphocyte apoptosis via engagement of CTLA-4 (cytotoxic T lymphocyte antigen-4) and PD1 (Programmed cell death 1), granzyme A/B, TNF related apoptosis-inducing ligand (TRAIL), FAS/FAS-ligand pathway, the galectin/TIM-3 pathway and through IL-2 deprivation. On the other hand, Treg's CTLA-4 binds with CD80/86 on APCs leading to the induction of indolamine-2,3-dioxygenase (IDO)[23,24] and LAG-3 binds with MHC-II preventing APC's ability to activate effector T cells[25]. Other mechanisms mediated by Tregs as TGF-β membrane-bound active expression, upregulation of ICER (inducible cAMP early repressor)[26] and the consequent inhibition of NFAT and IL-2 transcription by cAMP transference from Tregs to effector T cells, IL-10/IL-35/TGF-β production and miRNA exosome[27] transference are also suppressive physiological cues focused on diminishing immune response and rejection.

3. - T-immunotherapies (from Tregs to CARTregs)

Diverse therapies based on the use of immune-related cells to induce tolerance are currently undergoing clinical trials. Tolerance induction could be advantageous in different circumstances such as autoimmunity, in which control of self-reactive lymphocytes is defective, or transplantation. Even though Tregs are the cornerstone of this review, other cell strains are being considered and studied as tolerance inductors like myeloid-derived suppressor cells (MSDC), Mesenchymal Stem Cells (MSC), regulatory macrophages (Mreg), tolerogenic Dendritic Cells (Tol-DCs) or regulatory B lymphocytes (Breg).

3.1. Polyclonal Treg cells

Polyclonal Treg cells are non-antigen-specific cells (in contrast with antigen-specific Tregs we will describe later). Regulatory T cells are a well-defined subset that can be cultivated and expanded ex vivo and returned safely to patients. The low rate of Tregs in adults (less than 9% of CD4+) requires their expansion *ex vivo* before clinical use. Polyclonal expansion generates large numbers of Tregs from peripheral blood with potential use as adoptive cell therapy. First of all, cells can be sourced directly from the patient (autologous) or a third-party unrelated donor (allogeneic). The source of autologous Treg cells is limiting and current manufacturing conditions are demanding and costly. On the other hand, allogeneic Tregs offer exceptional opportunities when immune host-mediated elimination of transferred cells is overcome, allowing a durable response.

In terms of production and isolation, the best marker to characterize Treg cells is a nuclear transcription factor (FOXP3) and therefore is not suitable for isolation by flow cytometry since it is an intracellular complex. As described above, CD25 is highly expressed in most Treg cells but is transiently shared with effector T cells, so cannot be used by itself to avoid unwanted T-cells [28].

In the present day, there are different protocols for regulatory Treg production. One option is to use CD8, CD14, CD19 and CD127 negative selection to discard non-CD4 T-cells followed by CD25 positive selection[28]. Instead of selection, Treg induction protocol is based on FOXP3 expression promoters (IL-2, TGF-β activation and use of mTOR inhibitors). By using mentioned promoters together with TCR activation we could selectively stimulate Treg development[28]. Once we have selected/induced Treg subset, expansion and proliferation is required; IL-2 is used as a growth factor promoting expansion and survival of Tregs previously isolated [29].

Clinical trials to determine the safety and stability of this cell therapy have been carried out. In solid organ transplantation, the ONE study (NCT02129881) has shown that Treg cells can be grown and are safe for administration to transplant recipients in a dose-escalating approach from $0.5\text{-}3.0\text{x}10^6$ cells/kg. There is an attractive argument for combining Treg with rapamycin (RAPA) monotherapy, since rapamycin may facilitate the survival of Tregs. Starting from ONE study, the so-called TWO study (MR/N027930/1), which started in 2017 and will end in 2023, aims to elucidate if nTreg can actually control rejection. For this purpose, 34 renal transplant recipients will be recruited over three years and each receptor will be treated with conventional immunosuppressive drugs. However, after transplant, cellular therapy of Treg isolated from their own blood (autologous) will be administered. Then, the immunosuppressive drug dose will be reduced while renal function

monitoring is carried out. Thus, evidence of nTreg role in protecting grafts from damage could be tested[30].

3.2 Antigen-specific Treg therapies

Efficacy of antigen-specific Tregs should be higher than polyclonal Tregs [31,32] but their expansion is challenging due to low precursor rates. Some studies suggest that these alloantigen-expanded Tregs are 100-fold more potent at suppressing alloantigen-stimulated proliferation *in vitro* than polyclonal Tregs [33]. Different approaches to obtain antigen-specific Tregs should be taken into account: 1) purified antigen-specific Tregs; 2) specific TCR transduction; 3) CAR Tregs, in which the CAR (Chimeric Antigen Receptor) recognizes specific targets; and 4) specific effector T cells reconverted into Treg cells by FOXP3 overexpression.

3.2.1. Purified antigen-specific Tregs

The frequency of direct allo-reactive Tregs (darTregs) has been estimated to be between 1% and 10% [33]. Proof-of-principle researches have shown that antigen-specific Tregs can be cultured and expanded using donor APCs such as DCs, B lymphocytes[34] and mononuclear cells. *Qizhi et al*[35] group estimated that 5×10^9 polyclonal Tregs would be necessary to induce tolerance when combined with 90% deletion of endogenous T cells while even just 150×10^6 darTregs would be enough to achieve similar efficacy. When alloantigen was presented directly, the precursor frequency of darTregs in normal individuals was 1.02% but when alloantigen was presented indirectly (MHC-matched), the frequency of specific Tregs was approximately 100-fold less[33]. Isolated Tregs were expanded with APCs, rapamycin, IL-2, and IL-15 resulting in Tregs that were capable of selectively suppress responses to specific alloantigen. Clinical trials in transplantation are currently ongoing: for example, the National Institute of Allergy and Infectious Diseases (NIAID) is performing a study (NCT02188719) in liver transplantation by administering different doses (from $50x10^6$ to 800 $x10^6$) of darTregs previously exposed to cells from the liver donor; promoters expect that by this Treg therapy approach, immunosuppressive drugs could be reduced, or even withdrawn, without liver rejecting.

3.2.2. TCR transduction in Tregs

Through the transduction of a TCR that specifically recognizes the desired antigen, it is possible to obtain antigen-specific 'artificial' Treg cells. Engineered TCR has been examined in preclinical models in transplantation as well as in Type 1 Diabetes (T1D), colitis, rheumatoid arthritis or multiple sclerosis. Concerning transplantation trials, *Tsang et al*[36] explored whether mouse Tregs specific for allogeneic MHC molecules could be generated *in vitro*: Tregs were retrovirally transduced with TCR genes conferring specificity for MHC class II molecules presented by host APCs (via indirect recognition). Results show that TCR-transduced Tregs induced long-term survival of partially MHC-mismatched heart grafts when combined with short-term adjunctive immunosuppression, suggesting that Tregs specific for allogeneic MHC class II molecules are effective in promoting transplantation.

Considerable efforts have been made in other immune-mediated diseases to elucidate the feasibility of applying TCR-engineered Tregs adoptive therapy. For example, *Hull et al* [37] demonstrated the potential of TCR lentiviral-mediated gene transfer to develop islet-specificity on polyclonal human Tregs as a potential tool in T1D. Also, *Kim YC et al*[38] reported the outcomes of engineered factor VIII-specific Tregs obtained by TCR transduction, that efficiently suppress proliferation and cytokine release of FVIII-specific T-effector cells. Similarly, isolation of recombinant T-cell from a myelin-basic protein-specific T-cell clone of a multiple sclerosis patient and posterior TCR expression in human Tregs resulted in suppression of MBP-specific T effector cells[39].

4. Genetically engineered T-cells

T cells genetically engineered to express chimeric antigen receptors (CARs) are a new and revolutionary promising antitumoral immunotherapy especially in hematologic malignancies[40,41,42,43]. Two are the main proposals to induce suppressor/regulatory T-cells: CAR-Tregs and reconverted specific T-cells.

4.1 CAR-Treg

CARs are recombinant antigen receptors composed of an extracellular region of antigen recognition and intracellular regions that activate T cells. The antigen binding domain is usually a single chain variable region (scFv) from a monoclonal antibody and the intracellular domains are composed mainly by CD3ζ T-cell receptor next to other signalling domains, most commonly from CD28 or 4-1BB [44-47]. The major advantage of using a CAR instead of TCR-engineered cells is their ability to recognize surface antigen allowing to bypass HLA-I restriction[48].

Initial proof-of-concept studies in murine models of colitis with Tregs cells expressing CARs showed that they can be redirected and accumulated to the site where antigen is expressed and suppress effector T-cells[49]. However, the interest of regulatory T-cells in the context of solid organ transplantation is focused on redirecting these cells to donor HLA antigens. Recently, *MacDonald et al*. generated a CAR-Treg targeting the HLA-A2 antigen, the most common mismatch in transplantation, and demonstrated the capacity of preventing Graft Versus Host Disease (GVHD) in skin xenograft model[50]. Further studies showed also the capacity of similar CAR-Treg targeting the HLA-A2 antigen to suppress skin allograft rejection where the alloimmune-mediated response against HLA-A2+ skin allografts were inhibited, and the long persistence of the genetically engineered cells within the graft [51-53].

4.2 Reconverted specific-effector T cells

By forcing the expression of FOXP3 in CD4+ T cells, some research groups have aimed to reconvert antigen-specific CD4+ cells into Tregs-like cells by lentiviral transduction. It has been tested in patients with immune-related diseases such as IPEX syndrome[54], caused by FoxP3 deficiency, or rheumatoid arthritis[55] establishing an effective way to work with adoptive cell therapy using genetically engineered Tregs in patients with immune disorders of different origins.

5. No-T-immunotherapies

5.1 DCs-driven Tregs

Tregs can be induced or expanded by tolerogenic DCs (tol-DCs). *Banerjee et al* found that human myeloid-derived dendritic cells are more efficient than other APCs for the maintenance of Tregs in culture[56]. Coculture of tolDCs with autologous T cells leads to an increase in both the number of Tregs, as well as the expression of FOXP3 protein per cell both in healthy donors and myeloma patients. TolDC-mediated expansion of FOXP3high Treg is enhanced by endogenous IL-2.

TolDCs can be generated, for example, by exposing DCs to IL-4 and retinoic acid, dexamethasone or IL-10 and TGF-β. DCs are known to mediate Treg generation via several surface molecules, including CD80/CD86, ICOS-L, ILT3, and ILT4 and PD-L1 or PD-L2 [57]. In transplantation models, the induction of CD4+CD25+FOXP3 Treg has been showed by several groups. For example, the injection in a murine model of syngeneic Rapamycin-DCs pulsed with donor antigens induced

tolerance to heart allograft via CD4+CD25+FOXP3 Treg induction [58]. Also, recent studies in pancreatic islet allograft transplantation demonstrated that CD4+CD25+FOXP3hi Treg were increased in spleen, lymph nodes and graft of mice treated with autologous TolDCs and anti-CD3 [59].

5.2. Other suppressor/regulatory/tolerogenic cells:

- Mesenchymal stromal cells

Mesenchymal stromal cells (MSCs) have been proposed as an alternative strategy in transplantation; MSCs affect immunologic, inflammatory, vascular, and regenerative pathways with beneficial immunomodulatory and regenerative effects, making MSC-based therapy one of the most promising tolerance-promoting cell therapies in solid organ transplantation. One of the mechanisms suggested is based on the secretion of matrix metalloproteinase (MMP) 2 and MMP9 facilitating the cleavage of CD25 expressed on CD4+ T cells and inhibiting alloantigen driven proliferation preventing islet allograft rejection[60]. In animal models of transplantation, MSCs promote donor-specific tolerance through the generation of Tregs and APCs. In some settings, however, MSCs can acquire proinflammatory properties and contribute to allograft dysfunction. The available data from small clinical studies suggest that cell infusion in kidney transplant recipients is safe and well tolerated at a dose of $1–2\times10^6$ cells/kg[61].

Currently, ongoing clinical trials are trying to test if MSCs are able to promote tolerance and to improve graft survival with minimization of immunosuppression obtaining controversial results, probably because the characterization of these MSCs is unspecific and several cells can be used: while some preclinical studies with allogeneic MSCs, showed a precipitated graft rejection after their administration[62], other published studies support the clinical applicability of MSCs in transplantation by the induction of allograft-specific tolerance when administered in combination with rapamycin[63], cyclosporine [64] or mycophenolate mofetil[65].

- Myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSCs) are innate cells that act as a key factor regulating immune responses in many pathological situations associated with chronic inflammation. In recent years, substantial evidence supports a critical role of MDSCs in immune suppression in tumoral progression[66] and several transplantation research groups focused their works on MDSCs to induce graft tolerance. In fact due to the current complications of adoptive transfer of MSDCs, researchers are mainly working on MDSCs induction by M-CSF and TNF-alpha [67]. Monocytic MDSCs obtained have powerful immunosuppressive activity in an iNOS-dependent pathway, being able to promote immune tolerance to donor antigens in a murine skin transplant model [67].

- Regulatory B cells

B cells in transplantation have long been considered merely to serve as precursors of plasma cells, which produce alloantibodies and promote antibody-mediated rejection. However, a special subset of B lymphocytes may be useful to achieve immune tolerance in transplantation: regulatory B cells (Bregs). The main role of these Bregs is to negatively regulate the immune system and maintain immunological homeostasis by IL-10 dependent mechanism [68] or by other alternative ways, the so called IL-10-independent mechanisms, based on IL-35[69], TGF-β[70], Fas-L[71], and PD-L1[72] signalling.

Evidence regarding the critical role of Bregs in transplantation tolerance has been found comparing patients with stable graft function without clinical features of CR in the absence of any immunosuppressive drugs for >1 year, versus stable patients under immunosuppression[73]. Peripheral blood phenotype showed that these tolerant patients had a higher ratio of B cells displaying inhibitory signals (including decreased FcγRIIA/FcγRIIB ratio, an increased number of B-cells expressing CD1d and CD5 and an increase in TACI expression)[74]. Contrary to Tregs, there is no clinical trial using Bregs, although it has been proven effective in some animal models; the main concerns for their use arrive for the lack of knowledge on Bregs induction, expansion, maintenance, and function [73].

- Regulatory macrophages

Murine monocytes exposed to IFNγ and macrophage colony-stimulating factor (M-CSF) resulted in a novel-phenotype suppressor cell, regulatory macrophages (Mregs)[75]. Mregs express surface markers that differ from M0, M1 or M2 phenotype and suppress T cells in an allospecific way by oxide synthase (iNOS)-dependent mechanism. The capacity of allograft rejection prevention by Mregs has been evaluated, for example, in a heterotopic heart transplant model using unconditioned, fully allogeneic, non-immunosuppressed recipients. In this study, a single intravenous administration of 5×10^6 donor-strain Mregs before transplantation significantly prolonged allograft survival.

Another research group[76] infused $7.5\ x10^8$ viable donor-derived Mregs to two living-donor renal transplant recipients: despite the minimization to low-dose (under 2ng/mL) tacrolimus monotherapy, both patients displayed a stable renal function with creatinine levels under 2.5 mg/dl after 7 and 4 years after transplantation.

6.-Conclusion

In summary, new cell immunotherapies are appearing as options for control rejection in transplantation; probably the use of Tregs seems to be most promising, although other similar cell therapies are arriving to boost this option. The promise of a durable tolerance without unwanted immunosuppression is now a clear possibility in the near future.

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