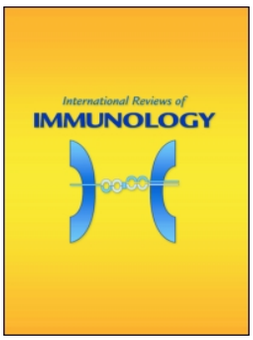

International Reviews of Immunology

ISSN: 0883-0185 (Print) 1563-5244 (Online) Journal homepage: https://www.tandfonline.com/loi/iiri20

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To cite this article: Amit Sharma & Dipayan Rudra (2019): Regulatory T cells as therapeutic targets and mediators, International Reviews of Immunology, DOI: 10.1080/08830185.2019.1621310

To link to this article: https://doi.org/10.1080/08830185.2019.1621310

Published online: 02 Jun 2019.

INTERNATIONAL REVIEWS OF IMMUNOLOGY
https://doi.org/10.1080/08830185.2019.1621310

Regulatory T cells as therapeutic targets and mediators

Amit Sharma[a,b] and Dipayan Rudra[a,b]

[a]Academy of Immunology and Microbiology, Institute for Basic Science (IBS), Pohang, Republic of Korea; [b]Division of Integrative Biosciences & Biotechnology, Pohang University of Science and Technology (POSTECH), Pohang, Republic of Korea

ABSTRACT

With the advent of the concept of dominant tolerance and the subsequent discovery of $CD4^+$ regulatory T cells expressing the transcription factor FOXP3 (Tregs), almost all productive as well as nonproductive immune responses can be compartmentalized to a binary of immune effector T cells and immune regulatory Treg populations. A beneficial immune response warrants the timely regulation by Tregs, whereas a nonproductive immune response indicates insufficient effector functions or an outright failure of tolerance. There are ample reports supporting role of Tregs in suppressing spontaneous auto-immune diseases as well as promoting immune evasion by cancers. To top up their importance, several non-immune functions like tissue homeostasis and regeneration are also being attributed to Tregs. Hence, after being in the center stage of basic and translational immunological research, Tregs are making the next jump towards clinical studies. Therefore, newer small molecules, biologics as well as adoptive cell therapy (ACT) approaches are being tested to augment or undermine Treg responses in the context of autoimmunity and cancer. In this brief review, we present the strategies to modulate Tregs towards a favorable clinical outcome.

ARTICLE HISTORY
Received 4 February 2019
Accepted 15 May 2019

KEYWORDS
Treg; Foxp3; regulatory T cells; cancer; tumor immunity; immune therapy

Enforcing dominant peripheral tolerance, a tiny subset of helper T cells – $CD4^+FOXP3^+$ regulatory T cells (Tregs) has been at the forefront of immunological research for almost last two decades. The singular importance of Tregs can be appreciated by the fact that both in mice and humans, a loss or defect of Tregs invariably leads to catastrophic breach of immune tolerance [1–3]. Tregs not only ensure tolerance to self-antigens and innocuous commensals but also regulate an ongoing immune response leading to resolution of inflammation [4–6], thus, maintaining overall immune homeostasis.

Emergence of Tregs as clinically relevant immune modulators

The idea of so called 'suppressor' cells of thymic origin started taking shape when it was demonstrated that B cells could not be made tolerant to Sheep Red Blood Cells (SRBC) in absence of thymus derived T cells [7]. Another group demonstrated development of various autoimmune disorders after thymectomy in 3-day old neonatal mice [8]. However, a lack of specific marker precluded efficient identification and characterization of these cell types with potential immune-suppressive properties. Sakaguchi et al. [9] showed that 3 day thymectomized mice had reduced number of $CD4^+CD5^{hi}$ (then called $Lyt\text{-}1^+Lyt\text{-}2,3^-$) T cells and reconstitution with such cells rescued the autoimmune reaction in thymectomized mice. Two subsets of $CD4^+$ T cells, $CD4^+CD45Rb^{hi}$ and $CD4^+CD45Rb^{lo}$ T cells were described in rats, wherein $CD45Rb^{hi}$ cells were IFN-γ producing Th1 type inflammatory T cells and $CD45Rb^{lo}$ cells were IL4 and IL10 producing T cells which protected against cell mediated immunity by reducing production of IFN-γ [10]. Expanding this characterization in mice, it was found that $CD4^+CD45Rb^{lo}$ cells did not produce IL-4 but protected against adoptively transferred $CD4^+CD45Rb^{hi}$ induced colitis in mice [11]. Nonetheless, the elusive specific identity of suppressive T cells could only be ascertained by seminal work of Sakaguchi et al. [12] after a decade of their earlier report. In two successive studies, the group showed that high affinity IL-2 receptor (IL-2Rα, CD25) was the specific marker on suppressive cells which were both $CD5^{hi}$ and $CD45Rb^{lo}$ [12]. These $CD4^+CD25^+$ T cells started appearing after day 3 of birth in mouse

CONTACT Dipayan Rudra rudrad@ibs.re.kr Academy of Immunology and Microbiology, Institute for Basic Science (IBS), Pohang 37673, Republic of Korea.

and expanded till day 10 [13]. The group named the cells regulatory T cells. However, CD25 as a marker of Tregs was not specific, owing to increased expression on large proportion of activated effector T cells.

In 1991, Godfrey et al. [14] characterized a mouse line which originated from a spontaneous X-linked recessive mutation at Oak ridge national laboratory under Manhattan project in USA. This mouse presents a severe lymphoproliferative phenotype with hypergammaglobulinemia, anemia, runting and so forth. leading to early death. The animals typically have severe skin inflammation resulting in a scaly appearance giving them the name 'Scurfy' mice. Crossing these mice with athymic nude mice resulted in complete protection from the disease indicating thymic T cell mediated defect. Extensive genomic analyses identified a 2-bp insertional mutation in a gene on X-chromosome of scurfy mice [15]. This gene had high homology with DNA binding domain of forkhead family of proteins and thus, was named as Forkhead box P3 – *Foxp3*. The mutations in homologous *FOXP3* gene were also found, almost simultaneously, [1,16] in the human patients of Immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome [17]. Phenotype of IPEX patients and Scurfy mice led to probing of *Foxp3* in Tregs. Finally, in 2003, three separate groups confirmed Foxp3 as a lineage defining transcription factor exclusively expressed in $CD4^+CD8^-CD25^+$ thymocytes and $CD4^+CD25^+$ peripheral T cells in mice [18–20]. $Foxp3^{eGFP}$ reporter mice established expression of Foxp3 in $TCR\beta^+$ T cells in the hematopoietic compartment and its conditional deletion in $CD4^+$ T cells led to scurfy phenotype in mice [18]. These discoveries paved the way for intense investigations into the field of Treg biology as well as maintenance and transcription program of Foxp3 [21–24].

Numerous mechanisms including direct cytolysis [25], production of inhibitory cytokines like TGF-β, IL-10 and IL-35 [26], inhibitory cell surface molecules like cytotoxic T lymphocyte antigen 4 (CTLA-4) [27], PD-1 [28], TIM-3 [29] and so forth. have been implicated as Treg mediated suppressive modalities hereafter. Tregs also limit supply of growth factors [30] as well as activating antigens [31] to effector T cells. Identification of Tregs in different non-lymphoid organs which adapt to the prevalent atmosphere makes them even more formidable in policing the immune system [32]. Thus, not surprisingly, a defect in number and/or function of Tregs is observed in a number of autoimmune and allergic diseases in humans [33]. On the other hand, sustained Treg response becomes a detrimental challenge in cancers. Therefore, manipulating Treg numbers and/or functions provides a two-way opportunity in ameliorating graft versus host diseases (GvHD) and other autoimmune disorders on one hand and cancers and other immunosuppressive disorders on the other. Here, we present the therapeutic strategies and approaches that are currently pursued to enhance Treg functions towards improvement of GvHD and other autoimmune disorders to establish an operational tolerance. We also discuss the modalities being explored to reduce or abrogate suppressive effects of Tregs to stop immune evasion by cancers. After enormous preclinical developments and discoveries in the field of Treg biology, Tregs are being pursued with lot of anticipation in clinic; hence, we also aim to describe various efforts with a clinical point-of-view.

(Re)establishing immune tolerance: strategies to strengthen Tregs

To heal the breach in immune tolerance in considerable number of autoimmune diseases and allergies or to reeducate the immune system for tolerance to alloantigens in transplant patients, strengthening the professional regulatory cells, that is Tregs is a compelling mode of remedial intervention. This can be achieved by increasing the brute number of Tregs and/or enhancing their suppressive functions (Table 1).

Adoptive cell (Treg) therapy – ACT-Treg

One of the simplest ways to increase Treg numbers is to infuse those in the patient, which creates an intended regulatory immune response. Here, we describe various approaches which are being utilized for adoptive transfer of Tregs in patients with pertinent indications.

Polyclonal Tregs

The preclinical proof of concept of legitimacy of adoptive Treg transfer was available at the onset of identification of the specific marker for Tregs, that is CD25. Asano et al., [13] showed that autoimmune reaction after neonatal thymectomy could be reversed by transfer of $CD4^+CD25^+$ T cells. Subsequently, several studies have documented immunosuppressive effect of adoptively transferred Tregs in various preclinical animal models [34–36]. In humans, decrease in Tregs was observed in acute transplant rejections and an increase was observed in patients developing

Table 1. Examples of clinical trials aiming to enhance Treg functions various clinical trials described in the text employing strategies to enhance Treg functions are listed.

Strategy	Study title	*ClinicalTrials.gov* identifier (NCT number)	Medical condition	Primary formulation/regimen	Primary objective	Phase	Status	Sponsor	References
Adoptive Cell Therapy	The ONE Study UK Treg Trial	NCT02129881	End-stage Renal Failure	In vitro expanded autologous regulatory T Cell Product (1–10 million cells/kg) infused intravenously 5 days post renal transplantation	safety and feasibility of regulatory T (Treg) cell therapy in renal transplantation	Phase 1 Phase 2	Completed	Guy's and St Thomas' NHS Foundation Trust, UK	44, 45
	Safety and Efficacy Study of Regulatory T Cell Therapy in Liver Transplant Patients (ThRIL)	NCT02166177	End-stage Liver Disease	In vitro expanded autologous regulatory T cell therapy infused intravenously (2 dose groups: low dose and high dose)	feasibility, safety and efficacy of TR002, a regulatory cell therapy	Phase 1 Phase 2	Completed	Guy's and St Thomas' NHS Foundation Trust, UK	45, 46
	T1DM Immunotherapy Using CD4 + CD127lo/-CD25+ Polyclonal Tregs (Treg)	NCT01210664	Type 1 Diabetes Mellitus	Ex vivo Expanded Human Autologous Polyclonal Regulatory T Cells by infusion. Four dose groups: 0.05×10^8 cells, 0.4×10^8 cells, 3.2×10^8 cells and 26×10^8 cells.	safety and feasibility of intravenous infusion of ex vivo selected and expanded autologous polyclonal Tregs in patients with type 1 diabetes (T1DM) to support dose selection for a future efficacy trial	Phase 1	Completed	University of California, San Francisco	52, 53
	T-Regulatory Cell Infusion Post Umbilical Cord Blood Transplant in Patients with Advanced Hematologic Cancer	NCT00602693	Leukemia Lymphoma Multiple Myeloma Plasma Cell Neoplasm Myelodysplastic Syndromes	HLA matched umbilical cord blood Tregs, in vitro expanded with artificial APCs. Dose escalation ranges included 1, 3, 10, 30, 100, 300 1000 and 300×10^5 Treg/kg.	Determination of the maximum tolerated dose (MTD) of umbilical cord blood (UCB)-derived T-regulatory (Treg) cells.	Phase 1	Completed	Masonic Cancer Center, University of Minnesota	54
IL-2 induced Treg expansion	Evaluation of Clinical Efficacy and Immunologic Response After IL-2 Therapy in HCV-related Vasculitis Patients	NCT00574652	Cryoglobulinemia Vasculitis	Four cycle of subcutaneous IL-2 (Proleukin) therapy (3 millions IU/day from day 1 to 5 every 21 days a Week 1, 3, 6 and 9).	Immunologic follow-up of Treg and of HCV cellular immune response before, during and after IL-2 therapy	Phase 1 Phase 2	Completed	French National Agency for Research on AIDS and Viral Hepatitis	77
	Ultra-Low Dose Interleukin-2 for Refractory Chronic Graft Versus Host Disease	NCT00529035	Graft Versus Host Disease	Three doses of IL-2 - 0.3×10^6 IU/m^2/day, 1.0×10^6 IU/m^2/day and 3.0×10^6 IU/m^2/day subcutaneously	The maximum tolerated dose of IL-2 over 8 weeks in cGVHD patients with an inadequate response to steroids	Phase 1	Active, not recruiting	Dana-Farber Cancer Institute	78
	Low-dose IL-2 for Treg Expansion and Tolerance (LITE)	NCT02949492	Transplantation Liver Diseases	4 weeks of low dose IL-2 (Proleukin) therapy followed by 4 weeks of low dose IL-2 and withdrawal of immunosuppressive therapy upon ascertaining at least 2-fold increase in peripheral Treg numbers, stable liver functions and no adverse reactions and sub-clinical graft rejection.	To determine the capacity of a short course of low-dose IL-2 to facilitate the complete discontinuation of immunosuppressive drugs in liver recipients 2–6 years after transplantation.	Phase 4	Recruiting	King's College London	

(*Continued*)

Table 1. Continued.

Strategy	Study title	*ClinicalTrials.gov* identifier (NCT number)	Medical condition	Primary formulation/regimen	Primary objective	Phase	Status	Sponsor	References
	Low-dose IL-2(Interleukin-2) Treatment in SLE	NCT02084238	Systemic Lupus Erythematosus	1 million units of recombinant human Interleukin-2 every alternate day for 14 days followed by 14 days rest (3–6 cycles)	Number of SLE responders in participants over course of therapy	NA	Completed	Peking University People's Hospital	79
	Biological Activity and Safety of Low Dose IL2 in Relapsing Remitting Multiple Sclerosis (MS-IL2)	NCT02424396	Relapsing Remitting Multiple Sclerosis	IL-2 or placebo each day for 5 days (induction), followed by a maintenance course at the same dose or placebo every two weeks over 6 months.	Treg response to low dose IL2 induction course period	Phase 2	Recruiting	Assistance Publique - Hôpitaux de Paris	
	Induction of Regulatory t Cells by Low Dose il2 in Autoimmune and Inflammatory Diseases (TRANSREG)	NCT01988506	14 autoimmune pathologies	Proleukin Induction: low-dose IL-2 (1 MUI/day, sc) for 5 days Maintenance: 1MUI once every 15 days (except systemic lupus erythematosus's patients every 7 days) for 6 months	Change in Treg percentage (percentages of Tregs within the CD4+ lymphocytes) at Day-8 after administration of low-dose of IL2 compared to baseline (Day-0)	Phase 2	Recruiting	Assistance Publique - Hôpitaux de Paris	
	Administration of Low-dose IL-2 in Established T1D (IL-2)	NCT03243058	Diabetes Mellitus, Type 1	Proleukin 0.5 million IU/m^2 (up to a maximum of 1 million IU), or placebo, will be given for 5 consecutive days (days 1–5), and then on day 15 and every 15 days thereafter, for one year. After 1-year treatment arm subjects will be randomized into two groups of continued treatment or placebo.	C-peptide response (preservation of insulin secretion) at 1 year	Phase 1 Phase 2	Not yet recruiting	University of Miami	
	Low Dose IL-2 for Ulcerative Colitis	NCT02200445	Ulcerative Colitis	8-week course of once daily IL-2 sc. 3 treatment dosage groups: 0.3×10^6 IU/m^2/day, 1.0×10^6 IU/m^2/day and 1.5×10^6 IU/m^2/day.	To identify serious and non-serious adverse drug events	Phase 1	Recruiting	Boston Children's Hospital	
	A Phase II Study with Low-dose Recombinant Human IL-2 for the Treatment of Primary Sjögren's Syndrome	NCT02464319	Primary Sjögren's Syndrome	hrIL-2 (1 million units) every other day sc for a period of 14 days. After a 14-day rest, another cycle started for 3 more cycles	Improvement in EULAR Sjögren's syndrome disease activity index (ESSDAI)	Phase 2	Completed	Peking University People's Hospital	
TNFα - TNFRII mediated Treg expansion	Regulatory T-cells in Psoriasis Patients as Targets for Therapy	NCT01233583	Psoriasis	Dovobet, neotigason, etanercept, adalimumab. Infliximab (observational, treatment allocation is not part of the study)	correlations of treatment outcome in psoriasis patients assessed by PASI score and PGA, with changes in the balance between Treg and effector T cells	–	Completed	University of Aberdeen	

All data are derived from https://clinicaltrials.gov.

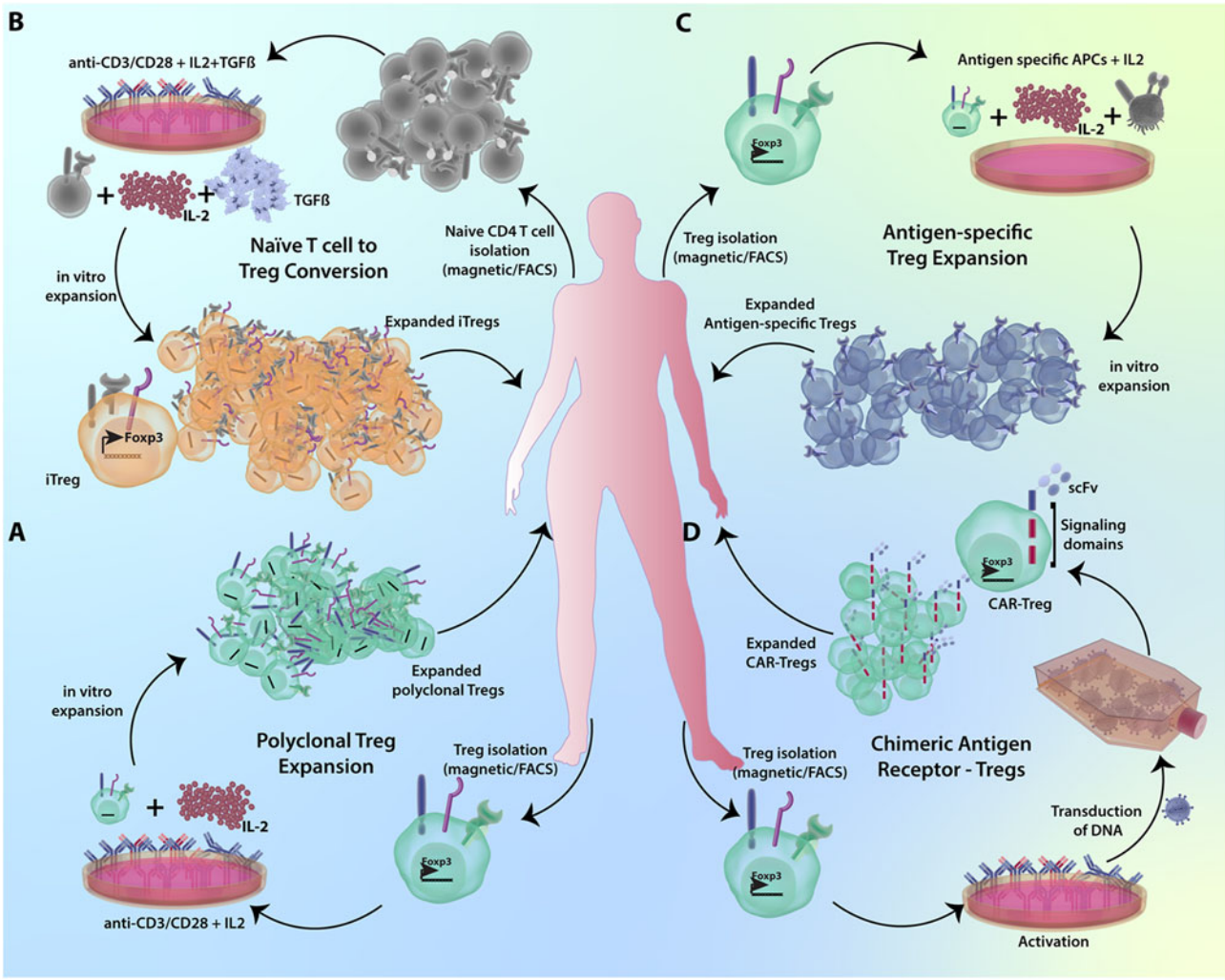

Figure 1. Strategies for ex vivo expansion of Tregs for adoptive cell therapy (ACT). (A) Autologous peripheral white blood cells are collected by leukapheresis. Natural Tregs ($CD4^{+}CD25^{hi}CD127^{low/-}$) (nTregs) are purified by magnetic enrichment or fluorescence associated cell sorting (FACS). Highly purified nTregs are activated by coated anti-CD3/CD28 antibodies in presence of high amount of IL-2. Activated Tregs are expanded ex vivo for several days and are ready for ACT after quality assessments. (B) Naïve T cells enriched from peripheral white blood cells can be induced into Tregs (induced Tregs, iTregs) by activation with anti-CD3/CD28 antibodies in presence of TGFβ and IL2. iTregs are further expanded for several days in presence of IL2 before those can be used for ACT. (C) For manufacturing alloantigen specific Tregs, purified nTregs are activated with donor antigen presenting cells (APC) or artificial APCs pulsed with given antigen in presence of high IL-2. Cells are expanded for several days before ACT post-quality assessment. (D) Chimeric antigen receptor – Tregs (CAR-Tregs) are manufactured by activation and subsequent transduction of chimeric DNA. Transduced cells are expanded ex vivo for several days before ACT. scFv- Single chain variable region fragment

spontaneous tolerance post-transplantation [37–39]. FOXP3, being a nuclear resident transcription factor, cannot be used for isolation of peripheral circulating Tregs in humans. First human Treg adoptive cell therapy (ACT) report [40] described clinical efficacy by transfer of $CD4^{+}CD25^{+}CD127^{-}$ Tregs in related acute and chronic GvHD patients (Figure 1A). FOXP3 and CD127 expressions inversely correlate in human Tregs [41]. Thus, $CD4^{+}CD25^{+}CD127^{low/-}$ Treg phenotype has largely been utilized for fluorescence assisted cell sorting. To establish tolerance to a graft, the extrapolated calculations from preclinical models suggest that there should be an increase in 33–50% of total Tregs [42]. This increase can only be achieved by infusion of about $3–5 \times 10^{9}$ Tregs after lymphodepletion in the host [42]. As a single leukapheresis can provide about 0.2×10^{9} Tregs, the required dose of Tregs can only be produced by expanding them ex vivo with anti-CD3/CD28 antibodies and high concentration of IL-2, taking about 2 weeks for requisite expansion [43]. While looking for stable Treg phenotype after expansion, it was found that $CD45RA^{+}$ naïve Tregs provide more stable and suppressive Tregs after ex vivo expansion [43].

A phase I/II dose escalation study for autologous ex vivo expanded Tregs in kidney transplant recipients

– The ONE study (NCT02129881) [44,45] and a phase I/II low and high dose autologous Treg adoptive therapy in liver transplant patients – ThRIL trial (NCT02166177) [45,46] are in patient follow up stage and no serious adverse effects have been observed, suggesting safety of adoptive therapy of autologous polyclonal Tregs. In type I diabetes mellitus (T1DM), autoimmune loss of pancreatic islet β cells is primary pathology [47]. In animal models of T1DM, it was shown that $CD4^+CD25^+$ Tregs are protective [48,49]. Initial clinical trial of Treg ACT in a cohort of recently diagnosed T1DM children provided safety as well as efficacy of the therapy, as no adverse effects were observed in two dose groups along with increase in C-peptide levels, an indicator of pancreatic β cell function, and a decrease in requirement for exogenous insulin [50,51]. Another phase I, dose escalation (0.05×10^8 to 26×10^8 cells) study in T1DM patients reported no adverse reactions with ex vivo expanded $CD4^+CD25^+CD127^{low/-}$ polyclonal Treg ACT (NCT01210664) [52,53]. The authors reported no serious infusion or cell therapy related adverse effects in any cohort. Also, the long term monitoring of transferred Tregs (by non-radioactively labeling them with [6,6-$2H_2$]glucose suggested that a subset (~25%) of Tregs was long lived and persisted up to 1 year post-transfusion [53].

Instead of peripheral blood Tregs, attempts have been made to isolate and expand Tregs from cryopreserved umbilical cord blood (UCB). In the first dose escalation study (NCT00602693) for safety and feasibility of partial human leukocyte antigen matched UCB derived $CD4^+/CD25^+$ Tregs, a median 211-fold expansion was reported after 18 days culture with anti-CD3/CD28 beads and human IL-2. The study reported no infusion related adverse effects in acute GvHD patients. For the higher yield of expanded Tregs, UCB derived Tregs were expanded with artificial APCs (K562 cells modified to express high-affinity Fc receptor (CD64) and CD86, natural ligand of CD28) [54] (NCT00602693). There was a median 13000-fold expansion in Tregs. Authors reported no infusion related adverse events and reduced risk of acute GvHD [54].

By appropriate treatment (Costimulation, TCR activation, TGFβ and IL-2), conventional naïve T cells can be converted to induced Tregs (iTregs) in vitro (Figure 1B). This protocol enables us to use naïve T cells from peripheral blood to manufacture iTregs for ACT as natural Tregs are comparatively rare. However, iTregs are known to be plastic [55] and under an inflammatory host environment, can convert to effector T cells and worsen the disease. Therefore, it is imperative to understand the molecular mechanisms which underlie such transmutations in iTreg phenotypes. Pharmacological agents which stabilize expression of *FOXP3* in iTregs may positively affect Treg mediated ACT. Recently, we have reported that sister transcription factor of Foxp3, Foxp1 [56] is essential for maintenance of Foxp3 expression in mouse iTregs. A loss of Foxp1 leads to gradual loss of Foxp3 and results in intestinal inflammation in aged mice. On the other hand, a major player promoting the plasticity of Foxp3 under inflammatory settings was found to be the transcriptional regulator Id2 [57], which when ectopically overexpressed in iTregs, sequesters the transcriptional regulator E protein E2A in the cytoplasm leading to generation of Th17 cells in Treg differentiation environment. A forced demethylation of *Foxp3* Treg specific demethylated region, which is methylated in conventional T cells by agents like DNMT1 inhibitors as 5-aza-2-deoxycytidine (5-Aza) can stabilize the FOXP3 expression in Tregs [58,59].

Antigen specific Treg and chimeric antigen receptor (CAR) Treg therapy

Unspecified and widespread specificities of polyclonal Tregs somewhat undermine their efficacy. In inducing transplant tolerance, it is found that donor alloantigen specific Tregs are five to ten times more potent than polyclonal Tregs [60,61]. The efficacy of these Tregs was comparable to the frequency of alloantigen specific Tregs in the polyclonal pool (Figure 1C). Thus, in theory an ACT with antigen specific Treg should require five to ten-fold less cells. Also, the property of bystander suppression by Tregs suggests that Tregs of a unique antigen specificity can also function against other possible MHC antigens for inducing operational tolerance. Several clinical trials with alloantigen specific Tregs towards liver and kidney transplant patients are being pursued at present [62].

A rather potent antigen specificity is offered by chimeric antigen receptor (CAR) Tregs (Figure 1D). Low number of Tregs in body, and even rare frequency towards specific alloantigens makes CAR-Tregs a suitable candidate. CAR-T cells can interact with antigens in an MHC independent manner like B cells and there is a high freedom of modulation with CARs. In a first, Elinov et al., developed a transgenic mouse with all T cells expressing a tripartite chimeric receptor made of single-chain variable region fragment (scFv) specific to hapten 2,4,6- trinitrophenol (TNP) as recognition unit linked to CD28 costimulatory domain and FcRγ

immune receptor tyrosine-based activation motifs-anti-TNP scFv-CD28-FcRγ tripartite chimeric receptor (TPCR) to activate the T cells specifically in response to model antigen TNP [63]. The TNP-CAR Tregs were specifically activated by TNP and were protective against TNBS induced colitis which generates TNP-haptenized cell surface antigens in colon [63]. CAR-Tregs were shown to accumulate at the site of inflammation in autoimmunity after systemic administration in an antigen dependent manner [64]. CAR-Tregs targeting human HLA-A2 antigen are also reported, which suppress GvHD upon allogenic hematopoietic stem cell transplantation [65]. Yoon et al. reported a CAR-Treg against factor VIII (Factor VIII's replacement therapy in hemophilic patients generally induces an immune response) [66]. These FVIII-CAR Tregs suppressed both T and B cell effector response to FVIII.

CAR-Tregs come with their own safety and survival issues. The dependence of Tregs for survival on paracrine IL-2 sourced from other cells is a constraint. Efforts are on to produce CAR-Tregs which can produce and utilize their own IL-2 in an autocrine fashion. Tregs tend to be plastic in an inflammatory environment and can turn into effector T cells. Therefore, engineering the cells by deleting inflammatory cytokine genes as a strategy is proposed. Also, these Tregs can be developed with inducible 'suicide switches' to eliminate all CAR-Tregs, on demand [67,68]. USA-based Sangamo Therapeutics is planning a CAR-Treg (TX200) clinical trial for Kidney transplant patients, wherein, Sangamo wants to apply their expertise in zinc finger nuclease gene editing technology towards increasing safety and survival of CAR-Tregs [69].

Strategies for in vivo Treg expansion

All the constraints of ACT can be overcome by suitable approaches to expand and enhance Tregs in vivo. The amenability of these methods to simpler clinical paraphernalia makes those even attractive.

IL-2 therapy for Treg expansion

Common γ-chain cytokine Interleukin-2 is a growth factor for various lymphocytes. Its therapeutic potential was initially utilized in co-therapy with autologous natural killer cells [70] and subsequent monotherapy for metastatic tumors in humans. The treatment established importance of immunotherapy for treating cancers. High dose IL-2 therapy presented several adverse effects like fluid extravasation due to capillary leak and so forth, however, most of those were reversible upon cessation of treatment [71]. Paradoxically though, deletion of units of IL-2 receptor (IL2Rα and IL2Rβ) resulted in proliferative immune disorders instead of expected immunosuppression [72,73] in mice, suggesting a role of IL-2 in immune regulation. While Treg development and suppression potential in IL-2 or IL-2 receptor deficient animals was largely intact, its maintenance at the periphery was subdued [21]. It was reported that IL-2 administration expanded the Treg pool in mice [74] as well as humans [75]. The higher expression of high affinity IL-2Rα (CD25) indicated a lower threshold requirement of IL-2 for Tregs [73]. Indeed, a short span (5 days), low dose IL-2 administration was found to expand and activate Tregs in pancreatic islets in diabetes onset NOD mice [76]. Subsequent, clinical trial NCT00574652 reported around 420% increase in Treg numbers in peripheral blood in HCV-Induced Vasculitis human patients [77] (Figure 2A). Daily low-dose IL-2 administration (NCT00529035) to glucocorticoid refractory active chronic graft versus host disease (GvHD) patients was found safe [78]. This therapy increased Treg numbers to more than 8 times of baseline numbers without changing the conventional T cell numbers leading to significant amelioration of chronic GvHD [78]. Currently, Low-dose IL-2 for Treg Expansion and tolerance (LITE) study NCT02949492, a phase 4 clinical trial to wean the liver transplant patients from immunosuppressive drugs by expanding the Treg pool by administering low dose IL-2 is being tested. Similarly, studies assessing the effect of IL-2 therapy in SLE (NCT02084238) [79], relapsing remitting multiple sclerosis (NCT02424396, MS-IL2 trial), a phase 2 trial in a set of 14 autoimmune and auto-inflammatory disorders (TRANSREG trial - NCT01988506), a multicenter phase I/II clinical trial to evaluate the safety as well as efficacy of low dose IL-2 in prevention of further loss of beta-cell function in patients with established T1D (NCT03243058), ulcerative colitis (NCT02200445), Primary Sjögren's Syndrome (pSS) (NCT02464319) and so forth are being conducted, as described in www.clinicaltrials.gov.

Boyman et al., [80] discovered that IL-2 and anti-IL-2 monoclonal antibodies when given in vivo can potentiate the IL-2 effect. It was observed that different monoclonal antibodies can expand either $CD8^+$ T cells (Clone S4B6) or Tregs (Clone JES6-1), presumably by increasing the biological activity of IL-2 by forming immune complexes (Figure 2A). Indeed, pre-formed IL-2 anti-IL-2 mAb immune complexes increased Tregs

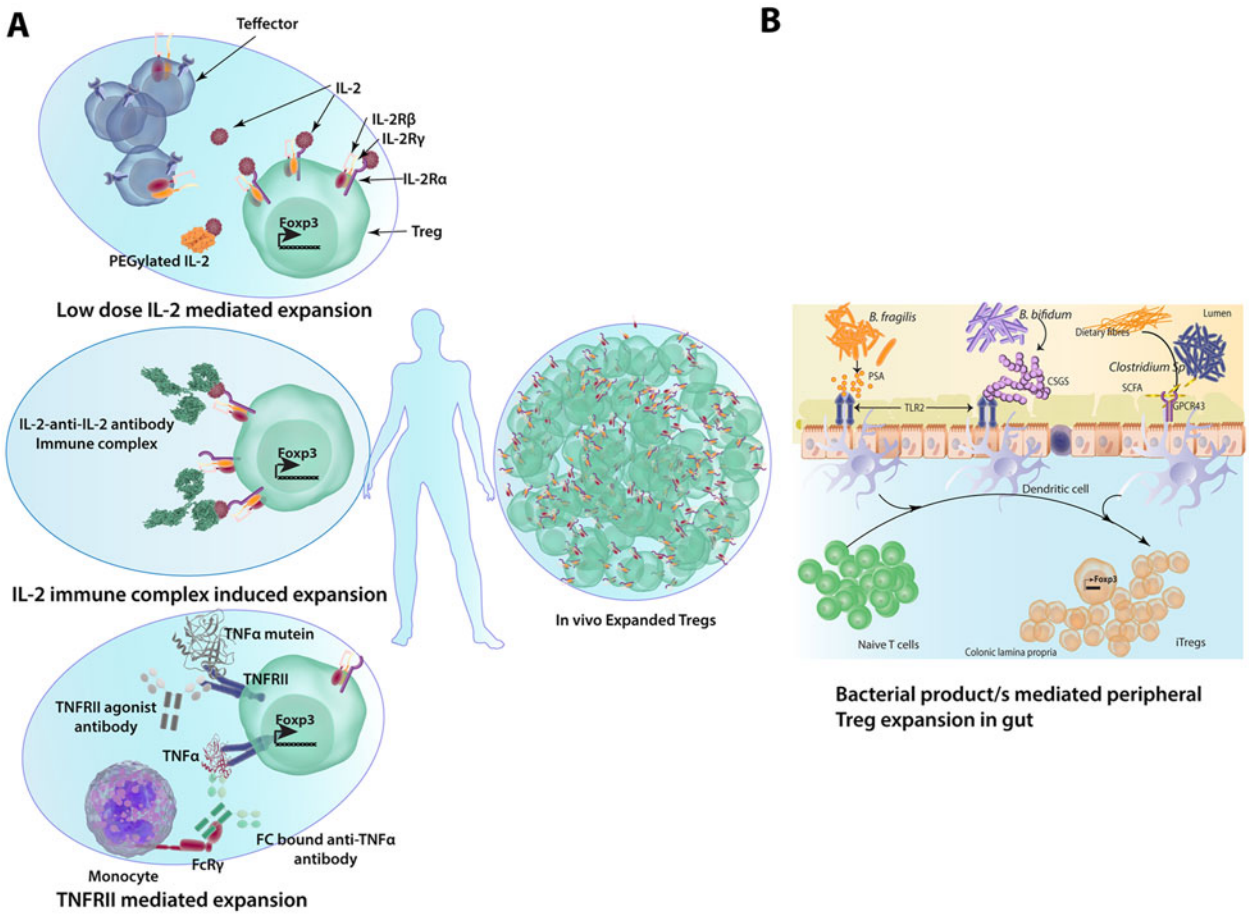

Figure 2. Approaches for in vivo expansion of Tregs. (A) Top, Low dose IL-2 therapy results in selective Treg expansion owing to high density of high affinity IL-2Rα on Tregs. Similarly, low dose long half-live IL-2 (PEGylated IL-2) also selectively induces Treg expansion. Middle, IL-2 and anti-IL-2 antibody immune complexes with certain monoclonal antibodies like JES6-1 selectively expands Tregs owing to their ability to sterically hinder IL-2 binding to low affinity IL-2 receptors on effector T cells. Bottom, Activation of TNFRII on Treg surface by membrane bound TNFα, TNFα muteins or agonist antibodies specifically expands Tregs. Also, certain anti-TNFα antibodies like adalimumab bind to Fc receptors on monocytes and thus make membrane bound TNFα available to TNFRII on Treg surface leading to their selective expansion. (B) Certain commensal bacteria and their products like polysaccharide A from *Bacteroides fragilis* or Cell surface β-glucan/galactan (CSGG) of *Bifidobacterium bifidum* selectively expand colonic Tregs via a dendritic cell TLR2 dependent process.

in mice. Recently, by solving the crystal structure of JES6-1 monoclonal antibody in complex with IL-2, Spangler et al., [81] demonstrated that JES6-1 binding with IL-2 sterically hinders it is binding to IL2Rβ and IL-2Rγ and at the same time lowers the affinity to IL-2Rα by an allosteric modulation – 'trigger exchange'. This favored the IL-2 availability to Tregs which have very high IL-2Rα expression and expanded their population. Several studies have demonstrated successful Treg expansion and amelioration of immune disorders in mouse models like lupus nephritis [82], collagen induced arthritis [83], systemic lupus erythematosus-like chronic graft-Versus-host Disease [84] and development of immune tolerance in mice receiving lung transplants [85] and so forth. For testing, this approach in clinic, it is important to produce such potentiating antibodies in humans. In fact, recently, a fully human anti- IL-2 antibody F5111.2 has been reported [86] that when in complex with human IL-2, stabilizes IL-2 in a conformation which selectively sustains downward signaling in Tregs. F5111.2-hIL-2 immune complexes improved Treg/T conventional and Treg/T cytotoxic cell ratio by almost four folds in a dose dependent manner and successfully reduced type 1 diabetes remission in NOD mice, disease severity in experimental autoimmune encephalitis and xenogeneic graft versus host disease in mice [86].

Recently, synthetic peptide mimics of IL-2 has been described which bind to IL-2Rβ and γ chain heterodimer but have no binding with IL-2Rα [87]. Such strategies can be utilized for selective signaling via IL-2Rα and other receptor complexes for Treg expansion.

Tumor necrosis factor α mediated Treg expansion

Tumor necrosis Factor-α (TNF-α) is produced as a 26 KD type II transmembrane protein which forms a functional homotrimeric molecule after getting cleaved into a 17 KD soluble monomer by metalloprotease TNF-α converting enzyme [88,89]. TNF initiates NFkB signaling by ubiquitous TNFRI or immune cell specific TNFRII [90]. TNFRI is activated by both membrane bound and soluble TNF [91], TNFRII is activated by membrane bound TNF [92].

TNF-α is a classic inflammatory cytokine that can inhibit Treg suppressive potential by increasing PKC-θ recruitment to immunological synapse [93]. It also inactivates FOXP3 by inhibiting its phosphorylation [94]. Rather paradoxically, it was found that TNF interaction with TNFRII receptor promotes the expansion of Tregs which are highly suppressive [95,96] (Figure 2A). This interaction was found to be instrumental for Treg mediated effects in various animal models like autoimmune colitis [97] and EAE [98] and so forth. Specific TNF mutein – selective mouse TNF-based agonist of TNF receptor 2 (STAR2) increased Tregs by 2–4 folds which successfully suppressed acute allogenic stem cell transplantation (allo-HCT) induced GvHD in mice [99]. This selective TNFRII stimulation did not induce any appreciable activation of conventional T cells. A human TNFRII-specific STAR-2 equivalent TNC-scTNF (143 N/145R) was shown to expand human Tregs in vitro [99]. Similarly, TNFRII antagonistic antibody TR7554.7 was shown to abolish the protective effect of Tregs on allo-HCT induced GvHD [100]. Rather unexpectedly, it was found out that adalimumab, a therapeutic anti-TNF antibody, crosslinked membrane bound TNF on monocytes with TNFRII on Tregs, both isolated from patients with rheumatoid arthritis. This, in turn, facilitated the expansion of functional Tregs in vitro [101].

Human Tregs isolated from PBMC produce their own membrane bound TNF-α which can act as autocrine growth factor for Tregs by cross-linking TNF receptor type II, resulting in enhanced proliferation particularly when IL-2 is limiting [102]. Several pharmaceutical agents targeting human TNFRII have been described as TNFRII agonist antibodies like MR2-1 [103] and TNF muteins like TNF07 [104] and so forth. A detailed account can be found in this review [105]. Thus, standalone or as a co-therapy TNFRII mediated Treg expansion is a potential treatment strategy for various autoimmune, GvHD and chronic inflammatory diseases. One clinical study NCT01233583 has been testing effect of adalimumab and other drugs on Treg modulation in psoriasis patients.

Peripheral Treg expansion by microbes and their products

Several bacteria have been shown to increase the frequency of Tregs in gut associated lymphoid tissues (Figure 2B). *Bacteroides fragilis* has been shown to induce functional IL-10 producing Tregs in mice gut which were protective in the models of experimental colitis [106]. Oral administration of bacterial polysaccharide A (PSA) produced by *B. fragilis* in mice also reversed experimental colitis [106]. Later, PSA was reported to induce FOXP3 and CD39 in human $CD4^+$ T cells, in vitro [107]. Similarly, *Clostridium* strains induce higher frequency of functional Tregs in mice colon by inducing a TGFβ1 enriched environment [108]. Colonization with *Clostridium* and subsequent increase in Tregs was protective in animal models of inflammatory bowel disease and food allergy [108]. Later studies found that bacteria producing short chain fatty acids butyrate and propionate by metabolism of dietary fibers induce Tregs by working via GPCR43 and histone deacetylase inhibitory properties [109–111]. Oral feeding of rationally selected mixture of 17 strains of *Clostridia* from human feces, based on Treg frequency and high IL-10 and ICOS expression, increased Treg abundance and were protective in experimental colitis and allergic diarrhea in mice, suggesting a possible therapy for similar human conditions [112]. Other lactic acid bacteria like *Bacillus coagulans* 09.712 [113] and *Lactobacillus plantarum WCFS1* [114] are reported to increase Treg frequency. Recently, human probiotic *Bifidobacterium bifidum* strain PRI1 has been shown to de novo generate Tregs in mouse gut [115]. The authors found that Cell surface β-glucan/galactan (CSGG) polysaccharide of *B. bifidum* are the main component which increased the TGFβ production from dendritic cells largely through a TLR2 dependent manner. The Tregs generated were functional and provided significant protection against experimental colitis [115].

While most of these studies provide a future perspective wherein these microbial mixtures or their components can be used alone or in combination with other existing therapies, a lack of very specific mechanisms and biological importance of increased Treg generation over the normally present Tregs in homeostasis are not very clear.

Targeting Tregs for tumor immunotherapy: inhibition of Tregs

Highly immunosuppressive intra-tumoral environment helps the tumor to escape anti-tumor immune

response. While several suppressive cell types contribute to this tumor environment, Tregs are probably the most important suppressive cells, enriched in many type of tumors [116–119] and can constitute more than half of $CD4^+$ T cell compartment [120]. In animal models it has been demonstrated that Tregs contribute to early tumor initiation and subsequent progression [121,122]. A high ratio of T effector cells to Tregs has been indicative of successful immunotherapy in both mice and humans [123,124].

Earlier it was reported that certain conventional chemotherapeutic agents enhance their anti-tumor effect by inhibiting Treg cell function and viability [125,126]. Indeed, low dose cyclophosphamide and paclitaxel, both chemotherapeutic agents were found to be suppressing $FOXP3^+$ Tregs [127–129] (Table 2).

Intratumoral Treg depletion

Antibody therapies to block the binding of immune checkpoint molecules, on effector T cells, with their ligands have revolutionized cancer immunotherapy. A high expression of checkpoint molecules like CTLA-4, PD-1, TIM-3, LAG-3 and so forth. makes them ideal target for Treg depletion via antibody dependent cellular cytotoxicity (ADCC). Other than checkpoint inhibitors, specific chemokine receptors and other surface markers specifically expressed by intratumoral Tregs can also be utilized for similar purposes.

Treg depletion by diphtheria toxin administration in mouse models of cancers where Tregs express a receptor for Diphtheria toxin has been shown to curb the tumor progression both in autochthonous [130] and transplanted tumors [131]. However, Treg depletion in human tumors is constrained by absence of an exclusive tumor specific Treg surface marker. It is desirable to deplete only the intratumoral Tregs so that adverse autoimmune reactions due to peripheral Treg depletion can be avoided. Nevertheless, in such cases a depletion therapy is aimed at markers with relatively higher expression in Tregs than conventional T cells, so that a therapeutic window could be achieved.

IL-2/IL-2R based Treg depletion

A most direct inhibition of Treg mediated suppression is possible either by depletion of Tregs within tumors or blockade of their recruitment and/or intratumoral induction of Tregs, if any. Based on TCR repertoire overlap analysis, several reports have suggested that there is little to no intratumoral conversion of naïve or effector T cells into induced Tregs [132–135].

A high expression of high affinity IL2 receptor α (CD25) makes it a natural target for selective depletion of Tregs (Figure 3A). Thus, humanized anti-CD25 antibodies (daclizumab and basiliximab) have been assessed in clinical trials. Daclizumab in a combination with dendritic cell vaccine in metastatic melanoma patients, although depleted most peripheral Tregs, did not provide any significant effect in either generation of vaccine specific effector T cells or in progression free survival [136]. Similarly, basiliximab, an IgG1 chimeric mouse-human antibody has been shown to deplete Tregs and reduce tumor growth during dendritic cell vaccination in chemotherapy induced lymphopenia in mice with malignant melanoma [137]. This regimen also depleted Tregs in humans with glioblastoma [137]. Further effect of basiliximab in patients is being evaluated in clinical study NCT00626483. Recently, anti-CD25 antibody optimized for FCγR binding and ADCC has been reported to have better intra-tumoral Treg depletion as well as better therapeutic profile in combination with anti-PD1 check-point inhibitor [138]. IL-2 fused with diphtheria toxin (Denileukin difitox, Ontak and E-7777) or exotoxin A of *Pseudomonas spp.* (LMB-2) have been used to selectively kill Tregs (Figure 3A). Ontak is approved for cutaneous T-cell lymphoma. Studies have reported mixed response from Ontak treatment in various cancers and as co-therapy for vaccination in mice and humans [139–141]. It was found to deplete activated Tregs while sparing the resting ones [140]. Several clinical studies are evaluating it in various cancerous indications. E-7777 is a variant of Ontak with better bioavailability. Its phase I clinical trial (NCT01401530) has provided a manageable adverse effect profile and clinically meaningful anti-tumor activity in relapsed/refractory peripheral and cutaneous T cell lymphoma [142]. A phase II clinical study of LMB-2 (NCT00924170) in combination with chemotherapy, cyclophosphamide and fludarabine, has shown complete remission in adult T cell leukemia [143]. Accumulated evidence suggest that Treg depletion based on CD25 can be a good strategy in lymphopenic conditions, when high CD25 expressing effector T cells can be spared. High expression of CD25 by tumor cells and Tregs can potentiate the effect of IL-2 and toxin fusion proteins.

Checkpoint therapy mediated ADCC of Tregs

Punctual deletion of CTLA-4 on adult mouse Tregs increases the immunosuppressive function of Tregs via higher IL-10 and other rebound immunosuppressive

Table 2. Examples of clinical trials aiming to undermine Treg functions various clinical trials described in text employing strategies to restrain Treg functions are listed.

Strategy	Study title	*ClinicalTrials.gov* identifier (NCT number)	Medical condition	Primary formulation/regimen	Primary objective	Phase	Status	Sponsor	References
IL-2/IL-2R mediated Treg depletion	Basiliximab in Treating Patients with Newly Diagnosed Glioblastoma Multiforme Undergoing Targeted Immunotherapy and Temozolomide-Caused Lymphopenia (REGULATe)	NCT00626483	Malignant Neoplasms Brain	Basiliximab 7 days before autologous DC vaccination (In combination with radiotherapy and temozolomide)	To determine if basiliximab inhibits the functional and numeric recovery of Tregs after temozolomide (TMZ)-induced lymphopenia in the context of dendritic cell vaccination of adult patients with newly diagnosed glioblastoma multiforme (GBM).	Phase 1	Active, not recruiting	Duke University National Cancer Institute	137
	E7777 for the Treatment of Patients with Peripheral T-Cell Lymphoma	NCT01401530	Peripheral T-Cell Lymphoma	Denileukin diftitox (E7777) will be administered by intravenous (IV) infusion for 5 days from Day 1 through 5 of each cycle (21 days/ cycle) with 8 cycles.	Finding Maximum Tolerated Dose (MTD) and Recommended Dose (RD)	Phase 1	Completed	Eisai Co., Ltd.	142
	Phase II Trial of LMB-2, Fludarabine and Cyclophosphamide for Adult T-Cell Leukemia	NCT00924170	Adult T-Cell Leukemia (ATL)	Drug: LMB-2, Fludarabine, Cyclophosphamide. Cycle 1 with Fludarabine and Cyclophosphamide (FC) alone. Two weeks after starting cycle 1, 6 cycles of FC plus LMB-2 at minimum 20-day intervals.	To determine safety of treatment regimen. To find out superiority of treatment over Alemtuzumab (CAMPATH).	Phase 1 Phase 2	Active, not recruiting	National Cancer Institute	143
Checkpoint therapy mediated ADCC of Tregs	Study of Nivolumab (BMS-936558) Plus Ipilimumab Compared with Ipilimumab Alone in the Treatment of Previously Untreated, Unresectable or Metastatic Melanoma (CheckMate 069)	NCT01927419	Unresectable Melanoma Metastatic Melanoma	Nivolumab or placebo + Ipilimumab therapy followed by Nivolumab or placebo alone therapy	To compare the objective response rate of Nivolumab combined with Ipilimumab versus Ipilimumab monotherapy in patients with untreated, unresectable or metastatic melanoma	Phase 2	Active, not recruiting	Bristol-Myers Squibb	151
Inhibition of Treg function	A Phase 3 Study of Pembrolizumab + Epacadostat or Placebo in Subjects with Unresectable or Metastatic Melanoma (Keynote-252 / ECHO-301)	NCT02752074	Melanoma	pembrolizumab + epacadostat pembrolizumab + placebo	Progression-free survival	Phase 3	Active, not recruiting	Incyte Corporation	169, 176

All data are derived from https://clinicaltrials.gov. NA: Not applicable; -: phase not defined. Studies where references are not included are not published yet.

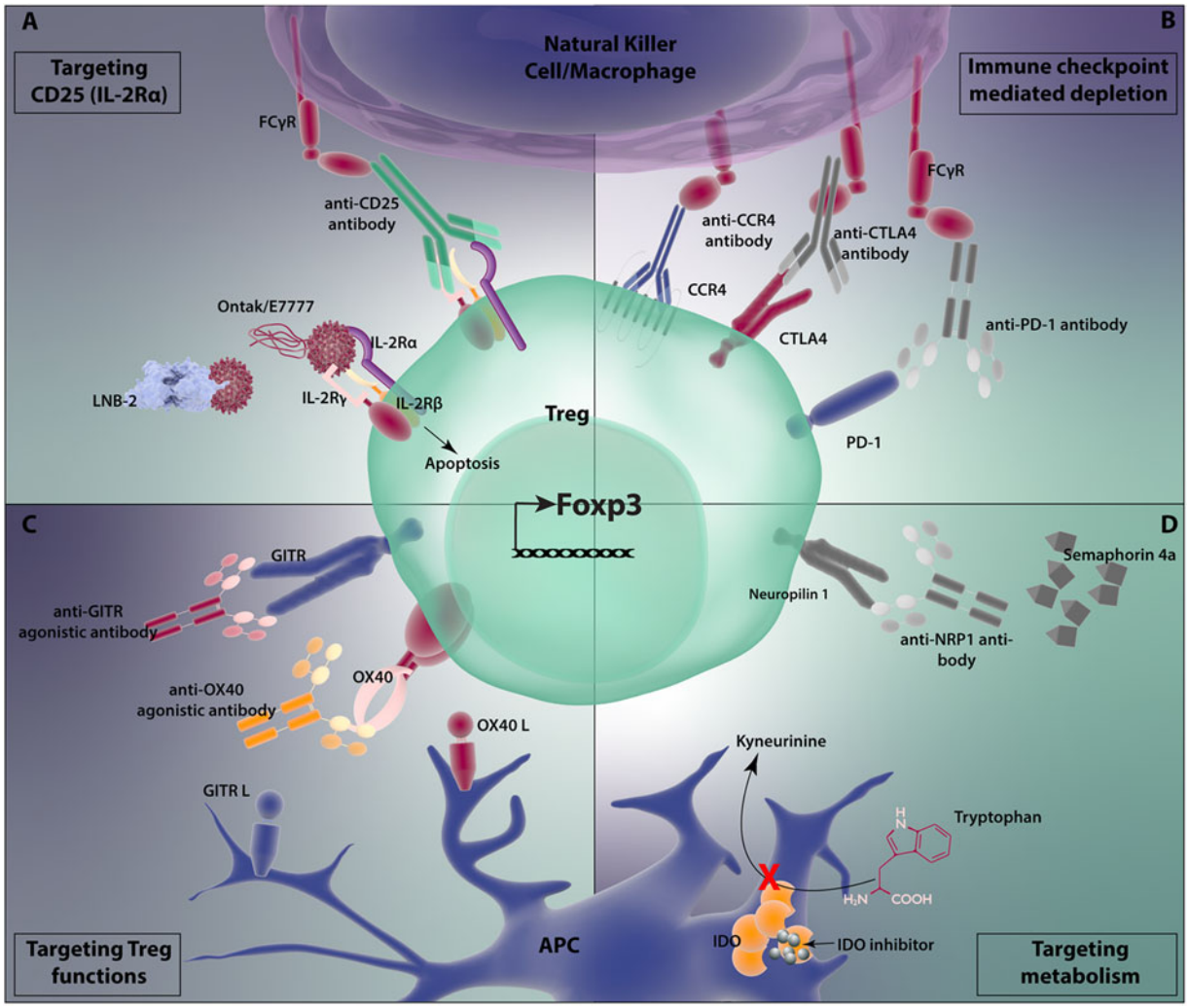

Figure 3. Subjugation of Tregs in tumor microenvironment. (A) CD25 (IL-2Rα) mediated Treg depletion can be achieved by natural killer (NK) cell mediated antibody dependent cell cytotoxicity (ADCC) by binding to anti-CD25 antibodies by Fc receptors. IL-2 fusion proteins with diphtheria toxin (Ontak, E7777) and exotoxin A of *Pseudomonas spp*. can deliver the toxins to Tregs via CD25 binding leading to the apoptosis of Tregs. (B) Immune check-point inhibitor antibodies like anti-CTLA4 and anti-PD-1 can lead to NK cell mediated Treg ADCC. Similarly, depleting anti-CCR4 antibodies also cause Treg ADCC. (C) Inhibiting Neuropilin-1 (NRP-1) signaling by blocking its binding to ligand Semaphorin A leads to conversion of Tregs into effector like T cells. Also, Indole deoxygenase inhibitors spare the tryptophan and reduce kyneurinine formation leading to decreased Treg frequency. (D) Blocking TNF family surface receptors on Tregs like OX-40 and GITR leads to decreased suppressive functions of Tregs.

molecules [144]. Thus, a mere inhibitory approach towards Treg CTLA-4 without Treg depletion might not be effective in anti-tumor therapy.

Antibodies against CTLA-4, PD-1 and PDL-1, while developed for rejuvenating exhausted T cells, are increasingly demonstrated to inhibit Tregs, owing to a higher expression of these molecules on Treg cell surface. Recent preclinical studies demonstrated that anti-CTLA-4 antibody isotypes especially IgG2a deplete Tregs in mouse tumor models [145,146]. This depletion was incumbent on presence of FCγRIV receptor expressing macrophages in the tumors (Figure 3B). Similarly, anti-human CTLA4 antibodies ipilimumab and tremelimumab also increase CD8/Treg ratio inside tumors and decrease the frequency of infiltrating Tregs [147]. However, this was observed in highly inflamed tumors presenting a high affinity polymorphism of CD16 receptor. A recent study tried to assess absolute depletion of Tregs, instead of frequency, by immunohistochemistry in prostate cancers, primary bladder cancers and metastatic melanoma [148]. This analysis indicated that though CD8/Treg ratio increases after ipilimumab therapy, this is largely attributable to increased number of CD8 T cells while Treg density remains rather unchanged. Thus, there is a scope of designing dual activity anti-CTLA4 antibodies, which can effectively block CTLA-4 signaling on effector T cells and simultaneously, deplete the

Tregs, particularly in tumors expressing ADCC sufficient cells.

Further, it is observed that though metastatic melanoma patients treated together with ipilimumab and radiation showed favorable response in some patients, majority of patients did not respond. Modeling the same treatment in mouse model of B16F10 melanoma revealed increased PD-L1 expression by tumor cells. Thus, adding a PD-L1 or PD-1 blockade reduced the resistance to radiation and ipilimumab co-therapy [149]. A combined treatment with nivolumab (anti-PD-1 antibody) and ipilimumab has provided favorable outcomes in melanoma patients in an ongoing Clinical Trial NCT01927419 [150].

Anti-chemokine receptor antibody mediated depletion

Tumor cells and infiltrating macrophages produce chemokines like CCL17 [151] and CCL22 [152]. Both of these chemokines are ligands for CCR4, expressed on effector Tregs infiltrating the tumors. A defucosylated humanized anti-CCR4 antibody KW-0761 (mogamulizumab) has shown effective intratumoral Treg depletion [153,154] (Figure 3B). This antibody was approved in Japan for relapsed or refractory CCR4-positive adult T-cell leukemia-lymphoma in 2012. Recently, FDA has approved mogamulizumab for rare non-melanoma skin cancers mycosis fungoides and Sézary syndrome, two subtypes of cutaneous T cell lymphoma. However, CCR4 is found to be expressed on effector T cells as well as resting Tregs, albeit lower than effector Tregs. Indeed, it was found that mogamulizumab depletes both Tregs and effector T cells [155], which might be the reason for adverse drug reactions seen with this therapy [156]. Recent genome wide transcriptome studies identified CCR8 as major chemokine receptor expressed selectively on intratumoral Tregs [157,158]. CCR8 ligands CCL1 and CCL18 are highly transcribed in tumor infiltrating myeloid cells [157]. Indeed, anti-CCR8 therapy in a mouse model of colon carcinoma induced protective immunity and enhanced *L. monocytogenes*-based vaccine response [159].

Inhibition of Treg functions

Certain TNFR family molecules like GITR and OX-40 are highly expressed on activated Tregs. GITR ligation with non-depleting agonistic antibodies destabilizes Tregs reducing their numbers resulting in reduced tumor growth [160,161] (Figure 3C). Anti-GITR antibodies also synergize with checkpoint therapy [162,163]. A review of current biologicals targeting TNFR molecules on Tregs can be found in [164].

The heme proteins TDO (tryptophan 2,3-dioxygenase) and IDO (indoleamine 2,3-dioxygenase) are specific enzymes catalyzing first rate limiting reaction in conversion of Tryptophan to kyneurinine. They are expressed by several tumors and dendritic cells in response to inflammation [165] (Figure 3D). Utilization of tryptophan by these pathways renders effector T cells ineffective and increases Treg generation [166]. Several mono as well as dual inhibitors are under various stages of clinical development [167,168]. However, recently a phase 3 clinical trial of IDO inhibitor epacadostat in a co-therapy with pembrolizumab has reported negative results [169].

It has been reported that intratumoral Tregs are stabilized by NRP-1 [170]. Binding of NRP-1 to its ligand semaphorin4a inhibits AKT phosphorylation and thus, increases FOXO1 and 3 nuclear localization. This stabilizes the Treg genetic program. Loss of NRP-1 expression from intratumoral Tregs led the Tregs to produce interferonγ and loss of suppressive phenotype [120]. Thus, inhibition of NRP-1 ligation can be utilized as a therapy to subdue tumor Tregs probably in combination with other therapies (Figure 3D).

Recently, it has been shown that human Tregs induce senescence in effector T cells via nutrient (glucose) deprivation via a TLR8 dependent mechanism, which is a mechanism of immunosuppression by tumor cells as well. A therapy directed against such mechanisms can provide a synergistic effect as it will inhibit both tumor cell as well as intratumoral Treg expansion [171,172].

Conclusion and future perspectives

Both autoimmunity and alloimmunity have long been clinically managed by general immunosuppression. With the efforts to utilize Tregs for these therapies, a specificity is being brought, which can protect against the ills of a global suppressive milieu. However, Treg therapy is in its infancy and several issues are still being addressed. For the adoptive Treg therapy, the process of manufacturing of Tregs is itself tricky. We still do not have markers which can be utilized for identifying Tregs with absolute surety. Also, a cell therapy requires Good Manufacturing Practice (GMP) manufacturing facilities which are scarce and costly. The rather non-feasibility of imparting these treatments outside research facilities is also a major hurdle. The individual nature of cell

therapy is a deterrent but is safe. This is being addressed by off-the-self 'universal Tregs' [173]. To suppress alloimmunity and establish an immune tolerance, the number of adoptively transferred Tregs is very high (and not yet totally defined), so better strategies for ex vivo expansion of Tregs and clinical studies looking at safety of transferred Tregs in the efficacy ranges of Tregs are a requisite. The ex vivo hyper-expansion strategies with artificial APCs need to be pursued as well [174]. Also, better understanding of mechanisms of Treg mediated 'infectious tolerance' can supplement this endeavor [175].

Manufacturing iTregs from naïve T cells precludes need for high numbers of rather 'rare' natural Tregs, but possible plasticity as well as survival of iTregs in vivo is a hurdle. Hence, there is scope for new strategies and protocols to ensure Treg lineage stability. All these goals, theoretically, are achievable by CAR-Treg therapy. Therefore, there is need for more definitive preclinical and clinical studies with CAR-Tregs. Although the enormous cost associated with such therapies is an impediment, the results could be really astonishing. With the FDA approval of CAR-T cell therapy for certain lymphomas, the possibility of therapy with CAR-Tregs is ever more exciting. However, one big issue with this therapy remains with identification and thorough investigation of unique antigens to be targeted. The CAR-Tregs will also be faced with similar questions of survival, stability and plasticity. However, approaches like providing them with their own autocrine IL-2, deleting effector cytokine genes from them, incorporating some suicide switches and so forth. are already being discussed. As demonstrated in preclinical animal model studies, tissue specific homing of CAR-Tregs based on specific antigen is a very interesting prospect [176] as this eliminates chances of a global suppression. The strategies to expand Tregs in vivo are rather manageable and can be easily applied in a clinical setting. However, the efforts to increase half-life of IL-2 shall be pursued more aggressively. PEGylation of IL-2 definitely has been shown as one way of doing that [177]. However, IL-2 mediated preferential induction of Tregs is a challenge. It has been shown that low to ultra-low doses of IL-2 (10 to 100 folds less than what required for memory T cells) can induce Tregs in type 1 diabetic patients [178]. Recently, effector T cell specific IL-2 mimics which selectively bind to IL-2Rβ and γ chains have been described [87]. Along these lines, identification of IL-2 mimics which can selectively bind to IL-2Rα shall be pursued.

The overabundance of Tregs in a tumor milieu is indeed a bottleneck in cancer therapy. Similarly, depletion of tumor specific Tregs while preserving the global immune homeostasis is a challenge. Here again, identification of specific markers can be a boon. As immunosuppression inside tumors is in a large part driven by cancer itself, probably Treg based approaches need to take into account the conglomeration of all type of cells. The tumor specific and probably personalized identification of cells capable of ADCC inside tumors should be a norm before Treg depleting immunotherapy. Also, there are other cells which can take on the suppression mantle from Tregs, so they all should be looked in totality. Tregs, probably, are the primary reason for epitope masking by cancers and given the enhanced capabilities of intratumoral Tregs it will be beneficial to identify antigen specific Tregs. As recently demonstrated, even neoantigen specific Tregs exist in the host, harnessing potential of antigen specificity could be important [135]. A recent setback with IDO inhibitors (NCT02752074) [169,179] notwithstanding, muting Treg functions by specific targets is a very valid approach in cancer therapy. The amenability of this approach to small molecule inhibitors can push drug discovery forward. Also, identification of metabolic aspects of Treg survival and trafficking in tumor environment could provide specific and sensitive targets for future Treg based therapies.

To sum up, burgeoning information about Treg biology and innovative strategies for Treg modulation foretell exciting prospects for establishing broken tolerance in transplantation and autoimmune diseases. Also, Tregs provide a better understanding of immune evasions by cancers and thus, the conundrum of highly effective cancer immune checkpoint therapy but only in a small subset of patients might get a key.

Funding

This research received grant from Institute for Basic Science, Korean Ministry of Science and ICT

ORCID

Amit Sharma http://orcid.org/0000-0002-8109-1690
Dipayan Rudra http://orcid.org/0000-0002-9667-5891

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