IMMUNOLOGY

Hurdles in therapy with regulatory T cells

Piotr Trzonkowski,[1]*† Rosa Bacchetta,[2] Manuela Battaglia,[3] David Berglund,[4] Hermann Richard Bohnenkamp,[5] Anja ten Brinke,[6] Andrew Bushell,[7] Nathalie Cools,[8] Edward K. Geissler,[9] Silvia Gregori,[10] S. Marieke van Ham,[6] Catharien Hilkens,[11] James A. Hutchinson,[12] Giovanna Lombardi,[13] J. Alejandro Madrigal,[14] Natalia Marek-Trzonkowska,[15] Eva M. Martinez-Caceres,[16] Maria Grazia Roncarolo,[10,17] Silvia Sanchez-Ramon,[18] Aurore Saudemont,[14] Birgit Sawitzki[19]

Improper activation of the immune system contributes to a variety of clinical conditions, including autoimmune and allergic diseases as well as solid organ and bone marrow transplantation. One approach to counteract this activation is through adoptive therapy with regulatory T cells (T_{regs}). Efforts to manufacture these cells have led to good maunfacturing practice–compliant protocols, and T_{reg} products are entering early clinical trials. Here, we report the stance of the European Union Cooperation in Science and Technology Action BM1305, "Action to Focus and Accelerate Cell-based Tolerance-inducing Therapies—A FACTT," which identifies hurdles hindering T_{reg} clinical applications in Europe and provides possible solutions.

INTRODUCTION

Dysregulated immune activation can lead to autoimmune diseases or allergies. Fortunately, the immune system self-regulates, and these regulatory mechanisms can be exploited for different types of immunotherapy (*1*). $CD4^+$ regulatory T cells (T_{regs}), a critical component of immune regulation, are the front-runners in the race for therapeutic immunoregulation (*2*). T_{regs} have been shown to effectively prevent autoimmune conditions and rejection of transplanted allografts (*3*). Two subsets of these cells, naturally occurring or thymus-derived T_{reg} cells (tT_{regs}, $CD4^+CD25^{high}FoxP3^+$) and T regulatory type 1 (Tr1) cells, have already been tested in the clinic as cellular therapeutics (hereafter referred to as T_{reg}-based therapies when aspects are common to both) (*4*). Small-scale clinical studies have been conducted in patients with type 1 diabetes (T1D) (*5*–*8*), graft-versus-host disease (GvHD) after stem cell transplantation (*9*–*14*), allogeneic solid organ graft rejection (*15*), and refractory Crohn's disease (Fig. 1) (*16*). In line with these early experiences, the first problems and solutions of applying T_{regs} (herein used when aspects are common to both types of cellular therapy) in the clinical setting have also appeared. This Perspective highlights the particular properties or challenges of tT_{reg} or Tr1 cell therapy as reported by the European Union (EU) Cooperation in Science and Technology (COST) Action BM1305 "Action to Focus and Accelerate Cell-based Tolerance-inducing Therapies—A FACTT."

T_{REGS} AS A CELLULAR THERAPEUTIC AGENT

Identification/isolation of T_{regs}. There are no perfect markers for either polyclonal tT_{regs} or antigen-reactive tT_{regs}, which means that isolation methods depend on a pragmatic compromise between what is desirable and what is possible. Most groups evaluating tT_{regs} have decided to use magnetic sorting devices, which are approved in Europe (Fig. 2A). One advantage of this approach is that it is almost a closed system, and the necessary CE-labeled reagents and validated protocols are available. Data already available from clinical trials in which tT_{regs} were purified with this system and used as GvHD prophylaxis are very promising (*10*, *12*). Nonetheless, optimal purification is difficult to perform with this bulk separation technique because T conventional cells (T_{conv}) express low levels of CD25 receptor used to sort $CD25^{high}$ tT_{regs}. Although titration of bead concentration or antibody loading on beads allows for some discrimination of expression intensity between $CD25^{high}$ tT_{regs} and $CD25^{low}$ T_{conv}, magnetic systems still result in postisolation product contamination with $CD25^{dim}$ T_{conv}. Therefore, they excel in debulking unwanted cells from crude starting cell sources but are not optimal when aiming for highly pure tT_{reg} product. Nevertheless, debulking is still critical to reduce the time and cost associated with second-step flow cytometry sorting.

Flow cytometry–based purification offers the advantage that tT_{regs} can be isolated to high purity based on the combined staining intensities of multiple surface markers, including CD127, CD25, CD62L, CD45RA, and CD27. Gating allows isolation on the basis of different patterns of CD25 receptor expression and so enhances the purity and functional properties of isolated tT_{regs} (*17*, *18*). On the downside, this method presents a considerable regulatory challenge in most EU countries because EU regulations (Directive 2003/94/EC and its Annex 2) require compliance to good manufacturing practice (GMP) for cellular product manufacturing. These conditions are particularly demanding for traditional flow cytometry droplet-sorters, in which the sorting process involves a fluid stream traveling freely in the

[1]Medical University of Gdansk, Department of Clinical Immunology and Transplantology, Debinki 7, 80-952 Gdansk, Poland. [2]Department of Pediatric Stem Cell Transplantation and Regenerative Medicine, Stanford School of Medicine, Standford, California, USA. [3]Diabetes Research Institute (DRI), Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) San Raffaele Scientific Institute, Milan, Italy. [4]Uppsala University, Department of Immunology, Genetics and Pathology; Section of Clinical Immunology, Rudbeck Laboratory, 751 85 Uppsala, Sweden. [5]Miltenyi Biotec GmbH, Clinical Business, Friedrich-Ebert-Straße 68, 51429 Bergisch Gladbach, Germany. [6]Department of Immunopathology, Sanquin Blood Supply, Division Research, Plesmanlaan 125, 1066 CX Amsterdam, Netherland and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, Amsterdam, Netherlands. [7]Nuffield Department of Surgical Sciences, University of Oxford, John Radcliffe Hospital, Headington, Oxford OX3 9DU, UK. [8]Laboratory of Experimental Hematology, Vaccine & Infectious Disease Institute, Faculty of Medicine and Health Sciences, University of Antwerp, Antwerp University Hospital (UZA), Wilrijkstraat 10, B-2650 Edegem, Belgium. [9]Division of Experimental Surgery, Department of Surgery, University Hospital Regensburg, Regensburg, Bavaria, 93053, Germany. [10]San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Division of Regenerative Medicine, Stem Cells and Gene Therapy, IRCCS San Raffaele Scientific Institute, Milan, Italy. [11]Institute of Cellular Medicine, Newcastle University NE2 4HH, UK. [12]Division of Experimental Surgery, Department of Surgery, University Hospital Regensburg, Regensburg, 93053, Bavaria, Germany. [13]Medical Research Council (MRC) Centre in Transplantation, King's College London, Guy's Hospital, Great Maze Pond, London SE1 9RT, UK. [14]Anthony Nolan Research Institute, University College London (UCL) Royal Free Hospital Campus, 77c Fleet Road, London NW3 2QG, UK. [15]Department of Family Medicine, Medical University of Gdańsk, Ul. Dębinki 2 80-210 Gdańsk, Poland. [16]Immunology Division, Germans Trias i Pujol University Hospital. Campus Can Ruti. Department of Cellular Biology, Physiology, and Immunology, Universitat Autònoma Barcelona 08916, Badalona, Barcelona, Spain. [17]Department of Pediatric Stem Cell Transplantation and Regenerative Medicine, Stanford School of Medicine, Stanford, California, USA. [18]Departamento de Inmunología Clínica, Hospital Clínico San Carlos, Calle Profesor Martín Lagos S/N, E- 28040 Madrid, Spain. [19]AG Transplantationstoleranz, Charite Universitätsmedizin, Institut für Med. Imunologie, Augustenburgerplatz 1, 13353 Berlin, Germany. †All authors equally contributed to this work.
*Corresponding author. E-mail: ptrzon@gumed.edu.pl

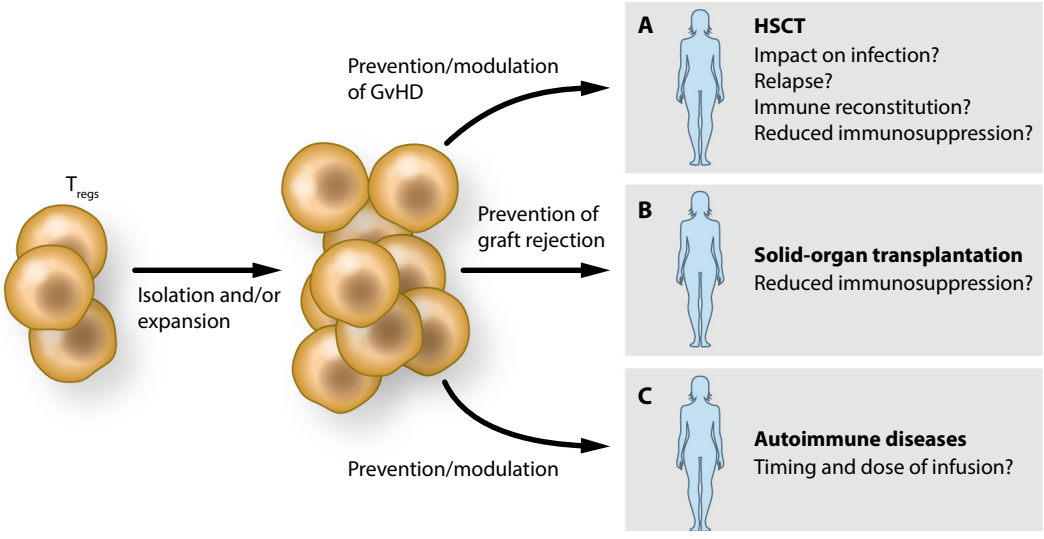

Fig. 1. Applications for T_{reg}-based therapies. T_{regs} could be used to induce tolerance for different indications. T_{regs} can be freshly isolated from a donor (peripheral blood, apheresis, or CB) or from the patient's blood and expanded under GMP conditions if required to obtain sufficient numbers or to generate antigen-specific cells. (**A**) In the case of HSCT, donor or CB T_{regs} (fresh, expanded polytT_{regs} or Tr1 cells) could be used for GvHD prophylaxis or the treatment of GvHD. This therapy could lead to the omission of immunosuppression; however, its effects on incidence of infection, relapse, or immune reconstitution need to be investigated further. (**B**) Therapies using the patient's T_{regs} (expanded polytT_{regs}, antigen-specific Tr1, or tT_{regs}) are currently being tested to prevent organ rejection after kidney or liver transplantation. T_{reg}-based therapies could minimize the immunosuppressive regimen used posttransplant. (**C**) Last, the patient's T_{regs} (expanded polytT_{regs} or antigen-specific Tr1 cells) could be used to treat certain autoimmune diseases such as recent T1D or refractory Crohn's disease; however, timing of infusion and doses to be used are questions that remain to be addressed.

air (Fig. 2B). So far, regulatory approval for flow-sorting has been given to one Polish group (Trzonkowski, Gdańsk) [also one U.S. group (Bluestone, San Francisco)]. The newest generation of droplet-based cell sorters attempts to fulfill GMP requirements but still put constraints on purification speed. (Fig. 3).

Microfluidic switch technologies offer another closed and sterile alternative for clinical cell purification, without droplet and associated aerosol formation, which makes a GMP-compliant purification possible in a grade C clean-room environment. Recent proof-of-principle data (*19*) clearly demonstrate that pure and viable tT_{regs} can be rapidly obtained (Fig. 2C). Other promising systems in development involve a sterile, closed, and disposable cartridge, in which cells are interrogated and sorted by a superfast mechanical sort valve (Fig. 2D). It can be reasonably envisaged that the powerful handpicking of cells offered by cytometry-based techniques could successfully enter clinical trials.

Recent attempts have been made to sort T_{regs} with reversible human lymphocyte antigen (HLA)/peptide multimers, termed streptamers. T_{regs} isolated in this way preserved functional activity because of complete dissociation and removal of isolation reagents after the sorting, which is not possible with standard antibody or tetramer technologies (*20*). This technique allows the cells to be classified as non–advanced-therapy medicinal product (non-ATMP), which requires considerably less clinical testing than advanced-therapy medicinal products (ATMPs) (*21*).

In contrast to tT_{regs}, the production and purification of Tr1 cells for clinical applications requires more individualized approaches related to disease and cell source. The first Tr1-based clinical product was the IL-10 DLI. This product consisted of donor peripheral blood mononuclear cells (PBMCs) anergized with irradiated patient T cell–depleted PBMC in the presence of exogenous interleukin-10 (IL-10) for 10 days (*13*). In another approach, dendritic cells (DCs) generated in vitro from circulating monocytes cultured with granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-4, and IL-10 were shown to have a tolerogenic phenotype, spontaneously release high levels of IL-10 and no IL-12, and act as potent antigen-presenting cells for the generation of Tr1 cells in vitro. Tr1 cells produced with these DC were named "DC-10 DLI" (*22*). Both IL-10 DLI and DC-10 DLI were used in a clinical trial in patients after hematopoietic stem cell transplantation (*13*, *22*). The third type of Tr1-enriched product has been developed only recently for recipients of allogeneic kidney transplants. In this situation, DC-10 are generated from living kidney donors, and PBMCs are collected from recipients (such as patients on dialysis). Unfortunately, this approach led to the generation of a Tr1 cell–enriched medicinal product with a low anergic phenotype, incompatible with their eventual clinical application (*23*). On the contrary, the use of purified $CD4^+$ T cells isolated from recipients, as opposed to that of total PBMCs, led to the generation of a highly anergic donor-specific medicinal product containing an average of 10% Tr1 cells named "T_{10} cells" (Table 1) (*24*).

Manipulation of T_{regs} under GMP conditions. The efficacy of T_{reg}-based therapy depends in part on the infusion of high numbers of these cells, especially polyclonal ones (*25*–*27*); yet, obtaining sufficient numbers of polyclonal T_{regs} from a single donor currently remains a challenge. Hence, T_{regs} need to be expanded ex vivo before administration, which requires GMP compliance. Many methods and materials in the appropriate GMP standard for clinical use were initially adopted from other existing applications and only later specifically developed for T_{regs}—for example, tT_{reg} culture technique (*28*, *29*). Currently, there is a focus on developing strategies to expand polyclonal tT_{regs} (*30*, *31*). Alternative approaches include (i) generation of antigen-specific tT_{regs} whose numbers can be substantially reduced without compromising efficacy (*32*, *33*), (ii) third-party cryopreserved cord blood (CB) units as a possible "off-the-shelf" cell source for T_{reg} isolation (*11*), and (iii) local delivery of small amounts of T_{regs} to the site of action (*34*, *35*).

Because of ever-improving insights into T_{regs} biology, there is a sustained gap in terms of available reagents and methods

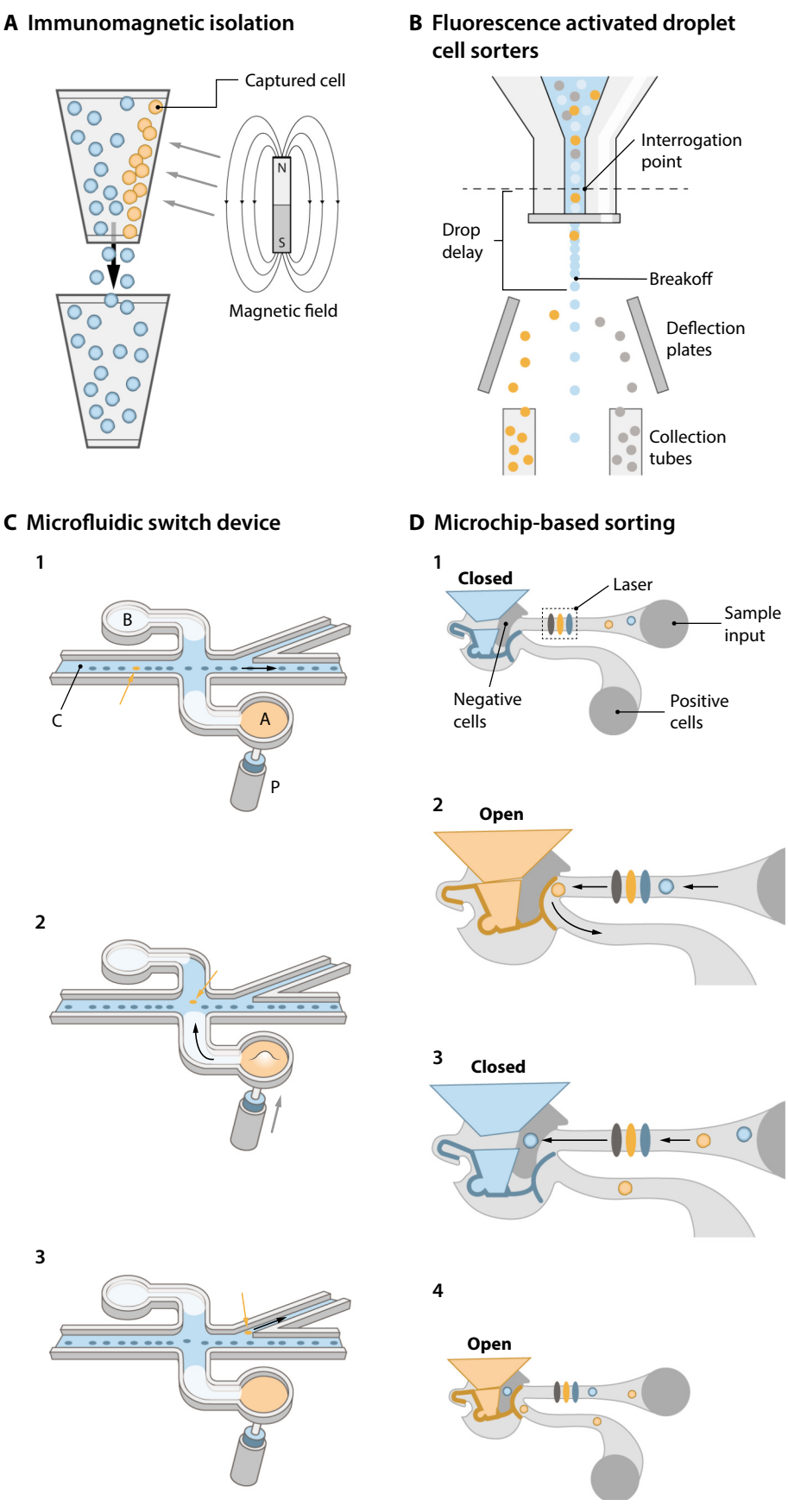

Fig. 2. Cell purification for cellular therapy. (**A**) Immunomagnetic isolation uses separation of cells coated with ferromagnetic-conjugated antibodies (orange). Such cells are captured in the magnetic field, whereas other cells (blue) are flushed out. (**B**) Fluorescence-activated droplet cell sorters separate cells labeled with fluorochrome-conjugated antibodies. The cells are detected at the interrogation point as the fluorochromes emit the light when excited by sorter lasers. Single cells are separated into discrete droplets freely in the air at the breakoff point. Droplets containing cells that fulfill the sorting criteria are electrically charged (orange and grey) and deflected in the electric field generated by charged deflection plates and drop into tubes, collecting populations of interest. (**C**) Microfluidic switch device (1) consists of two opposing chambers, the actuator chamber "A" and the damper chamber "B," that are filled with air, trapping a fluid plug in the main channel "C." The default state maintains the flow of cells into the unsorted fraction (black arrow). When a cell of interest (orange cell pointed with orange arrow) arrives in the fluid plug region, an external piezoelectric pin (P) under the actuator chamber is activated and pushes a flexible membrane, moving the air pocket toward the fluid plug (2). The fluid plug is deflected toward the damper chamber, redirecting the path of the cell from the default channel to the collection channel (black arrow in 2). The deflected cell (orange cell pointed with orange arrow) continues into the upper part of the Y-shaped structure (collection channel, black arrow in 3). Multiple switches (24 to 72) can be mounted in parallel on the same disposable chip, substantially reducing the time of sorting. (**D**) Microchip-based sorting. Labeled cells enter the chip from the sample input. As the cells approach the sort area, each cell is analyzed. When a selected cell (orange) is identified, a magnetic pulse opens the valve and the cell is redirected to a collection chamber (2 and 4). An integrated single-crystal silicon spring returns the valve to its original position, and undesired cells (blue) are allowed to flow through (3).

Table 1. The hurdles that must be addressed in T_{reg}-based therapies.

In manufacturing T_{regs} for clinical applications:
Difficult manipulation of T_{regs} under GMP conditions
Limited choice of single-use disposable systems that minimizes risk of cross-contamination
Limited choice of closed fluidic sorting systems to prevent contact with surrounding air
Need for high rate of purification of target cells (preferably in the magnitude of at least 50,000 events/s)
High cost of efficient reagents and disposables
Limited choice of operator-friendly procedures and equipment, not requiring extensive training of personnel
Overcomplicated legislation for academic trials
When T_{regs} are administered as cellular product in clinical trials:
Biological
Possibility that expanded T_{regs} revert to T_{conv} cells
Identification of unrecognized specific autoantigens, if antigen-specific T_{reg}-based therapy is considered
Risk of concomitant use of other immunosuppressants interfering with T_{regs}
Possibility that therapeutic levels of adoptively transferred T_{regs} may hamper protective immune responses against pathogens and cancer
Trial-related
Regulatory and ethics approval
Clinical protocol specifications, IMPD, and release criteria
Design of clinical protocols to assess T_{regs} efficacy
Timing and dose of infusion
In clinical monitoring after administration of T_{regs}:
In vivo T_{regs} survival and distribution
Safety of therapy by assessing in vivo stability of the infused T_{regs} and monitoring for signs of undesired immune activation
Analysis of T_{regs}-based therapy efficacy
Approaches to standardize assays used to monitor and analyze efficacy of T_{regs}-based therapy between different centers
For the commercialization of T_{reg}-based therapies:
Development of a Targeted Product Profile
Clinical studies demonstrating—besides safety and efficacy—a benefit over standard of care
Cost-effective manufacturing process
Business models suitable for T_{reg}-based cell therapies

between research laboratories and laboratories manufacturing T_{reg}-based clinical products that are on the path toward clinically safe and efficient cellular products available for the patient. Although the number of GMP-compatible reagents available for cellular therapy continues to grow, more discussion is required with regard to which standards should be mandatory in order to control the procedure at different levels of advancement (Table 1).

Regulatory barriers The translation of the adoptive transfer of T_{regs} from basic scientific models to the clinic is a good example of how a constructive and responsible dialogue between scientists, clinicians, and regulators can result in a therapeutic product for patients. Until recently, therapies with blood cells, including T_{regs}, were classified as cells for transplantation or blood-derived products. Recent changes now classify the majority of cell-based therapies as medicinal products. The manufacture and application of such products in Europe and the United States is strictly regulated so as to ensure an appropriate balance of risk and benefit to patients. Under EU law, cell-based medicinal products (CBMPs) are governed by a legislative framework enacted through EU Regulation 1394/2007/EC on ATMPs and an amendment of directive 2001/83/EC on the community code relating to medicinal products for human use (*36*, *37*). This legislation recognizes the inherent difficulties of studying cell-based therapies as pharmacological agents but also imposes exacting standards for preclinical and clinical characterization of cell products, comparable with those applied to conventional pharmaceuticals, including central registration through the European Medicinal Agency (EMA). Complying with these strict regulatory requirements is challenging, especially for academic institutions and small companies with limited resources, and may substantially delay clinical development of cell-based therapies, including T_{regs}. An example is the clinical development of CBMP, which has the same regulatory standard for commercial and noncommercial clinical trials. Although the former trials are usually executed by pharmaceutical companies aimed mainly at marketing authorization, the latter (including most T_{regs} trials) are performed by collaborations between clinicians and academics as basic proof-of-concept studies performed in academic centers. However, central and national regulatory authorities in Europe offer some concessions to noncommercial developers of CBMPs, such as reduced fees for scientific advice and assessment of clinical trial applications. European authorities also have a number of legal tools that could ease the burden of regulation for academics and small companies in the early phases of product development, including the Hospital Exemption Rule, granting of orphan drug status, and reclassification of certain CBMPs as transfusion or transplantation products.

Fig. 3. Droplet sorter for clinical use. GMP-compliant droplet sorter with exchangeable fluidics and accompanying equipment enclosed in a tailor-made laminar air flow bench maintaining grade A. The surrounding room is of grade B. (Photo from Uppsala University Hospital, Sweden)

Cooperation between academics and the Committee for Advanced Therapies (CAT) of EMA, as well as national authorities, may further ease the regulatory process. For example, sharing preclinical and clinical data among noncommercial agencies could lead to greater standardization of risk assessment, which would reduce bureaucratic

Table 2. Academic clinical trials with T_{regs} to assess efficacy and safety.

Study ID	Phase	Product	Indication	Effects	Centre	Reference
HSCT						
NKEBN/458-310/2008	I	Expanded polytT_{regs}	GvHD treatment	Safe/reduced immunosuppression in chronic GvHD; only transient improvement in acute GvHD	Gdansk	(*9*)
NCT00602693	I	Expanded CB polyt-T_{regs}	GvHD prophylaxis	Safe but increased occurrence of infections/reduced incidence of acute GvHD	Minnesota	(*11*, *41*)
01/08	I	Fresh polytT_{regs}	GvHD prophylaxis	Safe/reduced leukemia relapses/reduced incidence of GvHD	Perugia	(*10*, *12*)
T_{reg}002EudraCT: 2012-002685-12	I	Fresh polytT_{regs}	GvHD prophylaxis	Safe	Regensburg	(*38*)
EK 206082008	I	Expanded polytT_{regs}	GvHD treatment	Tumors diagnosed in 2 patients/stable chronic GvHD	Dresden	(*14*)
ALT-TEN	I	Tr1 (*IL-10 DLI* or *DC-10 DLI*)	GvHD prophylaxis	Safe/long-term disease-free survival in 4 patients	Milan	(*13*)
Organ transplantation						
One Study T_{reg}-based trials						
NCT02129881	I/II	Expanded polytT_{regs}	Living donor kidney transplant	Recruiting	London, Oxford,	(*15*)
NCT02371434	I/II	Expanded polytT_{regs}	Living donor kidney transplant	Recruiting	Berlin	(*15*)
NCT02244801	I/II	Donor-alloantigen-reactive T_{regs}	Living donor kidney transplant	Recruiting	San Francisco	(*15*)
NCT02091232	I/II	Belatacept-conditioned T_{regs}	Living donor kidney transplant	Recruiting	Boston	(*15*)
Planned	I/II	Antigen-specific Tr1 (T_{10} cells)	Living donor kidney transplant	Not yet recruiting	Milan	(*15*)
Other T_{reg}-based trials						
ThRIL NCT02166177	I	Expanded polytT_{regs}	Liver transplant	Recruiting	London	—
NCT02188719	I	Donor-alloantigen-Reactive T_{regs}	Liver transplant	Recruiting	San Francisco	—
NCT02088931	I	Expanded polytT_{regs}	Subclinical rejection in kidney transplantation	Recruiting	San Francisco	—
NCT02474199	I	Donor-alloantigen-Reactive T_{regs}	CNI reduction in liver transplantation	Not yet recruiting	San Francisco	—
Autoimmunity						
T_{reg}VAC ISRCTN06128462	I	Expanded polytT_{regs}	Recent T1D	Safe/reduced insulin doses (insulin independence in 2 out of 12 patients)	Gdansk	(*5*–*7*)
NCT01210664	I	Expanded polytT_{regs}	T1D	Safe	San Francisco	(*8*)
CATS1	I/II	Ovalbumin-specific Tr1	Refractory Crohn's disease	Safe/clinical response in 40% of patients	Lille	(*16*)
T_{reg}VAC2.0EudraCT: 2014-004319-35	II	Expanded polytT_{regs}	Recent T1D	Recruiting	Gdansk	—
T_{reg}SM EudraCT: 2014-004320-22	I	Expanded polytT_{regs}	Multiple sclerosis	Recruiting	Gdansk	—

costs and may ultimately avoid the need for extensive preclinical animal testing of new CBMPs. Notably, an initiative to compile an "academic investigational medicinal product dossier" (academic IMPD) available to all academic cell product developers is now organized through the A FACTT consortium (www.afactt.eu). Researchers also can limit their bureaucratic burdens during clinical trials, for example, by arguing for a risk-based approach to patient follow-up and trial management. Through collaborative efforts, academic developers can produce more accurate and comprehensive descriptions of the safety and efficacy of their products, which should eventually be reflected in less burdensome regulatory processes.

Table 3. Companies engaged in the clinical development of T_{reg}-based therapies.

Company	Country	Academic partner	T_{reg} product	Investigational therapy	Indication	Clinical development
NeoStem/ Caladrius	USA	UCSF, San Francisco, CA	Expanded autologous polyclonal $CD4^+CD25^+CD127^{dim}$ tT_{regs}	CLBS03 n/a n/a	T1D Steroid-resistant asthma Prevention of graft rejection following solid organ transplantation	Phase 2 to be initiated Phase I to be initiated n/a
TxCell	France	n/a	Antigen-specific Tr1 cells	Ovasave® Col-T_{reg}	Crohn's disease Autoimmune uveitis	Phase 2b started (CATS29 study) Phase 1 to be initiated
iReg Medical	Sweden	n/a	Autologous $CD4^+CD25^{hi}FoxP3^+$ tT_{regs}	n/a	Prevention of graft rejection following solid organ transplantation	n/a
TRACT Therapeutics	USA	Northwestern University, Chicago, IL	Expanded autologous tT_{regs}	TRACT platform	Prevention of graft rejection following solid organ transplantation	Phase 1 started

HURDLES IN CLINICAL TRIALS

T_{regs} in hematopoietic stem cell transplantation. Hematopoietic stem cell transplantation (HSCT) is used to treat different bone marrow disorders and hematological malignancies. Allogeneic donor T cells can attack the patient's malignant cells, providing a desirable graft-versus-leukemia (GvL) effect; however, they also react against the patient tissues, inducing one of the main complications post-HSCT, GvHD. Because of their ability to regulate aggressive T cell responses, tT_{regs} and Tr1 cells have been proposed as an adoptive therapy to prevent or modulate GvHD (Table 2 and Fig. 1) (*38*–*40*).

Martelli and colleagues reported reduced relapse rates in leukemic patients after adoptive transfer of tT_{regs} (*10*). These data are encouraging considering that T_{regs} could potentially suppress the functions of natural killer (NK) cells and T cells that drive the GvL effect. These investigators also suggested a positive impact of tT_{regs} cells on immune reconstitution, although data remain sparse (*12*). This effect might be dependent on the dose administered. A few groups demonstrated that the control of GvHD by tT_{regs} would depend on the infusion of an equal number of tT_{regs} and T_{conv} (*26*, *27*). These numbers can be achieved with third-party cryopreserved CB, which is better tolerated in HSCT (*11*). On the other hand, higher susceptibility to viral reactivation was observed in patients treated with CB-derived tT_{regs} compared with historical controls (*41*), and a recent trial with expanded donor-derived tT_{regs} in chronic GvHD revealed tumors in two treated patients (*14*). Importantly, both infections and tumors occurred in patients treated with conventional immunosuppressive drugs, and it is difficult to clearly associate these adverse effects with tT_{regs}. Moreover, no such effects were reported in other trials with tT_{regs} in GvHD. Therefore, the impact of T_{regs} on GvL as well as on the occurrence of infection post-HSCT and on hematopoietic stem cell function is still controversial. Analysis of available data will be a key to the design and monitoring of new clinical trials for which optimal dose and timing of T_{reg} infusion should be discussed (Table 1).

T_{regs} in transplantation. In humanized mouse models, expanded polyclonal human tT_{regs} or alloantigen-reactive human tT_{regs} can inhibit the rejection of human tissues (*32*, *42*–*44*). The observation that such populations can be isolated from patients with end-stage renal disease and manipulated in vitro (*33*, *45*, *46*) has led to their evaluation in clinical solid organ transplantation under the umbrella of FP7-supported grant "The ONE Study." Within this consortium, it is the intention of four groups to use expanded polyclonal tT_{regs}, whereas three other groups intend to use alloantigen-reactive T_{regs} or Tr1 cells (Table 2 and Fig. 1).

Of all the hurdles facing the introduction of T_{reg}-based therapy into clinical transplantation, the design of future clinical protocols appears to represent the greatest challenge. For example, 1- and 5-year survival of living-donor kidneys in the UK is currently of the order of 95 and 90%, respectively, indicating that detecting efficacy of any T_{regs} subset in this setting will either require long-term follow-up or a trial design in which T_{reg}-based therapy replaces one of the components of "gold-standard" immunosuppression. This latter option would appear to be ethically unacceptable. An alternative design might include accelerated drug minimization, but without reliable clinical indicators of reduced immune responses toward the donor organ, such minimization would be empirical rather than evidence-based. Furthermore, T_{regs} function and efficacy could be tested in presensitized patients characterized by high frequency of alloreactive memory T cells. Indeed, it has been shown that tT_{regs}, in conjunction with T cell depletion and transient low-dose calcineurin inhibitors (CNIs), can control reactivation, proliferation, and effector function of preformed memory T cells (*47*). One final option would be to introduce T_{regs} into transplant settings in which current graft outcome is poor. For example, in adult lung transplantation 1- and 5-year graft survival figures are ~76 and 50%, respectively (www.ishlt.org, 2012 statistics). Provided that current gold-standard immunosuppression is not detrimental to T_{regs} function [although this has been only partially investigated (*47*)], T_{regs} could be added to the current regimen. Such an approach might provide efficacy data in a relatively short time.

In the case of Tr1-enriched cells (T_{10} cells) in living-donor kidney transplantation within The ONE Study, dose and tim-

ing of cell infusion were decided on the basis of preclinical data and mostly theoretical reasoning. Over the past years of active research in the field of T_{regs}, it has been learned that to survive and to be effective in vivo upon transfer, Tr1 cells (i) need to "see" their specific antigens, (ii) have to be present at the time of T-effector–cell activation, and (iii) are less effective in controlling memory than naïve T cell responses (*40*). In addition, it is likely that Tr1 cells are sensitive to T cell–specific immunosuppressive drugs (Table 1) (*22*).

T_{regs} in autoimmunity. Autoimmune diseases affect a substantial proportion of the population and represent the second leading cause of chronic diseases (the first cause in women). Yet, current treatment modalities for autoimmunity are far from ideal, owing to unsatisfactory efficacy and toxicity. T_{reg} deficiency has been reported to be a leading cause of autoimmunity. Moreover, preclinical models have demonstrated efficacy of the administration of T_{regs}, mainly tT_{regs}, in autoimmune diseases such as T1D, systemic lupus erythematosus (SLE), and multiple sclerosis (MS) (*48*–*50*), which has been confirmed recently in first clinical trials (Table 2 and Fig. 1).

Clinical application of T_{regs} in autoimmune diseases faces specific challenges related to the nature of these conditions. An autoimmune inflammatory environment may affect the stability of these cells, leading to the possibility that administered T_{regs} may revert to T_{conv}, further worsening the condition instead of relieving it. In addition, identifying autoantigens that trigger the disease is another bottleneck that affects decisions about whether to treat with polyclonal or antigen-reactive T_{regs}. Although the use of antigen-reactive T_{regs} is tempting, our knowledge of the autoantigens that really trigger particular autoimmune diseases is limited. Hence, an incorrect choice of autoantigens or incomplete cover of autoantigens' spectrum can lead to ineffective therapy. A third concern lies in a lack of experience with the use of T_{regs} in autoimmune diseases. The protocols developed for production and administration of T_{regs} in humans have been shown to be clinically feasible, but several questions still remain, including the timing and dose of T_{reg} administration (*28*).

Autoimmune diseases are inevitably associated with progressive tissue damage; thus, T_{reg} administration early in disease course may be optimal for initiation of T_{reg}-based therapies. However, T_{regs} are not yet an approved "on-label" treatment—despite positive early reports of few adverse effects associated with the therapy—and early administration is not favored over approved therapies. If used in later phases of disease, T_{regs} are usually adjuvant to immunosuppressants, which may alter T_{reg} function. Combination therapies with tT_{regs}, including rapamycin or vitamin D, are known to induce T_{regs} or promote their expansion, and CNIs or depleting antibodies substantially inhibit tT_{regs} (*31*). Even with successful therapy, possible adverse effects may occur, highlighting the need for a narrow dosing window, and the dose at which T_{regs} can be safely administered may not necessarily achieve adequate efficacy (Table 1).

MONITORING SUCCESS AND FAILURE

Objective measures to monitor treatment success and failure of T_{reg} therapy are needed (*5*–*12*, *15*). Undesired induction of global immune suppression, immune activation through instability of the T_{reg}-based product, or contamination of the product with T_{conv} might all contribute to a failure of T_{reg}-based therapy. It is clear that control of the product quality—regardless of T_{reg} subset considered—is a first essential aspect needed to perform adequate immune monitoring for T_{reg}-based trials (Table 1).

Assessment of T_{reg}-based product quality. The heterogeneity of T_{reg}-based products requires different approaches to measure their quality and suppressive potential. In general, T_{reg}-based product purity is assessed through the analysis of the relative expression of cell product characteristic markers, such as expression of Foxp3 in case of tT_{regs} or Lag3/CD49b in case of Tr1 cells (*51*). Contamination of the cell product by potentially harmful T_{conv} cells is also analyzed—for example, by measuring the release of proinflammatory cytokines such as interferon-γ or IL-17A (*28*). Nevertheless, in addition to direct analysis of the phenotype, other assays are needed that assess (i) T_{regs} function by evaluating the in vitro suppressive capacity with regard to inhibition of proliferation or release of proinflammatory cytokines of cocultured effector T cells (*28*, *52*, *53*) and (ii) T_{reg} stability by assessing the demethylation of the "T_{regs}–specific demethylation region" (TSDR) in tT_{regs} (*54*, *55*). To assess the efficacy of T_{reg}–based therapy, T_{reg}-based product quality assays should be standardized or harmonized across different sites followed by interlaboratory validation efforts and the generation of standardized, common "quality control" standard operating procedures.

Analysis of in vivo survival and distribution. Monitoring the efficacy of T_{reg}-based therapy is often hampered by the difficulty of obtaining tissue samples before, during, and after treatment, limiting monitoring to the peripheral blood cell compartment. In vivo tissue tracking would allow us to estimate survival more precisely than would PBMC analysis alone, as well as simultaneously allow evaluation of the T_{regs}' homing potential and their eventual tissue distribution. In vivo T_{regs} tracking protocols have applied labeling techniques such as stable isotope labeling with deuterated water or glucose (*56*, *57*). However, most available technologies for in vivo imaging have a resolution too low to reveal single-cell properties, limiting their usability. In contrast, more recent techniques such as laser ablation–inductively coupled plasma mass spectrometry have been developed, which allow with a resolution of below 10 μm close to single-cell detection of gadolinium-labeled $CD4^+$ T cells in tissue specimens (*58*, *59*). Another way to visualize adoptive transferred T_{regs} is with full-body imaging. Single-photon emission computed tomography (SPECT)/CT imaging can be used in preclinical imaging studies of adoptively transferred T_{regs} without affecting the function and viability, allowing longitudinal studies within disease models (*60*). The last two approaches, however, have not been performed so far to track adoptively transferred T_{regs} in patients.

Studying safety aspects of T_{reg}-based therapy. T_{regs} populations may affect the patients' general immune competence and thus increase the risk for infections and development of malignancies (*41*, *54*). Therefore, monitoring of effects of T_{reg}-based therapy should always assess undesired tolerance induction against known harmful pathogens such as cytomegalovirus, Epstein-Barr virus, BK virus, certain fungi, and other opportunistic pathogens. Antigens such as tetanus toxoid included in the childhood vaccination programs could serve as good recall antigens in order to monitor off-target effects of therapy. Assays analyzing CD154 expression on pathogen-stimulated T cells (*44*) and expression of major histocompatibility complex II on monocytes shortly upon cell transfer may also help to evaluate general immune competence (*61*).

Assessment of T_{reg}-based therapy efficacy. The choice of assays or biomarkers to measure therapy efficacy strongly depends

on the disease treated. Organ- and disease-specific functional parameters such as c-peptide levels or insulin dependence in case of T1D or glomerular filtration rate (GFR) in case of kidney graft recipients may be compared with those of conventionally treated patients. However, some immunological mechanisms underlying the pathogenesis of different immune-mediated diseases may be common. Changes in numbers or function of pathogenic cells may thus be used across different clinical trials in order to assess the efficacy of T_{reg}-based therapy. For instance, "operationally" tolerant kidney transplant patients are characterized by low peripheral Toll-like receptor 5 (TLR5) expression (*43*). Successful T_{reg}-based therapy in patients receiving an umbilical CB transplant was also associated with low TLR5 expression, with $lin^-HLA\text{-}DR^-CD33^+CD16^+$ granulocyte-like cells being the major source of TLR5 (*62*).

Ideally, immunomonitoring assays should not only monitor clinical efficacy but also predict clinical efficacy during treatment. This would allow the identification of responding patients while simultaneously recognizing nonresponding patients who should no longer be subjected to ineffective T_{reg}-based treatment but receive enhanced conventional therapy. Identification of such correlates of protection (COPs) would have a strong impact on the efficacy and cost-effectiveness of T_{reg}–based therapy. The search for COPs for tolerance is ongoing. COPs may arise out of direct action of the transfused T_{regs} but may also result from the induction of infectious tolerance, in which transferred T_{regs} induce naïve T cells to become additional T_{regs} (*63*).

Efforts to standardize and quality control immune monitoring for T_{reg}-based trials. Researchers involved in T_{reg}-based trials should agree on specific methodologies and create international immunomonitoring platforms for tolerance-inducing therapies to evaluate the safety and efficacy of this novel form of cellular therapy. For example, the ONE Study has standardized whole blood flow cytometry protocol for immunomonitoring between participating centers (*64*), and this standardized approach could be applied to future T_{regs} studies.

TOWARD CLINICAL APPROVAL AND COMMERCIALIZATION

The success of clinical approval of novel therapies depends on several critical steps. Clearly, costs represent a substantial hurdle to the introduction of cellular therapies into larger clinical trials. At present, depending on the country in Europe, the cost of single preparation of clinical-grade polyclonal tT_{regs} is ~15,000 to 40,000 € per patient. The highest proportion of this sum relates to T_{regs} manufacturing and includes the costs of GMP-grade consumables, single-use equipment, facility charges, personnel, and product validation and release. These costs are even higher at the translational step, when the consumables are in the process of adjustment and validated to GMP standard in the first human trials. It is likely that costs may fall on a per-patient basis, particularly if GMP processes can be made less labor-intensive and the therapies are offered to a wider population of patients after clinical approval. Reimbursement by health insurance companies is another concern. There are several ways to increase the chance of reimbursement approval. One option is to ensure that the field of application of the cellular therapy is already optimally tuned for its specific field of application at the start of clinical trials. This not only will enhance the chance for clinical efficacy but may also lead to reimbursement approval with a prescription limitation; the therapy is only approved for reimbursement in a specific patient group, which limits the budgetary impact of the relatively costly therapy on health care costs in general. Another option might be the early application of standard development schemes that specifically focus on streamlining the complex multistep cell-manufacturing procedures into a cost-effective process. This will reduce the actual manufacturing cost of a designated cellular product but also diminish cost of development of the product. In both cases, close and early collaborations between academic institutions and industry may substantially increase a chance to achieve the goal of acceptable price of the eventual therapy for which reimbursement approval is requested (Table 1).

Cooperation of academic and industry partners. Realizing high-impact scientific ideas for clinical application requires a critical mass of translational scientists; committed clinicians; a state-of-the-art, GMP-compliant cell-manufacturing facility; the access to technologies for the manufacturing process; and applicable quality-control measures for the release of the manufactured cell products. For example, over the course of several years, a close partnership between King's College London and Miltenyi Biotec has led to the development of new technologies for the GMP-compliant production of human tT_{regs} in clinically relevant numbers (*65*). A second example is NeoStem/Caladrius T-Regulatory Cell Program for Immune Modulation. The company pursues a collaboration with the laboratories of the University of California, San Francisco, to develop T_{regs} for the treatment of T1D, steroid-resistant asthma, and organ transplant rejection.

T_{reg}-based therapies in commercial development. Currently, several companies are already pursuing commercial development of T_{regs}-based therapies (Table 3). Development speed—with the goal of being first to market (*66*)—and financial resources are two essential reasons for academic institutions to involve a potent industrial partner that takes the responsibility of several critical steps toward commercialization: (i) developing the Targeted Product Profile; (ii) sketching the clinical development path; (iii) implementing a regulatory strategy, such as orphan drug designation, breakthrough approval; and others (taking regional differences such as between EU or USA into account); (iv) analyzing the competitive landscape; (v) developing or licensing a protective IP portfolio; (vi) having insight in reimbursement systems; and (vii) designing the mass production setup and ideally automating the manufacturing process.

PERSPECTIVES

Adoptive immunotherapy with T_{regs} represents a viable strategy with a promise of efficacy and lower adverse effects as compared with that of the current clinical routine. Therapeutic efficacy and safety of T_{regs} have been shown in preclinical models and phase I studies, respectively. Nevertheless, more knowledge about the long-term behavior of these cells after transfer to humans and better definition of the particular subsets for each disease are essential in order to establish efficient treatment protocols. This may be accelerated with facilitation of legislation, at least for academic trials. With all the limitations and advantages, T_{reg}-based immunotherapy represents a real step toward personalized medicine. Academic hospitals and nonprofit institutions, such as blood banks, support efforts in the execution of clinical trials of these cellular products. Several companies are already under way to commercialize the potential of these cells for various therapies. However, for clinical ap-

proval of effective T_{reg}-based cell therapies, key critical steps of the clinical development path and cost-effective manufacturing processes should appear high on the agenda. Therefore, core academic groups that are experts in all phases of T_{reg}-based adoptive therapy and companies with an interest in developing T_{reg}-based cellular products need to join forces to accelerate the translation into clinical practice.

REFERENCES AND NOTES

1. R. K. Gershon, K. Kondo, Cell interactions in the induction of tolerance: The role of thymic lymphocytes. *Immunology* **18**, 723–737 (1970).
2. S. Sakaguchi, N. Sakaguchi, M. Asano, M. Itoh, M. Toda, Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* **155**, 1151–1164 (1995).
3. J. L. Riley, C. H. June, B. R. Blazar, Human T regulatory cell therapy: Take a billion or so and call me in the morning. *Immunity* **30**, 656–665 (2009).
4. A. K. Abbas, C. Benoist, J. A. Bluestone, D. J. Campbell, S. Ghosh, S. Hori, S. Jiang, V. K. Kuchroo, D. Mathis, M. G. Roncarolo, A. Rudensky, S. Sakaguchi, E. M. Shevach, D. A. Vignali, S. F. Ziegler, Regulatory T cells: Recommendations to simplify the nomenclature. *Nat. Immunol.* **14**, 307–308 (2013).
5. N. Marek-Trzonkowska, M. Mysliwiec, A. Dobyszuk, M. Grabowska, I. Techmanska, J. Juscinska, M. A. Wujtewicz, P. Witkowski, W. Mlynarski, A. Balcerska, J. Mysliwska, P. Trzonkowski, Administration of $CD4^+CD25^{high}CD127^-$ regulatory T cells preserves β-cell function in type 1 diabetes in children. *Diabetes Care* **35**, 1817–1820 (2012).
6. N. Marek-Trzonkowska, M. Myśliwiec, J. Siebert, P. Trzonkowski, Clinical application of regulatory T cells in type 1 diabetes. *Pediatr. Diabetes* **14**, 322–332 (2013).
7. N. Marek-Trzonkowska, M. Myśliwiec, A. Dobyszuk, M. Grabowska, I. Derkowska, J. Juścińska, R. Owczuk, A. Szadkowska, P. Witkowski, W. Młynarski, P. Jarosz-Chobot, A. Bossowski, J. Siebert, P. Trzonkowski, Therapy of type 1 diabetes with CD4(+)CD25(high)CD127-regulatory T cells prolongs survival of pancreatic islets - results of one year follow-up. *Clin. Immunol.* **153**, 23–30 (2014).
8. J. A. Bluestone, T1DM Immunotherapy Using $CD4^+CD127^{lo/-}CD25^+$ Polyclonal T_{regs}. *Clin. Trials* **NCT01210664** (2011).
9. P. Trzonkowski, M. Bieniaszewska, J. Juścińska, A. Dobyszuk, A. Krzystyniak, N. Marek, J. Myśliwska, A. Hellmann, First-in-man clinical results of the treatment of patients with graft versus host disease with human ex vivo expanded $CD4^+CD25^+CD127-$ T regulatory cells. *Clin. Immunol.* **133**, 22–26 (2009).
10. M. F. Martelli, M. Di Ianni, L. Ruggeri, F. Falzetti, A. Carotti, A. Terenzi, A. Pierini, M. S. Massei, L. Amico, E. Urbani, B. Del Papa, T. Zei, R. Iacucci Ostini, D. Cecchini, R. Tognellini, Y. Reisner, F. Aversa, B. Falini, A. Velardi, HLA-haploidentical transplantation with regulatory and conventional T-cell adoptive immunotherapy prevents acute leukemia relapse. *Blood* **124**, 638–644 (2014).
11. C. G. Brunstein, J. S. Miller, Q. Cao, D. H. McKenna, K. L. Hippen, J. Curtsinger, T. Defor, B. L. Levine, C. H. June, P. Rubinstein, P. B. McGlave, B. R. Blazar, J. E. Wagner, Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: Safety profile and detection kinetics. *Blood* **117**, 1061–1070 (2011).
12. M. Di Ianni, F. Falzetti, A. Carotti, A. Terenzi, F. Castellino, E. Bonifacio, B. Del Papa, T. Zei, R. I. Ostini, D. Cecchini, T. Aloisi, K. Perruccio, L. Ruggeri, C. Balucani, A. Pierini, P. Sportoletti, C. Aristei, B. Falini, Y. Reisner, A. Velardi, F. Aversa, M. F. Martelli, T_{regs} prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. *Blood* **117**, 3921–3928 (2011).
13. R. Bacchetta, B. Lucarelli, C. Sartirana, S. Gregori, M. T. Lupo Stanghellini, P. Miqueu, S. Tomiuk, M. Hernandez-Fuentes, M. E. Gianolini, R. Greco, M. Bernardi, E. Zappone, S. Rossini, U. Janssen, A. Ambrosi, M. Salomoni, J. Peccatori, F. Ciceri, M. G. Roncarolo, Immunological outcome in haploidentical-HSC transplanted patients treated with IL-10-anergized donor T cells. *Front. Immunol.* **5**, 16 (2014).
14. A. Theil, S. Tuve, U. Oelschlägel, A. Maiwald, D. Döhler, D. Oßmann, A. Zenkel, C. Wilhelm, J. M. Middeke, N. Shayegi, K. Trautmann-Grill, M. von Bonin, U. Platzbecker, G. Ehninger, E. Bonifacio, M. Bornhäuser, Adoptive transfer of allogeneic regulatory T cells into patients with chronic graft-versus-host disease. *Cytotherapy* **17**, 473–486 (2015).
15. E. K. Geissler, The ONE Study compares cell therapy products in organ transplantation: Introduction to a review series on suppressive monocyte-derived cells. *Transp. Res.* **1**, 11 (2012).
16. P. Desreumaux, A. Foussat, M. Allez, L. Beaugerie, X. Hébuterne, Y. Bouhnik, M. Nachury, V. Brun, H. Bastian, N. Belmonte, M. Ticchioni, A. Duchange, P. Morel-Mandrino, V. Neveu, N. Clerget-Chossat, M. Forte, J. F. Colombel, Safety and efficacy of antigen-specific regulatory T-cell therapy for patients with refractory Crohn's disease. *Gastroenterology* **143**, 1207–17.e1-2 (2012).
17. D. Berglund, M. Karlsson, A. R. Biglarnia, T. Lorant, G. Tufveson, O. Korsgren, B. Carlsson, Obtaining regulatory T cells from uraemic patients awaiting kidney transplantation for use in clinical trials. *Clin. Exp. Immunol.* **173**, 310–322 (2013).
18. C. Baecher-Allan, E. Wolf, D. A. Hafler, Functional analysis of highly defined, FACS-isolated populations of human regulatory $CD4^+$ $CD25^+$ T cells. *Clin. Immunol.* **115**, 10–18 (2005).
19. R. Hulspas, L. Villa-Komaroff, E. Koksal, K. Etienne, P. Rogers, M. Tuttle, O. Korsgren, J. C. Sharpe, D. Berglund, Purification of regulatory T cells with the use of a fully enclosed high-speed microfluidic system. *Cytotherapy* **16**, 1384–1389 (2014).
20. J. Neudorfer, B. Schmidt, K. M. Huster, F. Anderl, M. Schiemann, G. Holzapfel, T. Schmidt, L. Germeroth, H. Wagner, C. Peschel, D. H. Busch, H. Bernhard, Reversible HLA multimers (*Strep*tamers) for the isolation of human cytotoxic T lymphocytes functionally active against tumor- and virus-derived antigens. *J. Immunol. Methods* **320**, 119–131 (2007).
21. Anthony Nolan Institute, WP02 - Isolation of clinical grade umbilical cord blood regulatory T cells to treat Graft versus Host Disease, www.t-control.info/the-project (last assessed 31 March 2015).
22. S. Gregori, D. Tomasoni, V. Pacciani, M. Scirpoli, M. Battaglia, C. F. Magnani, E. Hauben, M. G. Roncarolo, Differentiation of type 1 T regulatory cells (Tr1) by tolerogenic DC-10 requires the IL-10-dependent ILT4/HLA-G pathway. *Blood* **116**, 935–944 (2010).
23. N. H. Litjens, M. Huisman, M. van den Dorpel, M. G. Betjes, Impaired immune responses and antigen-specific memory $CD4^+$ T cells in hemodialysis patients. *J. Am. Soc. Nephrol.* **19**, 1483–1490 (2008).
24. A. Petrelli, E. Tresoldi, B. G. Mfarrej, A. Paganelli, D. Spotti, R. Caldara, A. Secchi, M. Battaglia, Generation of donor-specific T regulatory type 1 cells from patients on dialysis for cell therapy after kidney transplantation. *Transplantation* **99**, 1582–1589 (2015).
25. Q. Tang, J. A. Bluestone, Regulatory T-cell therapy in transplantation: Moving to the clinic. *Cold Spring Harb. Perspect. Med.* **3**, a015552 (2013).
26. A. Trenado, M. Sudres, Q. Tang, S. Maury, F. Charlotte, S. Grégoire, M. Bonyhadi, D. Klatzmann, B. L. Salomon, J. L. Cohen, Ex vivo-expanded $CD4^+CD25^+$ immunoregulatory T cells prevent graft-versus-host-disease by inhibiting activation/differentiation of pathogenic T cells. *J. Immunol.* **176**, 1266–1273 (2006).
27. P. A. Taylor, A. Panoskaltsis-Mortari, J. M. Swedin, P. J. Lucas, R. E. Gress, B. L. Levine, C. H. June, J. S. Serody, B. R. Blazar, L-Selectin(hi) but not the L-selectin(lo) $CD4^+25^+$ T-regulatory cells are potent inhibitors of GVHD and BM graft rejection. *Blood* **104**, 3804–3812 (2004).
28. N. Marek, M. Bieniaszewska, A. Krzystyniak, J. Juścińska, J. Myśliwska, P. Witkowski, A. Hellmann, P. Trzonkowski, The time is crucial for ex vivo expansion of T regulatory cells for therapy. *Cell Transplant.* **20**, 1747–1758 (2011).
29. K. Gołąb, A. Krzystyniak, N. Marek-Trzonkowska, R. Misawa, L. J. Wang, X. Wang, O. Cochet, M. Tibudan, P. Langa, J. M. Millis, P. Trzonkowski, P. Witkowski, Impact of culture medium on $CD4^+$ $CD25^{(high)}CD127^{(lo/neg)}$ Treg expansion for the purpose of clinical application. *Int. Immunopharmacol.* **16**, 358–363 (2013).
30. P. Hoffmann, R. Eder, L. A. Kunz-Schughart, R. Andreesen, M. Edinger, Large-scale in vitro expansion of polyclonal human $CD4^+CD25^{high}$ regulatory T cells. *Blood* **104**, 895–903 (2004).
31. P. Trzonkowski, M. Szaryńska, J. Myśliwska, A. Myśliwski, Ex vivo expansion of $CD4^+CD25^+$ T regulatory cells for immunosuppressive therapy. *Cytometry A.* **75**, 175–188 (2009).
32. S. Landwehr-Kenzel, F. Issa, S. H. Luu, M. Schmück, H. Lei, A. Zobel, A. Thiel, N. Babel, K. Wood, H. D. Volk, P. Reinke, Novel GMP-compatible protocol employing an allogeneic B cell bank for clonal expansion of allospecific natural regulatory T cells. *Am. J. Transplant.* **14**, 594–606 (2014).
33. A. L. Putnam, N. Safinia, A. Medvec, M. Laszkowska, M. Wray, M. A. Mintz, E. Trotta, G. L. Szot, W. Liu, A. Lares, K. Lee, A. Laing, R. I. Lechler, J. L. Riley, J. A. Bluestone, G. Lombardi, Q. Tang, Clinical grade manufacturing of human alloantigen-reactive regulatory T cells for use in transplantation. *Am. J. Transplant.* **13**, 3010–3020 (2013).
34. N. Marek, A. Krzystyniak, I. Ergenc, O. Cochet, R. Misawa, L. J. Wang, K. Gołąb, X. Wang, G. Kilimnik, M. Hara, S. Kizilel, P. Trzonkowski, J. M. Millis, P. Witkowski, Coating human pancreatic islets with $CD4^+CD25^{(high)}CD127^-$ regulatory T cells as a novel approach for the local immunoprotection. *Ann. Surg.* **254**, 512–518, discussion 518–519 (2011).
35. A. Krzystyniak, K. Gołąb, P. Witkowski, P. Trzonkowski, Islet cell transplant and the incorporation of T_{regs}. *Curr. Opin. Organ Transplant.* **19**, 610–615 (2014).
36. The European Parliament and the Council, Regulation (EC) No 1394/2007 on advanced therapy medicinal products and amending Directive 2001/83/EC and Regulation (EC) No 726/2004. *Official Journal of the European Union* **324**, 121–137 (2007).
37. European Commission, "Report from the Commission to the European Parliament and the Council in accordance with Article 25 of Regulation (EC) No 1394/2007 of the European Parliament and of the Council on advanced therapy medicinal products and amending Directive 2001/83/EC and Regulation (EC) No 726/2004," European Commission, Brussels, 28 March 2014 COM(2014) 188 final (2014).
38. M. Edinger, P. Hoffmann, Regulatory T cells in stem cell transplantation: Strategies and first clinical experiences. *Curr. Opin. Immunol.* **23**, 679–684 (2011).
39. M. G. Roncarolo, S. Gregori, R. Bacchetta, M. Battaglia, Tr1 cells and the counter-regulation of immunity: Natural mechanisms and therapeutic applications. *Curr. Top. Microbiol. Immunol.* **380**, 39–68 (2014).

40. M. G. Roncarolo, M. Battaglia, Regulatory T-cell immunotherapy for tolerance to self antigens and alloantigens in humans. *Nat. Rev. Immunol.* **7**, 585–598 (2007).
41. C. G. Brunstein, B. R. Blazar, J. S. Miller, Q. Cao, K. L. Hippen, D. H. McKenna, J. Curtsinger, P. B. McGlave, J. E. Wagner, Adoptive transfer of umbilical cord blood-derived regulatory T cells and early viral reactivation. *Biol. Blood Marrow Transplant.* **19**, 1271–1273 (2013).
42. S. N. Nadig, J. Wieckiewicz, D. C. Wu, G. Warnecke, W. Zhang, S. Luo, A. Schiopu, D. P. Taggart, K. J. Wood, In vivo prevention of transplant arteriosclerosis by ex vivo-expanded human regulatory T cells. *Nat. Med.* **16**, 809–813 (2010).
43. P. Sagoo, N. Ali, G. Garg, F. O. Nestle, R. I. Lechler, G. Lombardi, Human regulatory T cells with alloantigen specificity are more potent inhibitors of alloimmune skin graft damage than polyclonal regulatory T cells. *Sci. Transl. Med.* **3**, 83ra42 (2011).
44. A. Schoenbrunn, M. Frentsch, S. Kohler, J. Keye, H. Dooms, B. Moewes, J. Dong, C. Loddenkemper, J. Sieper, P. Wu, C. Romagnani, N. Matzmohr, A. Thiel, A converse 4-1BB and CD40 ligand expression pattern delineates activated regulatory T cells (Treg) and conventional T cells enabling direct isolation of alloantigen-reactive natural Foxp3$^+$ Treg. *J. Immunol.* **189**, 5985–5994 (2012).
45. C. Scottà, M. Esposito, H. Fazekasova, G. Fanelli, F. C. Edozie, N. Ali, F. Xiao, M. Peakman, B. Afzali, P. Sagoo, R. I. Lechler, G. Lombardi, Differential effects of rapamycin and retinoic acid on expansion, stability and suppressive qualities of human CD4$^+$CD25$^+$FOXP3$^+$ T regulatory cell subpopulations. *Haematologica* **98**, 1291–1299 (2013).
46. B. Afzali, F. C. Edozie, H. Fazekasova, C. Scottà, P. J. Mitchell, J. B. Canavan, S. Y. Kordasti, P. S. Chana, R. Ellis, G. M. Lord, S. John, R. Hilton, R. I. Lechler, G. Lombardi, Comparison of regulatory T cells in hemodialysis patients and healthy controls: Implications for cell therapy in transplantation. *Clin. J. Am. Soc. Nephrol.* **8**, 1396–1405 (2013).
47. A. Siepert, S. Ahrlich, K. Vogt, C. Appelt, K. Stanko, A. Kühl, J. van den Brandt, H. M. Reichardt, H. Nizze, M. Lehmann, M. Tiedge, H. D. Volk, B. Sawitzki, P. Reinke, Permanent CNI treatment for prevention of renal allograft rejection in sensitized hosts can be replaced by regulatory T cells. *Am. J. Transplant.* **12**, 2384–2394 (2012).
48. X. Valencia, C. Yarboro, G. Illei, P. E. Lipsky, Deficient CD4$^+$CD25^{high} T regulatory cell function in patients with active systemic lupus erythematosus. *J. Immunol.* **178**, 2579–2588 (2007).
49. M. Battaglia, M. G. Roncarolo, Immune intervention with T regulatory cells: Past lessons and future perspectives for type 1 diabetes. *Semin. Immunol.* **23**, 182–194 (2011).
50. C. de Andrés, C. Aristimuño, V. de Las Heras, M. L. Martínez-Ginés, M. Bartolomé, R. Arroyo, J. Navarro, S. Giménez-Roldán, E. Fernández-Cruz, S. Sánchez-Ramón, Interferon beta-1a therapy enhances CD4$^+$ regulatory T-cell function: An ex vivo and in vitro longitudinal study in relapsing-remitting multiple sclerosis. *J. Neuroimmunol.* **182**, 204–211 (2007).
51. N. Gagliani, C. F. Magnani, S. Huber, M. E. Gianolini, M. Pala, P. Licona-Limon, B. Guo, D. R. Herbert, A. Bulfone, F. Trentini, C. Di Serio, R. Bacchetta, M. Andreani, L. Brockmann, S. Gregori, R. A. Flavell, M. G. Roncarolo, Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. *Nat. Med.* **19**, 739–746 (2013).
52. U. Schliesser, M. Chopra, A. Beilhack, C. Appelt, S. Vogel, J. Schumann, I. Panov, K. Vogt, S. Schlickeiser, S. Olek, K. Wood, C. Brandt, H. D. Volk, B. Sawitzki, Generation of highly effective and stable murine alloreactive T_{regs} by combined anti-CD4 mAb, TGF-β, and RA treatment. *Eur. J. Immunol.* **43**, 3291–3305 (2013).
53. P. Trzonkowski, E. Szmit, J. Myśliwska, A. Dobyszuk, A. Myśliwski, CD4$^+$CD25$^+$ T regulatory cells inhibit cytotoxic activity of T CD8$^+$ and NK lymphocytes in the direct cell-to-cell interaction. *Clin. Immunol.* **112**, 258–267 (2004).
54. S. N. Sherston, K. Vogt, S. Schlickeiser, B. Sawitzki, P. N. Harden, K. J. Wood, Demethylation of the TSDR is a marker of squamous cell carcinoma in transplant recipients. *Am. J. Transplant.* **14**, 2617–2622 (2014).
55. J. K. Polansky, K. Kretschmer, J. Freyer, S. Floess, A. Garbe, U. Baron, S. Olek, A. Hamann, H. von Boehmer, J. Huehn, DNA methylation controls Foxp3 gene expression. *Eur. J. Immunol.* **38**, 1654–1663 (2008).
56. M. Vukmanovic-Stejic, Y. Zhang, J. E. Cook, J. M. Fletcher, A. McQuaid, J. E. Masters, M. H. Rustin, L. S. Taams, P. C. Beverley, D. C. Macallan, A. N. Akbar, Human CD4$^+$ CD25^{hi} Foxp3$^+$ regulatory T cells are derived by rapid turnover of memory populations in vivo. *J. Clin. Invest.* **116**, 2423–2433 (2006).
57. M. K. Rahman, C. P. Offord, S. Khanna, G. C. Ford, X. M. Perrson, P. A. Svingen, Y. Xiong, Z. Bajzer, W. A. Faubion, Jr., Regulatory T cell kinetics in the peripheral blood of patients with Crohn's disease. *Hum. Immunol.* **74**, 145–150 (2013).
58. A. J. Managh, R. W. Hutchinson, P. Riquelme, C. Broichhausen, A. K. Wege, U. Ritter, N. Ahrens, G. E. Koehl, L. Walter, C. Florian, H. J. Schlitt, H. J. Reid, E. K. Geissler, B. L. Sharp, J. A. Hutchinson, Laser ablation-inductively coupled plasma mass spectrometry: An emerging technology for detecting rare cells in tissue sections. *J. Immunol.* **193**, 2600–2608 (2014).
59. A. J. Managh, S. L. Edwards, A. Bushell, K. J. Wood, E. K. Geissler, J. A. Hutchinson, R. W. Hutchinson, H. J. Reid, B. L. Sharp, Single cell tracking of gadolinium labeled CD4$^+$ T cells by laser ablation inductively coupled plasma mass spectrometry. *Anal. Chem.* **85**, 10627–10634 (2013).
60. E. Sharif-Paghaleh, K. Sunassee, R. Tavaré, K. Ratnasothy, A. Koers, N. Ali, R. Alhabbab, P. J. Blower, R. I. Lechler, L. A. Smyth, G. E. Mullen, G. Lombardi, In vivo SPECT reporter gene imaging of regulatory T cells. *PLOS ONE* **6**, e25857 (2011).
61. W. D. Döcke, C. Höflich, K. A. Davis, K. Röttgers, C. Meisel, P. Kiefer, S. U. Weber, M. Hedwig-Geissing, E. Kreuzfelder, P. Tschentscher, T. Nebe, A. Engel, G. Monneret, A. Spittler, K. Schmolke, P. Reinke, H. D. Volk, D. Kunz, Monitoring temporary immunodepression by flow cytometric measurement of monocytic HLA-DR expression: A multicenter standardized study. *Clin. Chem.* **51**, 2341–2347 (2005).
62. B. Sawitzki, C. Brunstein, C. Meisel, J. Schumann, K. Vogt, C. Appelt, J. M. Curtsinger, M. R. Verneris, J. S. Miller, J. E. Wagner, B. R. Blazar, Prevention of graft-versus-host disease by adoptive T regulatory therapy is associated with active repression of peripheral blood Toll-like receptor 5 mRNA expression. *Biol. Blood Marrow Transplant.* **20**, 173–182 (2014).
63. H. Waldmann, S. Cobbold, Regulating the immune response to transplants. a role for CD4$^+$ regulatory cells? *Immunity* **14**, 399–406 (2001).
64. M. Streitz, T. Miloud, M. Kapinsky, M. R. Reed, R. Magari, E. K. Geissler, J. A. Hutchinson, K. Vogt, S. Schlickeiser, A. H. Kverneland, C. Meisel, H. D. Volk, B. Sawitzki, Standardization of whole blood immune phenotype monitoring for clinical trials: Panels and methods from the ONE study. *Transp. Res.* **2**, 17 (2013).
65. N. Safinia, T. Vaikunthanathan, H. Fraser, C. Scotta, R. Lechler, G. Lombardi, PTU-122 A GMP T_{regs} expansion protocol restores Treg suppressor function and in end-stage liver disease; Implications for adoptive transfer therapy. *Gut* **63** (suppl. 1), A92–A93 (2014).
66. U. Schulze, M. Ringel, What matters most in commercial success: First-in-class or best-in-class? *Nat. Rev. Drug Discov.* **12**, 419–420 (2013).

Acknowledgments: The paper is supported by EU COST Action BM1305 www.afactt.eu. **Support:** P.T. received support from the National Centre for Research and Development, Poland (STRATEGMED1/233368/1/NCBR/2014); D.B. received support from the Swedish Medical Research Council (VR/K2011-65X-12219-15-6); M.G.R. received support from the EU-grant RISET for ALT-TEN trial; N.C. holds a postdoctoral fellowship from the Fund for Scientific Research (FWO)–Flanders (Belgium); M.B., A.B., G.L., and E.K.G. received support from the EU-FP7 grant The ONE Study. A.S. and J.A.M. received support from the EU-FP7 grant 601722. E.M.M. received support from the Instituto de Salud Carlos III (Spain), Projects PI11/02416 and PI14/01175. N.M.-T. received support from the National Science Centre, Poland (DEC-2011/01/D/NZ3/00262) and Polish Ministry of Science (JP2011 033771) **Conflict of interest:** P.T. and N.M.-T. are co-inventors of a patent related to the presented content. P.T. and the Medical University of Gdańsk received support from Neostem and Miltenyi Biotec. D.B. is one of the founders of iReg Medical. H.R.B. is employed by Miltenyi Biotec GmbH.

10.1126/scitranslmed.aaa7721

Citation: P. Trzonkowski, R. Bacchetta, M. Battaglia, D. Berglund, H. R. Bohnenkamp, A. ten Brinke, A. Bushell, N. Cools, E. K. Geissler, S. Gregori, S. M. van Ham, C. Hilkens, J. A. Hutchinson, G. Lombardi, J. A. Madrigal, N. Marek-Trzonkowska, E. M. Martinez-Caceres, M. G. Roncarolo, S. Sanchez-Ramon, A. Saudemont, B. Sawitzki, Hurdles in therapy with regulatory T cells. *Sci. Transl. Med.* **7**, 304ps18 (2015).