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REVIEW

T- and B-cell therapy in solid organ transplantation: current evidence and future expectations

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SUMMARY

Cell therapy has emerged as an attractive therapeutic option in organ transplantation. During the last decade, the therapeutic potency of Treg immunotherapy has been shown in various preclinical animal models and safety was demonstrated in first clinical trials. However, there are still critical open questions regarding specificity, survival, and migration to the target tissue so the best Treg population for infusion into patients is still under debate. Recent advances in CAR technology hold the promise for Treg-functional superiority. Another exciting strategy is the generation of B-cell antibody receptor (BAR) Treg/cytotoxic T cells to specifically regulate or deplete alloreactive memory B cells. Finally, B cells are also capable of immune regulation, making them promising candidates for immunomodulatory therapeutic strategies. This article summarizes available literature on cell-based innovative therapeutic approaches aiming at modulating alloimmune response for transplantation. Crucial areas of investigation that need a joined effort of the transplant community for moving the field toward successful achievement of tolerance are highlighted.

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Introduction

Organ transplantation has become the treatment of choice for most end-stage organ diseases. Since the achievement of valuable histocompatibility is not always feasible for multiple reasons, the complex task of immunosuppressive therapy in organ transplantation is a moving target. The high level of preoperative immunological mismatch in some transplants (i.e., heart and lung transplantation), in which clinical urgency's variables lead the allocation, drives the need for a greater burden of immunosuppressive therapies with all the related complications. In such a scenario, the possibility of inducing tolerance, even also temporary, represents an evident unmet clinical need. Many different strategies have been entrusted to immunomodulate more than immunosuppress, but there is the need to collect more data to bring such immunomodulation tools in the clinical field. Indeed, the massive activation of the recipient immune system against the foreign organ requires the life-long use of potent immunosuppressive drugs. These therapies have allowed optimal short-term graft survival but their side effects and the global immunosuppression expose the patients to unacceptable risks of life-threatening complications. In addition, the current therapeutics often fail to effectively prevent chronic rejection, which remains the leading cause of graft loss.

The establishment of donor-specific immunological tolerance to minimize or even eliminate the need of life-long immunosuppressive regimen remains the major objective of the transplant community.

It is now well established that transplantation of a major histocompatibility complex-incompatible organ triggers the activation of both anti-graft effector T cells as well as graft-protective regulatory T cells (Tregs) [1]. The same paradigm applies to B cells, which can show opposing activity in the alloimmune response from effector antibody-producing cells to cells with regulatory properties. Being able to skewing the balance of such opposing subsets toward regulation would drive the immune response toward tolerance rather than rejection. To this aim, several groups are developing adoptive Treg-based cellular therapy to regulate alloimmunity and promote tolerance. On the same line, though the role of B cells is less well defined than that of Tregs, strategies to expand B cells with immunoregulatory properties *in vivo* or *ex vivo* are matter of intense investigation.

Nowadays, despite the very low number of Tregs in the peripheral blood, GMP-compliant procedures for *ex vivo* expansion of polyclonal Tregs have been successfully developed and early phase 1 studies have shown the safety and feasibility of the infusion of a large number of Tregs [2–4].

Considering the preclinical evidence that antigen-specific Tregs are significantly more effective than polyclonal Tregs, several groups are building up protocols for expanding alloantigen-specific Tregs via stimulation with donor cells. However, they are facing major hurdles, such as the inefficiency of the expansion process. To overcome these barriers, genetic engineering technology is being employed to redirect specificity of therapeutic Tregs, possibly promoting also B cells with immunoregulatory properties [5]. The same technology is being applied to redirect cytotoxic T cells toward effector B cells as a strategy for depleting antibody-producer B cells.

Here, we summarized the completed and ongoing clinical studies with Treg cell therapy in solid organ transplantation, emphasizing major obstacles encountered by these strategies. We then provide an overview of the T-cell engineering approaches under development for tailoring antigen specificity of Tregs in order to promote tolerance and to control humoral alloimmune response. Finally, we highlight the emerging role of B cells with regulatory properties as a potential future cell therapy and the remaining outstanding questions that need to be solved to advance the field.

Polyclonal and donor-alloantigen-activated regulatory T cells

Regulatory T cells (Tregs) are a subset of $CD4^+$ T cells responsible of the maintenance of immune homeostasis and self-tolerance [6–8]. Human Tregs in the peripheral circulation are currently identified by the surface expression of a high level of the IL-2 receptor chain CD25 and low level of the IL-7 receptor (CD127) and by the intracellular expression of the specific transcription factor forkhead box P3 (Foxp3) [9,10]. Circulating Tregs are divided into two mains subpopulations: thymic Tregs or peripherally induced Tregs [11,12]. Thymic Tregs express Foxp3 constitutively and develop in the thymus through the recognition of self-antigen during their maturation. Peripheral Tregs develop in peripheral tissue from conventional $Foxp3^-$ T cells in response to non-self-antigens under pro-tolerogenic conditions. Therefore, the TCR repertoire of thymic Tregs is directed toward self-antigens while TCRs of peripheral Tregs recognize non-self-antigens with high affinity. The role of Tregs in inhibiting anti-graft effector T cells and in promoting tolerance toward solid organ transplant has been well established [13]. Studies using humanized mouse models have provided the additional evidence that the transfer of *ex vivo* expanded Tregs can control acute [14,15] and chronic [16] graft rejection, and laid the foundations for Treg-based therapy in clinical transplantation.

Several clinical trials involving *ex vivo* expansion of autologous Tregs and re-infusion into kidney and liver organ transplant patients have been performed worldwide (Table 1) or are currently underway (Table 2). Polyclonal Tregs have been administered as a single injection of $0.5–10 \times 10^6$/kg to kidney transplant patients with subclinical rejection [17], to living-donor kidney transplant recipients 2 months post-transplant after induction therapy with alemtuzumab [18] or to kidney transplant recipients not given induction therapy, five [19] or seven [20] days after transplant. A subset of patients of the two latter studies [19,20], belonging to the ONE study trial [21], had successfully withdrawn mycophenolate mofetil (MMF) and are currently on stable tacrolimus monotherapy. Polyclonal Tregs have been administered also to liver transplant recipients 3 or 6–12 months after transplant [22] even though the applicability and feasibility of the procedure were found to be poor. A non-GMP cell product enriched in donor-reactive Tregs has been given to splenectomized liver transplant patients after induction therapy with cyclophosphamide [23]. Immunosuppressive drugs were discontinued in 7 out of the 10 patients, documenting for the first time the possibility to taper immunosuppression after Treg-based cellular therapy. However, the cell product contained only 3–17% of Tregs, clouding any attribution of immunoregulation to Tregs. Overall, these phase 1 clinical studies using polyclonal Tregs showed a high safety profile of the procedure and a trend toward the possibility to safely minimize immunosuppression and reduce the incidence of opportunistic infections.

Despite the heterogeneity between them, these trials have also provided the feasibility of expanding Tregs *ex vivo* in GMP conditions to achieve the target clinical doses ($300–800 \times 10^6$ total Treg dose) from end-stage kidney disease and immunosuppressed transplant recipients. The protocols involved purification of Tregs through cell sorting technology by FACS- [17,24] or via magnetic beads (MACS)- [18–20,22,25,26] cell sorting using different panel of Treg markers such as $CD4^+CD25^+CD127^{low}$ [17,24] or of $CD4^+CD25^+$ [18–20,22,25,26] T cells. Purified cells were expanded by strong TCR stimulation (anti-CD3/anti-CD28 mAb-coated beads) and high-dose IL-2 alone [17,24] or with rapamycin [19,20,22,24–26] with the addition of TGFβ [18]. Expanded Tregs showed high expression of Foxp3 with stable demethylation in the promoter region, maintained TCR diversity, and exerted potent suppressive function *in vitro*. Tracking and *ex vivo* FACS analysis showed a transient increased in peripheral Tregs in treated patients [17–20]. Overall, feared complications such as over-immunosuppression, malignancies, or infections during the relatively long follow-up (2–3 years) have not been observed after the infusion of polyclonal Tregs. Also the conversion of Tregs into anti-graft effector T cells—a concern documented in non-human primates (NHP) given a high number of polyclonal Tregs [27]—did not occur in kidney and liver transplant recipients. These encouraging findings have allowed the advancement of Treg therapy to ongoing phase II clinical trials (Table 2).

However, GMP manufacturing of donor-alloantigen activated Tregs (darTregs) remains a major concern. Less encouraging appears to be the results on the feasibility of manufacturing donor-alloantigen-activated Tregs (darTregs). This approach is based on data demonstrating that donor-reactive Tregs inhibit the alloreactive T cells response better than polyclonal Tregs and requiring a lower number of cells [28,29]. To obtain the darTreg product, purified Tregs are *ex vivo* co-cultured with peripheral blood mononuclear cells form the donor organ under costimulation blockade with belatacept [30] or are stimulated with donor B

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Table 1. Published studies with Treg therapy in kidney and liver transplantation.

Authors, trial #	Title	Treg product, dose, timing of injection and number of patients	IS regimen	Main results
Chandran S NCT 02088931 (phase I)	Treg adoptive therapy for subclinical inflammation in kidney transplantation (TASKp)	Polyclonal Tregs 320 × 10^6 total dose, month 6, n = 3 patients	NA	Safe and feasible One patient developed acute cellular rejection Infused Tregs remained detectable for 1 month
Mathew JM NCT 02145325 (phase I)	Trial of adoptive immunotherapy with TRACT to prevent rejection in living donor kidney transplant recipients (TRACT)	Polyclonal Tregs day +60 0.5 × 10^6, 1 × 10^6, 5 × 10^6, n = 3 patients each dose	Induction: alemtuzumab Maintenance: TAC (converted to sirolimus from month 2) + MMF FU: 1 year	Safe and feasible Increased circulating levels of Tregs for the 1-year follow-up
Harden PN NCT02129881 (phase I)	The ONE study UK Treg trial (ONETreg1) Living-donor kidney tx	Polyclonal Tregs day +5 1 × 10^6; 3 × 10^6; 6 × 10^6; 10 × 10^6, n = 3 patients each dose	Induction: none Maintenance: MMF and TAC FU: 2 years	Safe and feasible MMF withdrawn and on TAC monotherapy (n = 4 patients) Lower incidence of opportunistic infections Transient increase in Treg cell number
Roemhild A NCT02371434 (phasel/IIa)	The ONE study nTreg trial (ONEnTreg13) Living-donor kidney tx	Polyclonal Tregs day +7 0.5 × 10^6; 1 × 10^6; 2.5 –3 × 10^6 n = 3 patients each dose	Induction: none Maintenance: MMF and TAC FU: 3 years	Safe and feasible IS tapering to low-dose tacrolimus (n = 8 patients) Transient increase in Treg cell number and post-transplant oligoclonality
Sanchez Fueyo (phase I) NCT02166177	Safety and efficacy study of regulatory T cell therapy in liver transplant patients (ThRIL)	Polyclonal Tregs month + 3 0.5–1 × 10^6/kg (n = 3) months +6–12 3–4.5 × 10^6/kg (n = 6)	Induction: thymoglobulin (only in low-dose cohort) + steroids Maintenance: TAC and rapamycin FU: 6–12 months	Safe (1 dose limiting toxicity episode with the high dose) Low applicability of the earlier injection Transient increase in Treg frequency
Todo et al.	Living-donor liver transplantation	Donor-reactive Treg-enriched cell product day +13 0.23–6.37 × 10^6 Tregs/kg (n = 10)	Induction: splenectomy and cyclophosphamide Maintenance: MMF and CNI	7/10 patients are IS drug free for >6 years

FU, follow-up; IS, immunosuppression; MMF, mycophenolate mofetil; TAC, tacrolimus; Treg, regulatory T cells.

cells previously activated with K562 cells expressing human CD40L and subsequently exposed to polyclonal restimulation [31]. The feasibility and safety of darTregs have been analyzed in pooled data of the regulatory cell-based medicinal product (CBMPs) arm in the ONE study trial [21]. This ONE study patient group received one of six CBMPs involving Tregs, DCs, or macrophages. The results of the cell therapy group revealed lower infections and lower requirements of immunosuppressive therapy over a 60-week follow-up period in comparison with the reference group trial administered standard-of-care immunosuppression. However, according to the results from each single trial available on the CORDIS website (cordis.europa.eu/project/id/260687/reporting), one of the two patients of the DART trial using B-cell-expanded darTregs showed signs of acute rejection shortly after infusion, and the trial has been suspended (Table 2). The challenges in

Table 2. Ongoing clinical studies with Treg therapy in kidney and liver transplantation.

Trial #	Title	Aim of the study	Phase	Status
NCT02244801 The ONE study – DART	Donor-alloantigen-reactive regulatory T cell (darTreg) therapy in renal transplantation	To evaluate the safety, and tolerability of darTreg infusion (300 million, 900 million) for adult, de novo, living-donor renal transplant recipients darTregs: Treg stimulated with donor B cells that had been activated with K562 cells expressing hCD40L	Phase I	Completed (6 participants)
NCT02091232 The ONE study	Infusion of T-regulatory cells in kidney transplant recipients	To examine in living-donor renal transplant recipients, the safety and feasibility of administering T regulatory cells derived from recipient PBMC stimulated with kidney donor PBMC in the presence of costimulatory blockade with belatacept	Phase I	Completed (5 participants)
NCT02711826 TASK	Treg therapy in subclinical inflammation in kidney transplantation	To determine the safety and efficacy of a single dose of autologous polyclonal Tregs in renal transplant recipients with subclinical inflammation in the 3–7 months post-transplant allograft protocol biopsy compared to control patients treated with CNI-based immunosuppression	Phase I/II	Recruiting
NCT02188719 deLTa	Donor-alloantigen-reactive regulatory T cell (darTregs) in liver transplantation	Safety of receiving one of three different doses of donor-alloantigen-reactive regulatory T cells (darTregs) while taking this specific combination of drugs	Phase I	Terminated
NCT02474199 ARTEMIS	Donor alloantigen-reactive Tregs (darTregs) for calcineurin inhibitor reduction	Safety of donor alloantigen-reactive Tregs (target dose of 400 $\times$ 10^6) to facilitate minimization and/or discontinuation of immunosuppression in adult liver transplant recipients	Phase I/II	Completed
NCT03577431 LITTMUS-MGH	Liver transplantation with Tregs at MGH	A drug withdrawal study of alloantigen-specific Tregs (target dose 2.5–125 $\times$ 10^6 total cells) in liver transplantation arTreg: alloantigen-reactive T regulatory cells under costimulatory blockade	Phase I/II	Recruiting
NCT03654040 LITTMUS-UCSF	Liver transplantation with Tregs at UCSF	A drug withdrawal study of alloantigen-specific Tregs (target dose 90–500 $\times$ 10^6 total cells) in liver transplantation arTreg: alloantigen-reactive T regulatory cells under costimulatory blockade	Phase I/II	Recruiting
ISRCTN11038572 TWO study	The TWO study: transplantation without over-immunosuppression	The TWO study aims to demonstrate the efficacy of polyclonal regulatory T cells, with the goal of allowing reduction of immunosuppression to a single drug by 6 months post-transplantation	Phase IIb	Recruiting
NCT03867617	Cell therapy for immunomodulation in kidney transplantation	This study investigates treatment with recipient regulatory T cells and donor bone marrow together with tocilizumab for immunomodulation in living-donor kidney transplant recipients	Phase I/IIa	Recruiting
NCT03943238	TLI, ATG & hematopoietic stem cell transplantation and recipient Tregs therapy in living donor kidney transplantation	This study will determine whether a preparatory regimen including total lymphoid irradiation (TLI), anti-thymocyte globulin (ATG), and infusion of the donor hematopoietic stem cells when given along with recipient regulatory T cells (Tregs) will allow for eventual discontinuation of anti-rejection drugs after living-donor kidney transplantation	Phase I	Recruiting

Table 2. Continued.

Trial #	Title	Aim of the study	Phase	Status
NCT03284242	A pilot study using autologous regulatory T cell infusion Zortress (Everolimus) in renal transplant recipients	This study is investigating/evaluating the safety and effectiveness of collecting, expanding, and infusing polyclonal Treg cells to renal transplant recipients who are using Zortress (Everolimus) as immunosuppressive therapy	N/A	Recruiting
NCT04817774	Safety and tolerability study of chimeric antigen receptor Treg cell therapy in living donor renal transplant recipients	A multicenter, first-in-human, open-label, single ascending dose, dose-ranging study of autologous chimeric antigen receptor Tregs (CAR Tregs) in HLA-A2 mismatched living-donor kidney transplant recipients	Phase I/II	Recruiting

manufacturing donor B-cell-expanded darTregs led also to the termination of Delta study after the enrollment of patients of the low-dose cohort (50×10^6; www.ClinicalTrail.gov NCT02188719). The MGH trial with darTregs expanded by donor cells and belatacept was able to infuse three patients without adverse events, but the trial has been suspended to improve the darTreg manufacturing protocol, as well. Due to similar manufacturing difficulties, the TASK trial has been updated and it does no longer foresee the arm with donor-specific Tregs as originally conceived and the study will assess the efficacy of polyclonal Tregs in kidney transplant patients developing graft inflammation (ClinicalTrials.gov NCT02711826). Finally, according to the last update of the ARTEMIS study, darTregs were successfully obtained for only 5 out of the 10 enrolled patients. These patients initiated IS withdrawal, but it appears that most of them experienced graft rejection (ClinicalTrials.gov NCT02474199). The complete results of darTregs studies are not yet published, but overall, it appears that the manufacturing of darTregs is more challenging than polyclonal Tregs with apparently less encouraging results regarding their efficacy. A suggestion of lack of efficiency of darTregs comes from a very recent report in a NHP cardiac transplant model. DarTregs expanded in vitro with donor B cells did not prolong graft survival when given as multiple infusions (range 20–120 $\times$ 10^6/kg each dose) either early (within 2 weeks) or late (6–8 weeks) after transplantation. Infused Tregs declined rapidly in the circulation and in secondary lymphoid and non-lymphoid organs, and downregulate the expression of Foxp3 and CTLA4Ig as well as the anti-apoptotic molecule Bcl2, suggesting that *ex vivo* expanded darTregs may lose regulatory signature and survival capacity when reinfused *in vivo* [32].

Considering the very recent pieces of evidence, it appears that the development of Treg-based therapy is facing more hurdles than initially expected.

From a basic science perspective, among the several questions that remain to be answered (i.e., dose, timing, concomitant immunosuppression) two main areas of Treg-research should be investigated:

1. Polyclonal Tregs seem to be a more feasible approach than darTreg. Nevertheless, this does not ensure that Tregs with the correct features are injected into the patients. Tregs should be able to survive in the new microenvironment and to maintain their identity. Tregs should exhibit cytokine, migratory, and transcription factor programs to allow them to traffic to lymphoid organs and to the graft along with their target effector T cells [33,34]. Therapeutic

cells should also maintain their suppressive functions robust enough in the most severe inflammatory environment. Hence, the challenge here is to enhance Treg survival, homing, and stability and to foster their effector program once reintroduced in the patient circulation.

2. The TCR repertoire of Tregs is different to that of conventional T cells but a fraction of Tregs is expected to recognize donor alloantigens through the direct pathway of antigen presentation, as well [35]. The concern is whether Tregs with a direct alloantigen specificity are strong enough, as a single agent, to control the activation of the large pool of alloantigen-reactive T cells with direct and indirect specificity. Tregs exert their regulatory effects via multiple mechanisms such as release of soluble factors, direct killing or disruption of metabolic pathways of target cells, and competition for growth factors. In addition, Tregs control the crosstalk between APC and T cells via inhibitory receptors and by affecting the APC antigen-presenting capacity [7,32,33]. The two remarkable and unique features of Treg mechanisms are bystander suppression [36] and infectious tolerance [37–39]. By exerting bystander suppression and infectious tolerance, Tregs with direct specificity could promote the emergence of peripherally induced Tregs with indirect specificity later on, which could contribute in the maintenance of original Treg-induced tolerance. Implementing these mechanisms of immune regulation would assure the durability of tolerance which can adapt to the dynamic and evolving post-transplant immune response.

New insight into the mechanisms of tolerance and function of Tregs as well as into the cellular network involved in the immunoregulation could lead to the development of new strategies to increase the efficacy of the Treg therapy and to delivering an optimal Treg product for the successful achievement of tolerance induction and maintenance.

T-cell engineering approaches for redirecting antigen specificity

Chimeric antigen receptor-Tregs

An attractive alternative to the difficult expansion of natural-occurring antigen-specific Tregs is genetic modification of polyclonal Tregs to redirect their specificity. Chimeric antigen receptors (CARs) are surface molecules genetically engineered to target a specific antigens and activate downstream signaling. CAR-engineered immune cells have been developed almost three decades ago as a novel therapy for cancers [40]. These chimeric fusion proteins consist of (T) cell stimulatory and costimulatory intracellular domains fused to a monoclonal antibody extracellular domains (scFV single chain). CAR T cells have demonstrated superior T effector efficacy in preclinical cancer models and clinical studies of hematologic malignancies [41], as they are able to recognize antigens without the need for presentation in context of MHC, which is also the major advancement over TCR transgenic Tregs.

Recent progress in the CAR technology in the context of cancer therapy led to a better understanding and development of more precise and potent CAR constructs. "First-generation" CARs, composed of only a single CD3ζ intracellular signaling domain, led to only suboptimal T-cell activation and insufficient proliferation; therefore, "second-generation" CARs contained an additional intracellular costimulatory domain, leading to a breakthrough in cancer immunotherapy [42].

The application of these rather "basic" CAR Tregs is currently being investigated in the treatment of autoimmune diseases, GVHD, and transplant rejection. The development of the third- and fourth-generation (also known as TRUCK) CAR constructs, also including various additional costimulatory domains and co-expression of cytokines or transcription factors, would likely maximize the potential of CAR Treg immunotherapies to suit the individual disease [5] (Figure 1). Details about design and important functional findings in CAR Treg engineering are reviewed in [5].

It has been shown that generation of allo-specific human CAR Tregs targeting the HLA-A2 molecule is possible; moreover, these CAR Tregs maintained their expected phenotype and suppressive function *in vitro* and have been shown to prevent xenogeneic GVHD in a humanized murine HSCT model *in vivo* [43]. Additional studies have proven the efficacy and stability of suppressive phenotype of 2,4,6-trinitrophenol-specific CAR Tregs in colitis and of HLA-A2-specific CAR Tregs in a human skin xenograft transplant model and several mechanisms of CAR Treg mediated suppression have been suggested [44]. As CAR Tregs can recognize antigen not only in the context of MHC on the surface of (antigen-presenting) cells, but also recognize soluble antigen, both contact-dependent and -independent mechanisms of suppression have been suggested. This offers the opportunity of using bystander suppression in inflammatory settings where direct targeting of antigen-expressing cells might be detrimental due to cytolytic activity of CAR Tregs [45].

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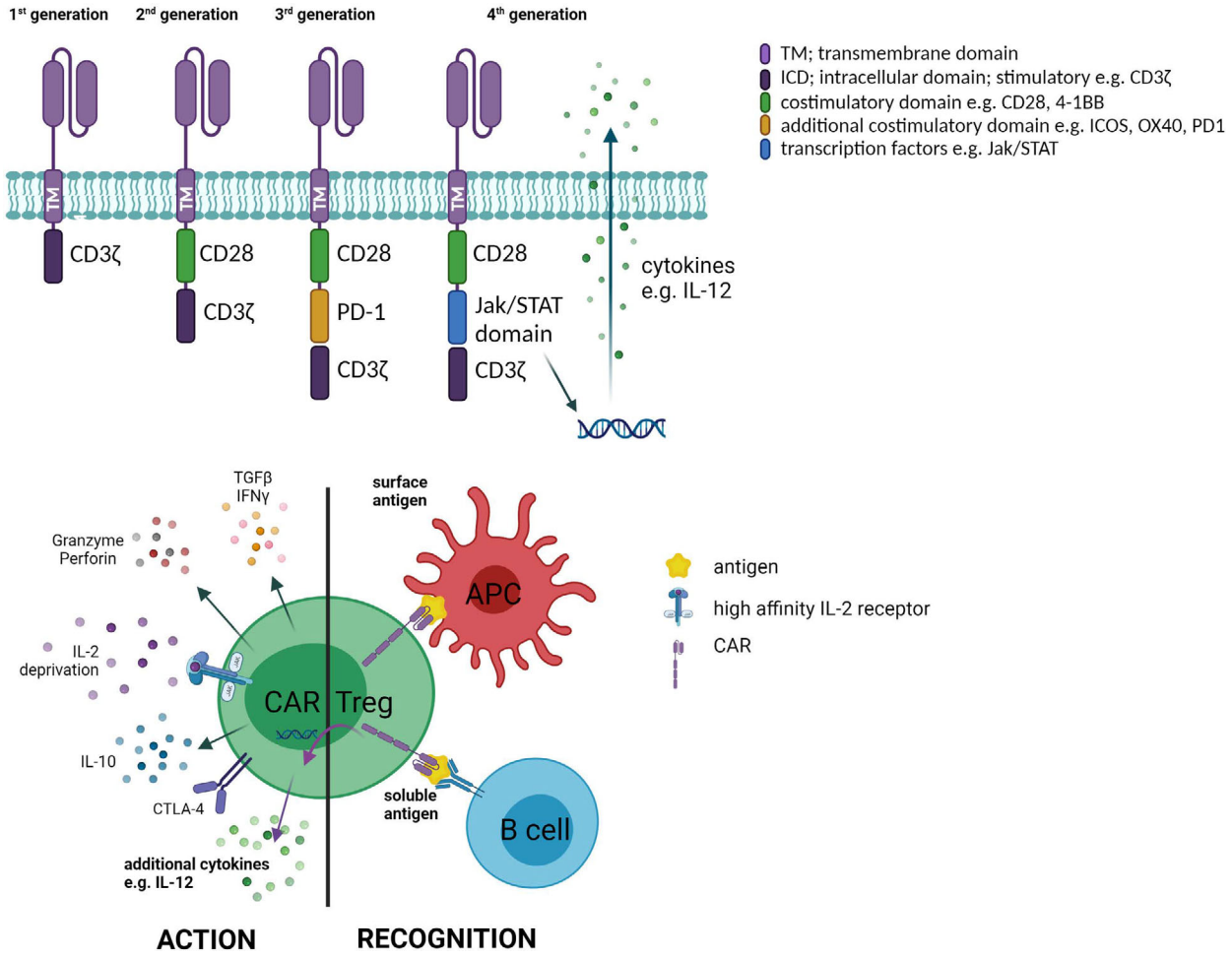

Figure 1 Schematic overview of CAR Treg structures, antigen-recognition and function. (a) Different CAR Treg generations varying in signaling domains, fourth generation CAR constructs contain additional constitutive or inducible factors such as cytokines or transcription factors for enhanced effector function. (b) CARs are engineered receptors which are able to bin specific antigen in an MHC-independent way, enabling them to recognize surface-expressed and soluble antigens. CARs expressed in Tregs initiate Treg-mediated suppressor mechanisms as well as additional mechanisms, depending on the CAR construct (created with BioRender.com).

Specific homing of adoptively transferred Tregs has been shown to be necessary for therapeutic Treg-mediated suppression in autoimmunity [46] and transplantation [34]. Donor-specific CAR Tregs have been shown to migrate into the target tissue in both a humanized mouse model [47] and in immunocompetent mice [48]. Moreover, donor-specific CAR Tregs could not only delay skin graft rejection but also attenuate B-cell responses and DSA production. However, CAR Tregs failed to control memory alloreactivity and graft rejection in sensitized mice [48], a well-known barrier to tolerance induction.

Nevertheless, safety of CAR Tregs has still to be confirmed as their production requires viral transduction techniques, mostly utilizing γ-retroviral or lentiviral vectors. Importantly, CAR manufacturing relying on viral transduction is not only associated with safety concerns but also with high manufacturing costs and limitations in vector capacity; therefore, the development of non-viral transfection approaches might be relevant for next-generation T-cell therapeutics [49].

Although CAR technology offers the possibility to generate antigen-specific Tregs in high numbers, there are still many open questions in CAR Treg design, keeping in mind the differences in TCR stimulation, co-receptor ligation, or cytokine production with regard to Treg and Teff biology.

Despite this uncertainty, a first-in human clinical trial with HLA-A*02 CAR-Treg product is currently ongoing in HLA-A2-mismatched living-donor kidney transplant transplantation (Table 2). This study is evaluating safety and tolerability of three single ascending dose cohorts of Tregs ($CD4^{+}CD45RA^{+}CD25^{+}CD127^{low/neg}$) that have been expanded *ex vivo* and transduced with a lentiviral

vector encoding for CAR to recognize HLA-A2*02 molecule (NCT04817774).

B-cell antibody receptor Tregs/T cells

New chimeric immune receptors (CIR) T cells have been recently developed to target specific antibody-producer B cells: Genetically engineered B-cell antibody receptor (BAR) T cells are composed of an antigen or its domains expressed on the cell surface and fused with intracellular costimulatory and T-cell signaling domains.

BAR-T cells represent an attractive therapeutic strategy for antibody-mediated diseases and were initially developed for the autoimmune disease [reason for which they are sometimes also refer as chimeric autoantibody receptors (CAAR) T cells]: Pemphigus Vulgaris [50] and Hemophilia A [51]. Both BAR-engineered CD8 T cells and Treg are able to silence the production of antibodies by directly depletes or regulates Ag-specific B cells, respectively.

The insufficient control of the humoral alloimmune response by immunosuppressive drugs armamentarium [52] results in the generation of *de novo* donor-specific antibodies (DSA), which promote damages to graft vasculature [53]. It is now widely accepted that antibody-mediated rejection accounts for a least 2/3 of late graft loss [54]. HLA-BAR Tregs represent an attractive option to prevent *de novo* DSA generation in non-sensitized recipients, through direct tolerogenic interactions with allo-specific B-cell clones.

Another major problem with humoral alloimmune response is that some patients on the waiting list for transplantation have preformed DSA against a wide range of allogeneic HLA, severely limiting their access to transplantation [55]. In the latter, global plasma cell depletion with proteasome inhibitors has not proven to be effective [56], likely because of replenishment of plasma cell by allogenic memory B cells [57]. Combining proteasome inhibitors with $CD20^+$ B cells depletion with Rituximab could prevent this problem but would then generate the total loss of humoral memory (including vaccinal protection), resulting in an unacceptable risk of major infectious complications. In this context, coupling drug-based global plasma cell depletion with HLA-encoding BAR-T $CD8^+$cells could represent an attractive option to desensitize patients with preformed DSA by selectively eliminating anti-donor HLA memory B cells, restoring sensitized transplant candidates access to transplantation while preserving their vaccinal protection. One could question the impact of circulating DSA on the life span of HLA-encoding BAR-T $CD8^+$ cells. This point was addressed in the experimental study, which investigated the efficiency of BAR-T cells in a murine model of Pemphigus Vulgaris. In this model, the presence of autoantibodies directed against the BAR increased the cytolytic effect of chimeric immune receptors T cells, resulting in a drastic decrease in serum autoantibody titer. These data therefore suggest that soluble DSA rather than impeding HLA-encoding BAR-T $CD8^+$ cells efficiency could instead enhance their therapeutic effects and persistence due to CD137-mediated costimulatory signals [50].

Finally, a major uncertainty regarding the use of the BAR-T cells in transplantation is due to the fact that these genetically engineered cells express an allogeneic HLA molecule as an extracellular domain. Allogeneic HLA can be recognized by 1–10% of T-cell receptors expressed by recipient's lymphocytes [58] through the direct allorecognition pathways. This could not only induce the destruction of BAR-T cells, but may also trigger a "cytokine storm" (i.e., a systemic inflammatory syndrome due to excessive T-cell activation [59], threatening patients' life).

In conclusion, BAR Tregs/T-cell approach combines cell and gene therapy and could be an effective therapeutic alternative to the non-specific treatments currently available to tame humoral alloimmune response. However, many unsolved questions, some specific to transplant immunology, remain to be addressed to determine whether this innovative immunotherapy approach will hold its promises in the field of transplantation.

B cells with regulatory properties

Whereas B lymphocytes are mainly known for their ability to produce antibodies, B cells also display regulatory functions via cytokine production, cell-to-cell contact, and by promoting regulatory T cells. Some subsets of B cells have thus been shown to display suppressive properties in different models of inflammation in rodent but also in humans in different clinical settings. Such B cells with regulatory properties (Bregs) are increased in patients with long-term graft acceptance. These Bregs are able to block effector T-cell proliferation via production of granzyme B or IL-10 and in a contact-dependent manner [60–67]. Other Bregs have been shown to display suppressive functions on different cell types and through multiple mechanisms, and the efficiency of adoptive transfer of B cells has been demonstrated in cancer field, mediating tumor regression and host T-cell antitumor immunity [68]. All these points render Bregs attractive for therapy in different conditions and particularly in transplantation [69,70].

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However, the use of B cells as a cell therapy first requires some important Go-noGo tasks. First, their usage will require their easy identification and isolation from one or several compartments and, to date, Bregs are mainly classified by their suppressive mechanisms and no consensual phenotype has been proposed thus far either in humans or mice. No specific transcription factor(s) has been identified yet for Breg cells and how they acquire their suppressive function remains to be elucidated. Key Breg markers or signaling molecules are still missing. Another point is their stability and the possibility to produce them easily, using reproducible protocols. Regarding the stability, it seems not to be the case since one of the most promising markers, CD9, has been shown to be highly modulated [71]. Moreover, since no cell lineage commitment has been identified yet for Bregs cells, it remains unclear from which B cells and which level of development we have to start with to produce the most efficient population.

When these obstacles will be overtaken, enrichment and GMP suitable protocols allowing not only their efficient expansion but also the maintenance of their suppressive properties would be needed to envision their interest as a cell therapy. Different strategies involving a combination of different ligands have already been tested efficiently but these protocols are also dependent from the Breg cell type to expand [72–74]. Finally, antigen specificity is also an important point to study [75], considering the experience in Treg cell therapy where donor-specific Tregs result in increased suppressive properties compared with polyclonal Tregs [28,29].

Chimeric antigen receptor (CAR) technology is one of the most exciting possibilities considering that it has revolutionized T-cell therapy and has shown promising clinical outcome. Different constructs may be included to increased efficiency of such therapeutic approach. Another possibility is the reprogramming of B cells, a technique that has been shown to be effective in chronic experimental autoimmune encephalomyelitis [76], but that remains challenging in human. Human-induced pluripotent stem cells (hiPSC) generation from differentiated cell types also represents a real opportunity for disease modeling as well as for cell-based therapy [76,77]. B-cell generation from hiPSC would offer several advantages including an unlimited cell source, the choice of cell donor origin, and the possibility to modulate and control their gene expression [78,79]. However to date, B-cell generation needs specific environment and gene activation to trigger their differentiation from hematopoietic stem cells and assays were more or less conclusive [80–83].

Thus, Breg cell therapy represents an exciting possibility in transplantation in the future and clinical trials would likely emerge in complementary to already existing studies. The main issue that requires an extensive basic research remains the clear identification of common and stable markers for Breg cells. Existing therapy that favor Breg cell generation or expansion should be considered, as well.

Conclusion

The potential of Treg cellular therapy in enabling immunosuppression minimization in transplant patients is undoubtedly high but the clinical translation of this strategy is proven challenging.

The generation of CAR Tregs recognizing alloantigens could help overcoming many of the obstacles encountered by natural Tregs, but there remain many outstanding questions that need to be solved. In addition, engineered BAR Tregs/T cells hold a promise for an antigen-specific cellular therapy for preventing or reversing humoral alloimmune response. Moreover, the ability of Bregs to inhibit the adaptive immune response while expanding Tregs makes them a promising therapeutic tool to complement each other in settings of successful tolerance.

From a basic science perspective, a concrete understanding of the complex mechanism of action of Tregs and Bregs and their control over adaptive alloimmune response is mandatory for designing successful engineered T- and B-cell therapies.

Much work remains to be done, but the field is evolving rapidly. The next decade will see major advancements in the genetic engineering of specific, stable, safe, and potent cell-based tolerogenic therapies.

Authorship

Nina Pilat: Wrote and critically revised the paper, final approval of manuscript. Katia Lefsihane: Wrote and critically revised the paper, final approval of manuscript. Sophie Brouard: Wrote and critically revised the paper, final approval of manuscript. Katja Kotsch: Critically revised the paper, final approval of manuscript. Christine Falk: Critically revised the paper, final approval of manuscript. Romy Steiner: Draft the figure and critically revised the paper, final approval of manuscript. Olivier Thaunat: Wrote and critically revised the paper, final approval of manuscript. Floriane Fusil: Wrote and critically revised the paper, final approval of manuscript. Nuria Montserrat: Critically revised the paper, final

approval of manuscript. Cristiano Amarelli: Wrote and critically revised the paper, final approval of manuscript. Federica Casiraghi: Wrote and critically revised the paper, final approval of manuscript.

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Conflict of interest

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REFERENCES

1. Ingulli E. Mechanism of cellular rejection in transplantation. *Pediatr Nephrol* 2010; **25**: 61.
2. Atif M, Conti F, Gorochov G, Oo YH, Miyara M. Regulatory T cells in solid organ transplantation. *Clin Transl Immunol* 2020; **9**: e01099.
3. Fortunato M, Morali K, Passeri L, Gregori S. Regulatory cell therapy in organ transplantation: achievements and open questions. *Front Immunol* 2021; **12**: 641596.
4. Oberholtzer N, Atkinson C, Nadig SN. Adoptive transfer of regulatory immune cells in organ transplantation. *Front Immunol* 2021; **12**: 631365.
5. Rosado-Sánchez I, Levings MK. Building a CAR-Treg: going from the basic to the luxury model. *Cell Immunol* 2020; **358**: 104220.
6. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 1995; **155**: 1151.
7. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 2003; **4**: 330.
8. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003; **299**: 1057.
9. Seddiki N, Santner-Nanan B, Martinson J, *et al.* Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med* 2006; **203**: 1693.
10. Ohkura N, Kitagawa Y, Sakaguchi S. Development and maintenance of regulatory T cells. *Immunity* 2013; **38**: 414.
11. Shevach EM, Thornton AM. tTregs, pTregs, and iTregs: similarities and differences. *Immunol Rev* 2014; **259**: 88.
12. Dhamne C, Chung Y, Alousi AM, Cooper LJN, Tran DQ. Peripheral and thymic foxp3(+) regulatory T cells in search of origin, distinction, and function. *Front Immunol* 2013; **4**: 253.
13. Zwang NA, Leventhal JR. Cell therapy in kidney transplantation: focus on regulatory t cells. *J Am Soc Nephrol* 2017; **28**: 1960.
14. Issa F, Hester J, Goto R, Nadig SN, Goodacre TE, Wood K. Ex vivo-expanded human regulatory T cells prevent the rejection of skin allografts in a humanized mouse model. *Transplantation* 2010; **90**: 1321.
15. Wu DC, Hester J, Nadig SN, *et al.* Ex vivo expanded human regulatory T cells can prolong survival of a human islet allograft in a humanized mouse model. *Transplantation* 2013; **96**: 707.
16. Nadig SN, Więckiewicz J, Wu DC, *et al.* In vivo prevention of transplant arteriosclerosis by ex vivo-expanded human regulatory T cells. *Nat Med* 2010; **16**: 809.
17. Chandran S, Tang Q, Sarwal M, *et al.* Polyclonal regulatory T cell therapy for control of inflammation in kidney transplants. *Am J Transplant* 2017; **17**: 2945.
18. Mathew JM, H.-Voss J, LeFever A, *et al.* A phase I clinical trial with ex vivo expanded recipient regulatory t cells in living donor kidney transplants. *Sci Rep* 2018; **8**: 7428.
19. Harden PN, Game DS, Sawitzki B, *et al.* Feasibility, long-term safety, and immune monitoring of regulatory T cell therapy in living donor kidney transplant recipients. *Am J Transplant* 2021; **21**: 1603.
20. Roemhild A, Otto NM, Moll G, *et al.* Regulatory T cells for minimising immune suppression in kidney transplantation: phase I/IIa clinical trial. *BMJ* 2020; **371**: m3734.
21. Sawitzki B, Harden PN, Reinke P, *et al.* Regulatory cell therapy in kidney transplantation (the ONE study): a harmonised design and analysis of seven non-randomised, single-arm, phase 1/2A trials. *Lancet* 2020; **395**: 1627.
22. Sánchez-Fueyo A, Whitehouse G, Grageda N, *et al.* Applicability, safety, and biological activity of regulatory T cell therapy in liver transplantation. *Am J Transplant* 2020; **20**: 1125.
23. Todo S, Yamashita K, Goto R, *et al.* A pilot study of operational tolerance with a regulatory T-cell-based cell therapy in living donor liver transplantation. *Hepatology* 2016; **64**: 632.
24. Putnam AL, Brusko TM, Lee MR, *et al.* Expansion of human regulatory T-cells from patients with type 1 diabetes. *Diabetes* 2009; **58**: 652.
25. Fraser H, Safinia N, Grageda N, *et al.* A Rapamycin-based GMP-compatible process for the isolation and expansion of regulatory T cells for clinical trials. *Mol Ther Methods Clin Dev* 2018; **8**: 198.
26. Landwehr-Kenzel S, Zobel A, Hoffmann H, *et al.* Ex vivo expanded natural regulatory T cells from patients with end-stage renal disease or kidney transplantation are useful for autologous cell therapy. *Kidney Int* 2018; **93**: 1452.
27. Ezzelarab MB, Zhang H, Guo H, *et al.* Regulatory T cell infusion can enhance memory T cell and alloantibody responses in lymphodepleted nonhuman primate heart allograft recipients. *Am J Transplant* 2016; **16**: 1999.
28. Sánchez-Fueyo A, Sandner S, Habicht A, *et al.* Specificity of CD4+CD25+ regulatory T cell function in alloimmunity. *J Immunol* 2006; **176**: 329.
29. Raimondi G, Sumpter TL, Matta BM, *et al.* Mammalian target of rapamycin inhibition and alloantigen-specific regulatory T cells synergize to promote long-term graft survival in immunocompetent recipients. *J Immunol* 2010; **184**: 624.
30. Guinan EC, Cole GA, Wylie WH, *et al.* Ex vivo costimulatory blockade to generate regulatory T cells from patients

awaiting kidney transplantation. *Am J Transplant* 2016; **16**: 2187.
31. Putnam AL, Safinia N, Medvec A, *et al*. Clinical grade manufacturing of human alloantigen-reactive regulatory T cells for use in transplantation. *Am J Transplant* 2013; **13**: 3010.
32. Ezzelarab MB, Zhang H, Sasaki K, *et al*. Ex vivo expanded donor alloreactive regulatory T cells lose immunoregulatory, proliferation and anti-apoptotic markers after infusion into ATG-lymphodepleted, nonhuman primate heart allograft recipients. *Transplantation* 2020. https://doi.org/10.1097/TP.0000000000003617.
33. Sawant DV, Vignali DAA. Once a Treg, always a Treg? *Immunol Rev* 2014; **259**: 173.
34. Zhang N, Schröppel B, Lal G, *et al*. Regulatory T cells sequentially migrate from inflamed tissues to draining lymph nodes to suppress the alloimmune response. *Immunity* 2009; **30**: 458.
35. Veerapathran A, Pidala J, Beato F, Yu X-Z, Anasetti C. Ex vivo expansion of human Tregs specific for alloantigens presented directly or indirectly. *Blood* 2011; **118**: 5671.
36. Thornton AM, Shevach EM. Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. *J Immunol* 2000; **164**: 183.
37. Gershon RK, Kondo K. Infectious immunological tolerance. *Immunology* 1971; **21**: 903.
38. Jonuleit H, Schmitt E, Kakirman H, Stassen M, Knop J, Enk AH. Infectious tolerance: human CD25(+) regulatory T cells convey suppressor activity to conventional CD4(+) T helper cells. *J Exp Med* 2002; **196**: 255.
39. Andersson J, Tran DQ, Pesu M, *et al*. CD4+ FoxP3+ regulatory T cells confer infectious tolerance in a TGF-beta-dependent manner. *J Exp Med* 2008; **205**: 1975.
40. Sadelain M, Rivière I, Riddell S. Therapeutic T cell engineering. *Nature* 2017; **545**: 423.
41. Sommermeyer D, Hudecek M, Kosasih PL, *et al*. Chimeric antigen receptor-modified T cells derived from defined CD8+ and CD4+ subsets confer superior antitumor reactivity in vivo. *Leukemia* 2016; **30**: 492.
42. Mohseni YR, Tung SL, Dudreuilh C, Lechler RI, Fruhwirth GO, Lombardi G. The future of regulatory T cell therapy: promises and challenges of implementing CAR technology. *Front Immunol* 2020; **11**: 1608.
43. MacDonald KG, Hoeppli RE, Huang Q, *et al*. Alloantigen-specific regulatory T cells generated with a chimeric antigen receptor. *J Clin Invest* 2016; **126**: 1413.
44. Rana J, Biswas M. Regulatory T cell therapy: current and future design perspectives. *Cell Immunol* 2020; **356**: 104193.
45. Grossman WJ, Verbsky JW, Barchet W, Colonna M, Atkinson JP, Ley TJ. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity* 2004; **21**: 589.
46. Jaeckel E, von Boehmer H, Manns MP. Antigen-specific FoxP3-transduced T-cells can control established type 1 diabetes. *Diabetes* 2005; **54**: 306.
47. Dawson NA, Lamarche C, Hoeppli RE, *et al*. Systematic testing and specificity mapping of alloantigen-specific chimeric antigen receptors in regulatory T cells. *JCI Insight* 2019; **4**: e123672.
48. Sicard A, Lamarche C, Speck M, *et al*. Donor-specific chimeric antigen receptor Tregs limit rejection in naive but not sensitized allograft recipients. *Am J Transplant* 2020; **20**: 1562.
49. Raes L, De Smedt SC, Raemdonck K, Braeckmans K. Non-viral transfection technologies for next-generation therapeutic T cell engineering. *Biotechnol Adv* 2021; **49**: 107760.
50. Ellebrecht CT, Bhoj VG, Nace A, *et al*. Reengineering chimeric antigen receptor T cells for targeted therapy of autoimmune disease. *Science* 2016; **353**: 179.
51. Pohl ADP, Venkatesha SH, Zhang A-H, Scott DW. Suppression of FVIII-specific memory B cells by chimeric BAR receptor-engineered natural regulatory T cells. *Front Immunol* 2020; **11**: 693.
52. Thaunat O, Koenig A, Leibler C, Grimbert P. Effect of immunosuppressive drugs on humoral allosensitization after kidney transplant. *J Am Soc Nephrol* 2016; **27**: 1890.
53. Chen C-C, Pouliquen E, Broisat A, *et al*. Endothelial chimerism and vascular sequestration protect pancreatic islet grafts from antibody-mediated rejection. *J Clin Invest* 2018; **128**: 219.
54. Gaston RS, Cecka JM, Kasiske BL, *et al*. Evidence for antibody-mediated injury as a major determinant of late kidney allograft failure. *Transplantation* 2010; **90**: 68.
55. Bestard O, Couzi L, Crespo M, Kessaris N, Thaunat O. Stratifying the humoral risk of candidates to a solid organ transplantation: a proposal of the ENGAGE working group. *Transplant Int* 2021; **34**: 1005.
56. Kirk AD. What should work, may not. *Am J Transplant* 2010; **10**: 447.
57. Kwun J, Burghuber C, Manook M, *et al*. Humoral compensation after bortezomib treatment of allosensitized recipients. *JASN* 2017; **28**: 1991.
58. Marino J, Paster J, Benichou G. Allorecognition by T lymphocytes and allograft rejection. *Front Immunol* 2016; **7**: 582.
59. Fajgenbaum DC, June CH. Cytokine storm. *N Engl J Med* 2020; **383**: 2255.
60. Beckett J, Hester J, Issa F, Shankar S. Regulatory B cells in transplantation: roadmaps to clinic. *Transpl Int* 2020; **33**: 1353.
61. Shen P, Roch T, Lampropoulou V, *et al*. IL-35-producing B cells are critical regulators of immunity during autoimmune and infectious diseases. *Nature* 2014; **507**: 366.
62. Mauri C, Gray D, Mushtaq N, Londei M. Prevention of arthritis by interleukin 10–producing B cells. *J Exp Med* 2003; **197**: 489.
63. Fillatreau S, Sweenie CH, McGeachy MJ, Gray D, Anderton SM. B cells regulate autoimmunity by provision of IL-10. *Nat Immunol* 2002; **3**: 944.
64. Sagoo P, Perucha E, Sawitzki B, *et al*. Development of a cross-platform biomarker signature to detect renal transplant tolerance in humans. *J Clin Invest* 2010; **120**: 1848.
65. Newell KA, Asare A, Kirk AD, *et al*. Identification of a B cell signature associated with renal transplant tolerance in humans. *J Clin Invest* 2010; **120**: 1836.
66. Chesneau M, Michel L, Dugast E, *et al*. Tolerant Kidney Transplant Patients Produce B Cells with Regulatory Properties. *J Am Soc Nephrol* 2015; **26**: 2588–2598.
67. Pallier A, Hillion S, Danger R, *et al*. Patients with drug-free long-term graft function display increased numbers of peripheral B cells with a memory and inhibitory phenotype. *Kidney Int* 2010; **78**: 503.
68. Li Q, Lao X, Pan Q, *et al*. Adoptive transfer of tumor reactive B cells confers host T-cell immunity and tumor regression. *Clin Cancer Res* 2011; **17**: 4987.
69. Menon M, Rosser EC, Mauri C. Identification and isolation of regulatory B cells in mouse and human. *Methods Mol Biol* 2019; **1899**: 55.
70. Mauri C, Menon M. Human regulatory B cells in health and disease: therapeutic potential. *J Clin Invest* 2017; **127**: 772.
71. Mohd Jaya FN, Garcia SG, Borras FE, Guerrero D, Chan GCF, Franquesa M. In vitro characterization of human CD24hiCD38hi regulatory B cells shows CD9 is not a stable breg cell marker. *Int J Mol Sci* 2021; **22**: 4583.

72. Chesneau M, Le Mai H, Brouard S. New method for the expansion of highly purified human regulatory granzyme B-expressing B cells. *Methods Mol Biol* 2021; **2270**: 203.
73. Chesneau M, Mai HL, Danger R, *et al*. Efficient expansion of human granzyme B-expressing B cells with potent regulatory properties. *J Immunol* 2020; **205**: 2391.
74. Mauri C, Menon M. The expanding family of regulatory B cells. *Int Immunol* 2015; **27**: 479.
75. Boonyaratanakornkit J, Taylor JJ. Techniques to study antigen-specific B cell responses. *Front Immunol* 2019; **10**: 1694.
76. Calderón-Gómez E, Lampropoulou V, Shen P, *et al*. Reprogrammed quiescent B cells provide an effective cellular therapy against chronic experimental autoimmune encephalomyelitis. *Eur J Immunol* 2011; **41**: 1696.
77. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006; **126**: 663.
78. Nagasawa T. Microenvironmental niches in the bone marrow required for B-cell development. *Nat Rev Immunol* 2006; **6**: 107.
79. Dubois F, Gaignerie A, Flippe L, *et al*. Toward a better definition of hematopoietic progenitors suitable for B cell differentiation. *PLoS One* 2020; **15**: e0243769.
80. Hystad ME, Myklebust JH, Bø TH, *et al*. Characterization of early stages of human B cell development by gene expression profiling. *J Immunol* 2007; **179**: 3662.
81. Crane GM, Jeffery E, Morrison SJ. Adult haematopoietic stem cell niches. *Nat Rev Immunol* 2017; **17**: 573.
82. French A, Yang C-T, Taylor S, Watt SM, Carpenter L. Human induced pluripotent stem cell-derived B lymphocytes express sIgM and can be generated via a hemogenic endothelium intermediate. *Stem Cells Dev* 2015; **24**: 1082.
83. Carpenter L, Malladi R, Yang C-T, *et al*. Human induced pluripotent stem cells are capable of B-cell lymphopoiesis. *Blood* 2011; **117**: 4008.