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Immune modulation via T regulatory cell enhancement: Disease-modifying therapies for autoimmunity and their potential for chronic allergic and inflammatory diseases—An EAACI position paper of the Task Force on Immunopharmacology (TIPCO)

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Abbreviations: Ab, antibody; ACT, adoptive cell therapies; AD, atopic dermatitis; Ag, antigen; AMPK, AMP-activated protein kinase; APC, antigen-presenting cell; BAR, B cell–targeting Ab receptor; BCR, B-cell receptor; CAAR, chimeric autoantigen receptor; CAR, chimeric antigen receptor; CEA, carcinoembryonic antigen; COPD, chronic obstructive pulmonary disease; CRISPR, clustered regularly interspaced short palindromic repeats; darTreg, donor-alloantigen-reactive Treg; Dsg3, desmoglein 3; EAE, experimental autoimmune encephalomyelitis; EMA, European Medicines Agency; FCεRI, high-affinity IgE receptor; FDA, United States Food and Drug Administration; FoxP3, forkhead box P3; GATA3, GATA binding protein 3; GMP, good manufacturing practice; GVHD, graft-versus-host disease; IFN, interferon; IL, interleukin; IL-2R, interleukin 2 receptor; iTr1, induced Tr1 cell; iTreg, in vitro-generated Treg; LAP, latency-associated peptide; mIgE, transmembrane form of IgE; mTOR, mammalian target of rapamycin; OIT, oral immunotherapy; PC, plasma cells; pTreg, peripherally induced Treg; PV, pemphigus vulgaris; RA, rheumatoid arthritis; scFv, single-chain variable fragment; SLE, systemic lupus erythematosus; SMD, small-molecule drug; T1D, type 1 diabetes; Tconv, conventional T cell (non-Treg); TCR, T-cell receptor; TGF-β, transforming growth factor β; Th1, T helper 1 cell; Th17, T helper 17 cell; Th2, T helper 2 cell; TIPCO, Task Force of Immunopharmacology; TMD, transmembrane domain; Tr1, type 1 regulatory cell; Treg, regulatory T cell; tTreg, thymus-derived Treg; UniCAR, universal CAR.

The Task Force of Immunopharmacology (TIPCO) within the Basic and Clinical Immunology Section of EAACI was established in 2017 to connect scientists and clinicians with different scientific backgrounds—physicians and basic scientists, pharmacologists, and computational biologists—with the task of examining recent breakthroughs on basic mechanisms of immune regulation and review their application in current, upcoming, and paradigm-shifting nonbiological therapeutic approaches for allergy and clinical immunology-related diseases. The topic for this second position paper was chosen unanimously, and specific parts were drafted by authors' subgroups. The first draft was compiled by first and corresponding authors and discussed in a general TF meeting in Vienna (March 29-30, 2019). The resulting draft was thereafter recirculated and critically appraised until the final version was approved by all Task Force members.

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Abstract

Therapeutic advances using targeted biologicals and small-molecule drugs have achieved significant success in the treatment of chronic allergic, autoimmune, and inflammatory diseases particularly for some patients with severe, treatment-resistant forms. This has been aided by improved identification of disease phenotypes. Despite these achievements, not all severe forms of chronic inflammatory and autoimmune diseases are successfully targeted, and current treatment options, besides allergen immunotherapy for selected allergic diseases, fail to change the disease course. T cell–based therapies aim to cure diseases through the selective induction of appropriate immune responses following the delivery of engineered, specific cytotoxic, or regulatory T cells (Tregs). Adoptive cell therapies (ACT) with genetically engineered T cells have revolutionized the oncology field, bringing curative treatment for leukemia and lymphoma, while therapies exploiting the suppressive functions of Tregs have been developed in nononcological settings, such as in transplantation and autoimmune diseases. ACT with Tregs are also being considered in nononcological settings such as cardiovascular disease, obesity, and chronic inflammatory disorders. After describing the general features of T cell–based approaches and current applications in autoimmune diseases, this position paper reviews the experimental models testing or supporting T cell–based approaches, especially Treg-based approaches, in severe IgE-mediated responses and chronic respiratory airway diseases, such as severe asthma and COPD. Along with an assessment of challenges and unmet needs facing the application of ACT in these settings, this article underscores the potential of ACT to offer curative options for patients with severe or treatment-resistant forms of these immune-driven disorders.

KEYWORDS
adoptive cell therapies, allergy, autoimmunity, CAR-Treg cells, immunoregulation

1 | INTRODUCTION

Alterations in immune tolerance toward proteins of either self or foreign infectious and noninfectious origin are critical in the pathogenesis of autoimmune and allergic diseases, respectively, through an imbalance between antigen (Ag)-triggered activating signals and the ensuing suppressive responses. Diverse alterations in the number or function of regulatory T cells (Tregs) have been documented in these conditions. This T-cell subpopulation suppresses other T cells and immune cells using both soluble molecules and contact-dependent mechanisms, in order to maintain self-tolerance and regulate effector responses during immunity.[1-4]

The mechanistic complexity of autoimmune and allergic diseases is reflected by their heterogeneous clinical presentations and disease courses and by the challenges in developing therapeutic strategies beyond the first-line broad immunosuppressive approach. Building upon the use of small-molecule drug (SMD)—compounds obtained by chemical synthesis such as synthetic glucocorticoids, beta-agonists, and leukotriene receptor antagonists—the advent of biologicals has ushered in the application of targeted therapies, which are increasingly successful due to improved understanding of disease phenotypes and endotypes.[5,6] Yet, with the exception of allergen immunotherapy for some IgE-mediated responses, disease-modifying strategies remain to be identified for most severe allergic and/or immune-driven chronic diseases such as food allergy, atopic dermatitis, severe asthma, and severe chronic obstructive pulmonary disease (COPD) and for autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). Cell-based immunotherapies, aided by cell engineering, have been described as the "third pillar" of therapeutics, the other two being SMD and biologicals,[7] and represent the only pillar with the potential for curative therapy for these diseases.

Oncology has seen dramatical improvement of clinical outcomes with adoptive cell therapies (ACT) using T cells engineered to express either Ag-specific T-cell receptors (TCRs) or chimeric Ag receptors (CARs) targeting specific tumor antigens that redirect T cells toward killing cancerous cells with high selectivity. CAR-T cell–based treatments recently received FDA/EMA approval for

the treatment of acute lymphoblastic leukemias and advanced lymphomas, while clinical trials for other hematologic and solid cancers are underway. Preclinical models have also demonstrated the efficacy of CAR-T/TCR-transferred cells in autoimmune settings in "killing" specific pathogenetic players such as B cells producing auto-Abs.

Besides targeted cell elimination, ACT in non-neoplastic diseases can be used to regain appropriate and stable Treg function against inflammatory responses while preserving protection against infection. Using different strategies, Treg-based ACT have been pursued for the treatment of autoimmune diabetes (type 1 diabetes, T1D) and for pathologic immune responses toward alloantigens as in graft-versus-host disease (GVHD) and transplant rejection prevention or protein replacement therapies, such as factor VIII in hemophilia.[8-11] Clinical trials of Treg-based ACT in SLE, discussed in the following paragraphs, indicate that this approach should be efficacious in other complex immune diseases where the causative Ag is unknown by re-establishing a tolerogenic microenvironment,[1,4,12-17] also taking advantage of the ability of Tregs to traffic to tissues and exert local immunoregulatory activities.[15]

This EAACI position paper of the Task Force of Immunopharmacology (TIPCO) reviews the various T cell–based strategies, their current application in preclinical and clinical autoimmunity settings, and the limitations encountered along with the strategies applied to overcome them. On these premises, it brings then the focus on studies currently supporting or testing the application of T cell–based approaches, especially Treg-based, in severe IgE-mediated responses and in severe phenotypes of asthma and COPD, providing up-to-date tables of preclinical and clinical studies. By framing the potential of ACT through critical revision of current challenges and unmet needs, this review aims at underscoring the groundbreaking potential of cell-based therapies, as a curative approach is still missing in most allergic and immune-based chronic inflammatory disorders.

2 | TREG CELLS IN ACT: RECRUITING THE LONG-TERM ARM OF IMMUNE REGULATION IN THE THERAPEUTIC BATTLEGROUND

Tregs constitute a subpopulation of T cells (Figure 1) that regulate the function of T cells and other immune cells using both soluble factors and contact-dependent mechanisms during immune responses.[1,2] Alterations in Treg numbers and/or function are critical determinants in the pathogenesis of allergic, autoimmune, and chronic inflammatory disorders, as well as in reactions to allotransplants.[15] Due to their potent suppressive capacity, even at very low Treg-to-T effector cell ratios,[18] the potential of Tregs as therapeutic tools in restoring immunological tolerance has been actively pursued.[19] To this end, studies uncovering the complex dynamics of Treg differentiation and interplay with T cells, as well as the mechanisms of their immunosuppressive function have been of utmost importance in the design of Treg-based ACTs.

Tregs constitute about 1%-3% of circulating CD4+T cells, and they are usually characterized by high expression of the interleukin (IL)-2 receptor (IL-2R) α chain (IL-2Rα) and forkhead box P3 (FoxP3), the master transcription factor for Treg differentiation. They are further characterized by their site of differentiation, namely thymus-derived Tregs (tTregs) and peripherally induced Tregs (pTregs), alongside their in vitro counterparts, commonly referred to as iTregs (Table 1). A phenotypic distinction between tTregs and pTregs has not been fully established, and various surface and intracellular markers define Tregs based on their functional characteristics and plasticity (Table 1).[20,21] In the context of allergic diseases, generation of pTregs is favored under suboptimal activation of Ag-presenting cells (APCs), high Ag doses, and a pro-tolerogenic environment rich in IL-10, transforming growth factor (TGF)-β, and retinoic acid.[22,23]

When activated by their cognate Ag through TCR interaction, Tregs can implement their immunoregulatory function by multiple molecular mechanisms (Figure 2). When residing outside of lymphoid organs, Tregs play a key role in tissue homeostasis and repair through the release of the growth factor amphiregulin.[24-28]

However, Treg phenotypes in vivo go beyond this dichotomous classification, as their specific immunosuppressive functions are determined by a complex interplay between the genetic background and the contextual cellular/molecular milieu in which they reside.[13] Human Tregs in fact display phenotypic plasticity and assume functional heterogeneity by expressing, along with FoxP3, other transcription factors—such as T-bet, GATA3, and RORγt—and chemokine receptors in analogy to associated effector subsets—for example, Th1, Th2, and Th17. This shift results in enhanced suppression of local, specific responses [29-37] and possibly limits detrimental bystander suppression.[38] However, the stability of Treg functions can be undermined within an inflammatory environment, where TCR-mediated stimulation skews them toward effector phenotypes with loss of FOXP3 and the production of pathogenic cytokines, such as interferon (IFN)-γ and IL-17.[39,40] These dynamic features (Figures 1 and 2) underscore the importance of phenotype stability and plasticity as limiting factors currently under intense scrutiny for the successful development of Treg-based therapies [41-44] (see paragraph 5).

3 | T CELL–BASED THERAPIES IN IMMUNE-DRIVEN DISEASES: ACT PATHFINDERS IN NON-NEOPLASTIC DISORDERS

3.1 | The basics: multiple strategies for T cell–based therapies

Different approaches exist to redirect altered immune responses by restoration of Treg function: either indirectly boosting Treg expansion in vivo, or isolating Tregs from peripheral blood and subsequent reinfusion as ACT, without or following ex vivo expansion. In the latter case, isolated T cells are expanded ex vivo to high purity either as

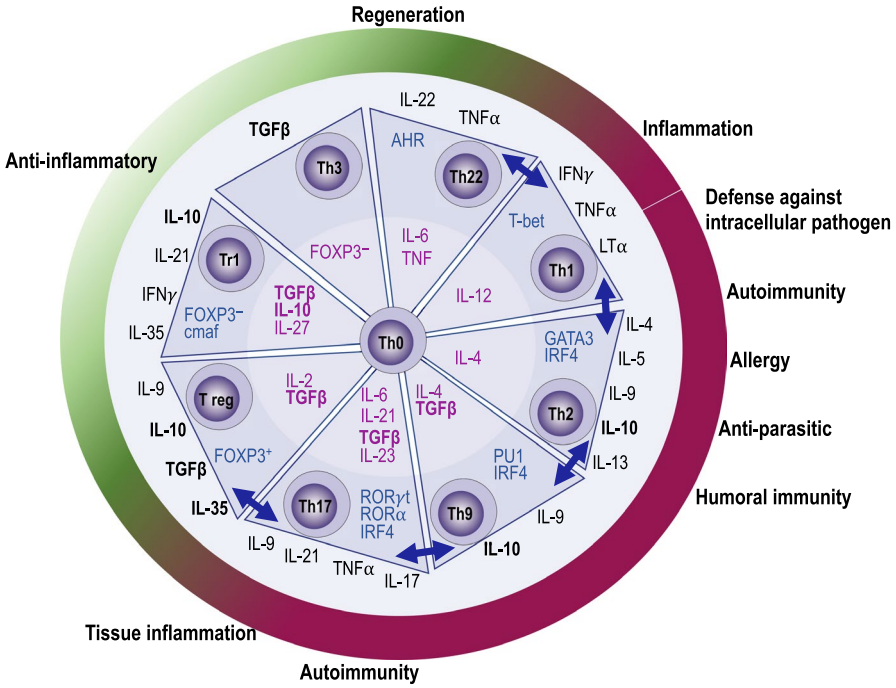

FIGURE 1 Interplay among Tregs and other CD4$^+$ T cells. Upon contextual stimulation, naïve CD4$^+$ T cells can differentiate into several T helper (Th) and different suppressive T cell subsets, for example, Treg expressing FoxP3 constitutively (Treg) or not expressing FoxP3, such as Tr1 and Th3 cells (colored circles), all exerting relatively distinct roles. These differentiation programs are controlled by different cytokines (in red) and each CD4$^+$ T-cell subset can be identified by lineage-specific transcription factors (in blue, italic) that support their function (as listed) and cytokine secretion pattern (in black: those with immunosuppressive functions are in bold). Importantly, T-cell lineages harbor a certain plasticity that allows lineage shifts across the different subsets (double-headed arrows)

polyclonal pool or as an Ag-specific population: this cellular pool can be generated by Ag-driven clonal expansion or by different types of cell engineering [45] (Figure 3). Initial therapeutic applications of Treg ACT were performed in the field of transplantation that prevented GVHD after allogeneic stem cell transplantation [46-48] and solid organ (kidney and liver) transplant rejection.[49,50] Cells are then re-administered to the patient and the entire procedure is implemented through standardized, good manufacturing practice (GMP)-compliant procedures [45,51-55] (Figure 4A). The use of polyclonal Treg activation through the TCR was the initial strategy for ACT in immune-driven diseases and the one currently most established in clinical use, employing enrichment of CD25$^+$ cells by magnetic beads followed by expansion of Tregs with anti–CD3/CD28-coated beads or antigen-presenting cells in the presence of IL-2.[51,56,57] To achieve targeted suppression using a smaller number of Tregs, the administration of ex vivo-expanded allo-Ag-specific Tregs was pursued. This approach is also based on findings in humanized mouse models showing that Ag-specific Tregs were more potent than polyclonal Tregs in controlling local inflammation and that they inhibit the priming of T cells in secondary lymphoid tissues.[36] This method has been used or planned in clinical trials for hematopoietic and solid organ transplantation and GvHD treatment/prophylaxis, while pursued in autoimmune diseases for juvenile and adult T1D.[58] The procedures to isolate low-frequency, Ag-specific cells are complex and expensive, with estimation of optimal dose for efficacy being a complex feature including considerations on the Treg/effector T cell ratio,[59] but also on Treg trafficking and retention in tissues [discussed in 58]. Antigen-specific T cells are also evaluated in preventing viral infection in primary immunodeficiency disorders using conventional T cells.[60] The use of CD8+cytotoxic T cells bearing Ag-specific TCR or CAR, designed to redirect cancer patient's T cells to specifically target tumor cells, is the main ACT strategy exploited for cancer therapy.[61,62]

The use of engineering Ag-specific TCRs or CARs provides also for Tregs efficient gene-transfer platforms in order to reliably produce Tregs of defined Ag specificity and sufficient number [24] (Figure 4B and C). Structurally, CARs are recombinant receptors targeting specific surface antigens, featuring an Ag-binding single-chain variable fragment (scFv) with a hinge region and a transmembrane domain,[63,64] capable of redirecting the specificity and function of the CAR-bearing T cells.[9] This MHC-independent strategy to produce Ag-specific T cells has resulted in four different generations of CAR-T cells (Figure 4D): (1) CAR containing only the CD3ζ activating domain; (2) CAR containing an additional costimulatory domain (either CD28 or CD137); (3) CAR combining both CD28 and CD137 costimulatory domains; and (4) the so-called universal CAR (UniCAR), which is engineered to link the hinge region to a P1 domain (a peptide or protein) that binds to another peptide or protein P2 fused to an scFv recognizing a surface molecule on target cells. Recently, CARs were developed that carried Ag domains for targeting B cells with specific surface Ig receptors (BCR) that secrete pathogenic antibodies: either auto-antibodies (chimeric auto-Ag receptor T or

TABLE 1 Major human T regulatory phenotypes

Human regulatory T cells			References
		Generally defined as CD4 + CD25+CD127-, expressing FoxP3+	175
tTregs		CD4 + cells expressing FoxP3 + constitutively, derived from the thymus, with Treg-specific demethylated regions (TSDRs)	176,177
		Site: Lymphoid organ, peripheral blood, tissues	
tTreg subsets			
CD4+FoxP3^{lo}CD45RA+		Naive, resting Treg	175,178,179
CD4+FoxP3^{hi}CD45RA^{-}		Activated T cells or activated Treg, subsets with markers, such as HLA-DR, GITR, TIGIT, LAG3, or CD39, with superior suppressive functions	175,176,180
pTregs		CD4+FoxP3-, inducible FoxP3 expression upon activation with, for example, IL-2, TGF-β, retinoic acid	175,178,179,181
		Site: Peripheral tissues	
iTregs		In vitro-generated, inducible FoxP3 expression, similar to pTreg	176
Markers of CD3 + CD4+functional Tregs			
Markers discriminating between tTregs and pTregs			
HELIOS		Suitable but not perfect marker to separate tTregs (HELIOS+) from pTregs (HELIOS^{-})	176
TIGIT		T-cell immunoreceptor with Ig and ITIM domain, surface marker	182
Markers	**Ligand**	**Function**	**References**
Surface			
CD25	IL-2	Promotes Treg differentiation, survival, expansion, and function (1)	74,183
CCR6	CCL20	Memory Tregs and IL-17–producing suppressive Tregs	175
CD39		Hydrolyzes ATP and ADP equally well to AMP; expression by >60% of FoxP3+ cells (1)	183
CD73		Degrades AMP to adenosine (1)	
CTLA4 (CD152)	CD80/CD86	Constitutively/preferentially intracellular; inhibits T-cell activation through competition for costimulation with CD28	175,178,183
GARP	TGF-β	Also defined as LLRC32 binds latent TGF-β complex, not detectable on freshly isolated Tregs, expressed upon in vitro activation	178-1
GITR (CD357)	GITR-L	Promotes Treg differentiation and expansion, highly expressed in effector Tregs; inhibition of Treg activity in short term	184-186
HLA-DR	TCR	Expressed on one third of effector Tregs in peripheral blood; subset with superior suppressive function	175
ICOS	ICOS-L	Costimulatory receptor of TCR, expressed by memory-like Tregs, stimulates IL-10 synthesis	175,178,179
LAG-3 (CD223)	FGL-1, MHC II	Binds MHC class II, identifies activated and terminally differentiated Treg, negatively regulates T cells proliferation and activation	175,187
LAP	TGF-β	TGF-β binding forms an inactive latent LAP-TGF-β complex; late-stage Treg activation marker, expressed upon in vitro activation	175,176,180
PD-1 (CD279)	PD-L1/2	Anti-apoptotic on Tregs, expressed in Treg subsets	178
TNFR2 (CD120b)		Constitutively and preferentially expressed by all human thymic Tregs and approx. 70% of pTregs	175,178,179
Intracellular			
FoxP3		Master regulator, essential for Treg generation, function, and survival	20
HELIOS		Ikaros zinc finger transcription factor	176

Note: (1) It mediates Tregs' immunosuppressive activity (see Figure 2).

Abbreviations: ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; CCL20, chemokine ligand 20; CCR6, chemokine receptor 6; CD, cluster of differentiation; CTLA4, cytotoxic T lymphocyte–associated protein 4; GARP, glycoprotein A repetitions predominant; GITR, glucocorticoid-induced TNFR-related protein; GITR-L, GITR-ligand; HELIOS, Ikaros zinc finger transcription factor; HLA-DR, human leukocyte Ag-DR isotype; ICOS, inducible T-cell COStimulator; ICOS-L, ICOS ligand; IFN, interferon; IL interleukin: iTreg, in vitro-generated, induced Treg; LAG-3, lymphocyte-activation gene 3; LAP, latency-associated peptide; LRRC32, leucine-rich repeat-containing protein 32; MHC, major histocompatibility complex; PD1, programmed cell death protein 1 receptor; PD-L, programmed death ligand; pTreg, peripheral Treg; TCR, T-cell receptor; TGF-β, transforming growth factor beta; TIGIT, T-cell immunoreceptor with Ig and ITIM domains; TNFR2, tumor necrosis factor receptor 2; tTreg, thymus-derived Treg.

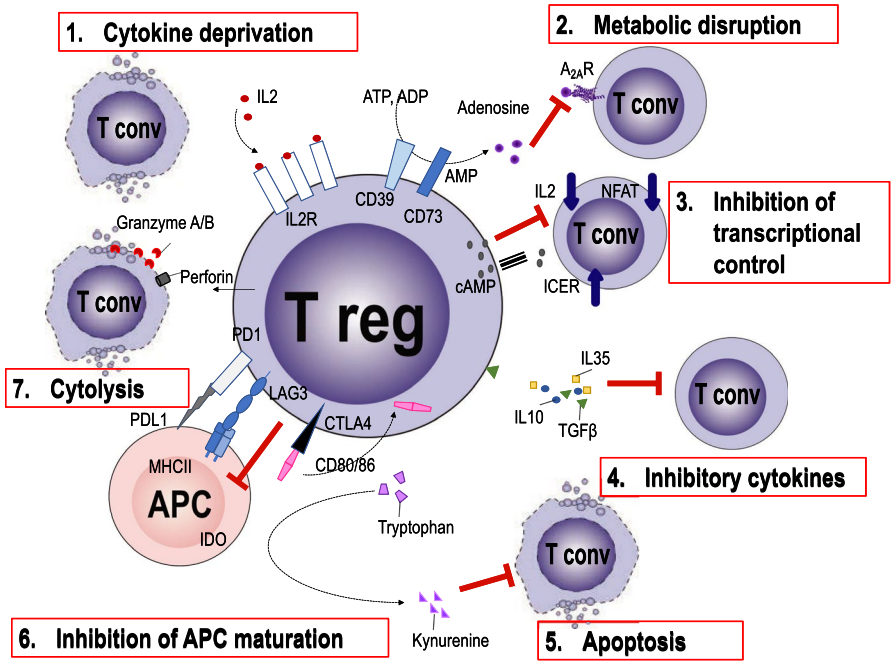

FIGURE 2 Molecular mechanisms of Treg Immunomodulatory functions on T cells. Tregs can suppress effector immune responses by several means: (1) deprivation of microenvironment from essential cytokines (ie, IL-2); (2) metabolic disruption, by degrading ATP to immunosuppressive adenosine; (3) inhibition of NFAT and IL-2 production by ICER (inducible cAMP early repressor) (207); (4) secretion of inhibitory cytokines such as IL-10, IL-35, and TGF-β; (5) kynurenine-mediated apoptosis by the tryptophan-catabolizing enzyme IDO; (6) modulation of Ag-presenting cell (APC) maturation toward a tolerogenic phenotype, through binding to MHC class II via LAG3 and by engagement/expression of the costimulatory molecules CTLA-4 and PD1; and (7) death of T cells by cytolysis mediated by perforin and granzyme B

CAAR) or anti-drug antibodies developed during replacement therapies (as for factor VIII in hemophilia, termed B cell–targeting Ab receptors or BAR).[8,17,65]

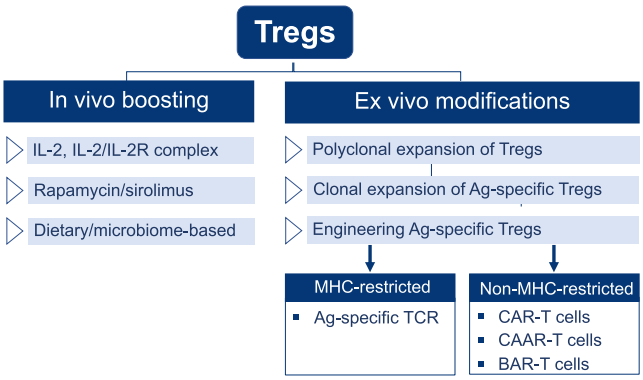

FIGURE 3 Overview of Treg-based therapeutics. Current preclinical studies and clinical trials of Treg-based therapies are based on two major approaches: in vivo boosting of Treg number and function via a series of Treg-promoting interventions (see Section 3.2.1), or adoptive transfer of purified, GMP-compliant Tregs previously modified ex vivo (see Section 3.1). In the latter case, cells are reinfused either following expansion with polyclonal or Ag-specific stimulation or after engineering with Ag-specific T cell receptors (TCR, MHC-restricted) or chimeric T-cell receptors (non–MHC-restricted) of different types [chimeric Ag receptor (CAR), chimeric auto-Ag receptor (CAAR), or B-cell Ab receptor (BAR) (see Section 3.1)]

Engineered Tregs were employed in immune disease models initially using TCR-transduced cells [9,66,67]; CAR-Treg technology has been tested more recently, in models of colitis and autoimmune encephalomyelitis.[10,63,65,68]

There are important structural and functional features distinguishing CAR-Tregs versus synthetic TCR-Tregs, which expand, rather than duplicate, the application of antigen-specific targeting strategies. These can function as relative strengths or limitations, guiding their application for best clinical efficacy.[69] Overall, both approaches represent a step forward compared to polyclonal Tregs expanded in vitro in terms of specificity and potency, while they significantly increased cell yield compared to in vitro expansion of Ag-specific Tregs derived from patients. In particular, some studies on CAR effector T cells indicated that their efficacy is dependent upon high Ag density on cell target, while low Ag levels are sufficient to activate TCR-mediated stimulation.[1-3] To this end, Ags overexpressed on diseased cells could be better targeted by CAR-Tregs while cells bearing recombinant TCRs could efficiently target systemic, low-level antigens. Moreover, CAR-Tregs have the following characteristics over TCR-Tregs [29]: (1) They are non–MHC-restricted and less dependent on IL-2; (2) besides Ag-specific function, they exploit Tregs function of dominant "bystander" suppression, which is the ability to suppress effector T cells with different Ag specificities [69,70]; and (3) they maintain all Treg features including high surface expression of CTLA-4, latency-associated peptide (LAP) and the inactive precursor of TGF-β. Second-generation CAR-Tregs include a CD28 costimulatory domain.[71] The CD28 domain affects

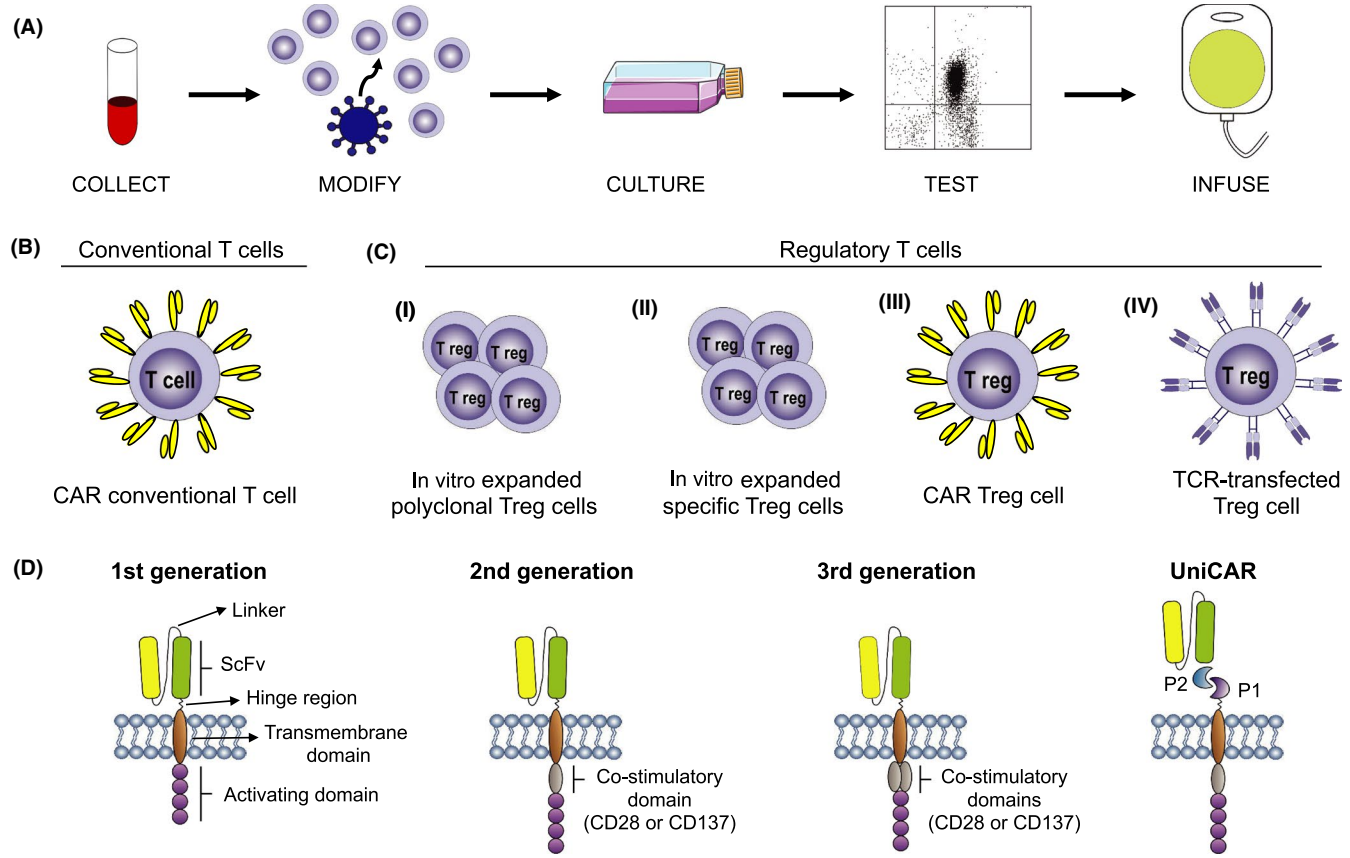

FIGURE 4 Adoptive Cell Therapies: overview of procedures, T cell types, and constructs. A, Peripheral blood from a patient is collected, white blood cells are enriched by leukapheresis, and $CD8^+$ cytotoxic T cells or Tregs are isolated to undergo either viral transduction with Ag-specific T cell receptors of different types (B, C. III - IV.), or stimulation and subsequent reinfusion as polyclonal/Ag-specific Tregs (C.I-II). Cells are then expanded in vitro with specialized culture conditions, sorted, tested to meet stringent GMP standards, and subsequently infused back into the patient [Modified with permission from (16)]. D, Schematic structures of successive CAR generations. See Section 3.1 for description. [Modified with permission from (63)]

CAR-Treg surface phenotype and functions and only CAR-Tregs with CD28 signaling domains induced significant suppression of T cell–mediated graft rejection in vivo.[72] Therefore, careful selection of chimeric receptors is needed to enable prolonged immunomodulatory effects of primary human cells to increase the chances for translation into human therapies.[73] In general, another potentially problematic aspect of recognition of specific molecules on target tissues by engineered Tregs could be ascribed to the Ag-specific suppression exerted by Tregs via cytotoxic activity, through secretion of granzyme and perforin (see Figure 2).[6] Although cytotoxicity has not been so far reported in preclinical settings, CAR-Treg cells have shown cytotoxic activity in vitro.[4,5] The impact of this mechanism needs to be investigated, to identify the disease settings in which may occur preferentially over other suppressive mechanisms.

3.2 | Current applications: T cell–based approaches in preclinical and clinical studies

Predominantly Treg-based cell therapies (Figure 3) have been tested in preclinical models (Table 2), and clinical trials are expanding their evaluation to a growing number of immune-mediated diseases (Table 3) using various in vivo and ex vivo T-cell expansion/engineering methodologies.

3.2.1 | In vivo induction and expansion of Tregs

Several protocols have been assessed that promote in vivo the expansion of Tregs or depletion of effector cells, thus increasing the Treg-to-T cell ratio. These include administration of IL-2, the mammalian target of rapamycin (mTOR) inhibitor rapamycin/sirolimus, certain anti-CD3 monoclonal antibodies [reviewed in 20], and dietary or microbe-derived pro-tolerogenic stimulations (Tables 2.1 and 3.1). Stimulation with IL-2 is crucial for Treg function and homeostasis.[74] Tregs recognize IL-2 via the trimeric IL-2R composed of IL-2Rα (CD25), IL-2Rβ (CD122), and IL-2Rγ (CD132). Improved IL-2 therapies, such as CD25-biased IL-2/anti-IL-2 Ab complexes (briefly, IL-2 complexes), are able to selectively and potently stimulate Tregs.[75] An immune cell-specific bias is achieved in IL-2 complexes by temporal interference with the IL-2R epitope, thus favoring either preferential activation of Tregs or $CD8^+$ T cells and natural killer cells.[76,77]

TABLE 2 Treg-based adoptive therapies in preclinical models of inflammatory disease

Part 1. In vivo-expanded Tregs		
Disease models and Treatment	**Biological effect**	**References**
Airway inflammation		
Ovalbumin/allergen-induced		
All-trans-retinoic acid, o.g.	CD4+CD25+ FoxP3+ Tregs induction dampened allergic inflammation; administration of retinoic acid with allergen reduced airway inflammation	82
B cell–induced Tregs i.v.	B cell–induced CTLA4+OX40+PD1+TNFR2+IL-10+ producing Tregs, suppressed Th2 cytokine production and eosinophilic infiltration	188
CTLA4-Ig i.p.	Bone marrow–derived macrophages/Tregs induction; CTLA4-Igs block T-cell activation via NO/Treg/TGF-β-dependent pathways	189
IL-2/anti-IL-2 Ab complexes	Expansion of FoxP3+ Tregs leads to suppression of murine airway inflammation induced by chicken egg ovalbumin or Schistosoma mansoni egg Ag	75,190
IVIG Ag-specific pTregs	CD4+CD25+ Tregs, inhibition of airway hyperresponsiveness and inflammation	191
IVIG	Ag-specific pTregs expansion, as IVIG induces tolerogenic DCs	192
Luteolin i.p.	Promotion of luteolin-induced CD4+CD25+ Tregs leads to reduction of airway hyperresponsiveness, airway eosinophilia, lower IgE and Th2 cytokines	87
Maresin-1 i.v.	CD4+FoxP3+ Tregs/Type 2-innate lymphoid cells de novo generation, restrained allergic lung inflammation	88
Streptococcus pneumoniae cell wall components i.t.	S.pneumoniae-induced CD4+CD25+ Tregs reduce airway inflammation that blocked NK T-cell activity	90
Bacterial/viral-induced		
DNA methylation inhibitor 5-aza-2'deoxycytidine (DAC) i.t.	DAC-induced lung CD4+CD25+ FoxP3+ Tregs reduced ongoing lung inflammation	193 135
Food Allergy		
Ovalbumin/allergen-induced		
OVA-Treg+ Insulin-like growth factor 2 (IGFR2)-induced i.p.	Enhances TGF-β–producing Tregs, reduces food allergy symptoms	194
Cow milk allergy		
Allergen with nondigestible oligosaccharides (NDO) o.g.	CD4+CD25+ Tregs induction, NDO improves whey-induced OIT	85
Hydrolyzed whey protein	CD4+CD25+ FoxP3+ Tregs induction by whey specific OIT prevents allergic symptoms to cow's milk	84
Type 1 Diabetes (T1D)		
T1 Diabetes (NOD mouse)		
D-Mannose oral	FoxP3+ Tregs induction; suppression of autoimmune diabetes	86
DNA vaccine GAD65 fragment and IL-10, im	CD4+CD25+ FoxP3+ Tregs induction, delayed diabetes onset, anti-CD25 abolished protective effect	195
IL-2/anti-IL-2 Ab complexes	Expansion of FoxP3+ Tregs results in significant delay of disease onset in NOD mice	75,196
IL-13Rα1 gene knockout	TGF-β+FoxP3+ Tregs induction; delay in rise of blood glucose levels and development of T1D	197
Inflammatory bowel disease (IBD)		
TNBS-induced IBD		
Lactobacillus o.g.	Tregs (iTregs) induction; suppression of TNFα and IL-17A and disease development	91
TNBS o.g./TNCB epicutaneous	CD4+CD25+ FoxP3+ Tregs induction; abrogation of intestinal inflammation	198
DSS-induced IBD		
Galectin-3, o.g.	Tregs induction and inhibition of intestinal inflammation	199
Multiple sclerosis		
MOG-induced experimental autoimmune encephalomyelitis, EAE		
Adiponectin i.p.	Adiponectin induces FoxP3+TGF-β+Treg expansion and reduction of EAE symptoms	200
Chloroquine treatment i.p.	Expands Tregs and decreases DCs resulting in suppression of inflammation and EAE development	201 154

(Continues)

TABLE 2 (Continued)

Part 1. In vivo-expanded Tregs		
Disease models and Treatment	**Biological effect**	**References**
IL-2/anti-IL-2 Ab complexes	Expansion of FoxP3+ Tregs before EAE induction results in significant delay of disease onset	75,202
Probiotic strains, o.g.	Promoted Tregs; adoptive transfer of probiotic-induced Tregs reduced EAE development by IL-10 increase and by decrease of Th1 and Th17 cytokines	92
Violacein i.p.	CD4+FoxP3+ Tregs induction; milder EAE symptoms	89
Zymosan i.p or zymosan-primed CD4 T cells iv	Promoted Tregs and prevented or reversed clinical development of EAE	203
Rheumatoid arthritis (RA)		
Collagen-induced arthritis		
M tuberculosis HSP65 peptide 1 i.n.	FoxP3 Tregs induction; Peptide 1 or adoptive transfer of CD4+ cells protected against RA	204
IL-2/anti-IL-2 Ab complexes	Expansion of FoxP3+ Tregs dampens disease severity	75,205
rIL-35 s.c.	Regulatory Treg CD25+CD39+FoxP3+/CD25$^-$CD39+ CD4+FoxP3+ induction; dampened arthritis development	206
Part 2. In vitro expanded Tregs		
Disease models and Treatment	**Biological effect**	**References**
Pulmonary arterial hypertension		
CD4+CD25+ Tregs iv	Reduced hypoxia-induced pulmonary hypertension and pro-inflammatory cytokines, increased IL-10	207
LPS-induced lung injury		
CD4+CD25+ Tregs iv	Reduced lung inflammation in neonatal mice	208
Ovalbumin/allergen-induced airway inflammation		
CEA-specific CAR-Tregs iv	Reduced severity of asthma by homing to CEA-expressing airways	102
Food allergy		
Cow milk/house dust mite/peanut/ovalbumin allergy		
CD5+CD19+CX3CR1+ B cells iv	Induced Tregs and suppressed food allergy	209
EPIT CD4+CD25+ Tregs iv	Milk-specific EPIT Tregs prevent sensitization to HDM and peanut	210
Cerebral inflammation		
Intracerebral hemorrhage		
CD4+CD25+ Tregs iv	Reduction pro-inflammatory cytokines, neuroprotection	211
Subarachnoid hemorrhage		
CD4+CD25+ Tregs iv	Reduction of cerebral inflammation by suppression of TLR4/NF-κB pathway	212
LPS-induced inflammation		
CD4+CD25+ Tregs iv	Reduced perinatal brain inflammation after maternal LPS exposure	213
Diabetes (T1D)		
T1 Diabetes (NOD mouse)		
MHC class II peptide-specific CAR-Tregs i.v.	CAR-Tregs homed to pancreatic lymph nodes and delay (not prevent) development of T1 diabetes	214
Inflammatory bowel disease (IBD)		
T cell–induced colitis		
CEA-CAR-Tregs i.v.	CEA-specific CAR-Tregs home to CEA-expressing colon and reduce severity of colitis	99
FoxP3+Tregs i.v.	Expression of ubiquitinase USP7 is important for Treg capacity to resolve inflammation	215
RA treated Thy1.1+Tregs i.v.	RA-Tregs suppress acute intestinal inflammation, but not an established chronic inflammation	83
Tregs or B cells i.v.	IL-10–independent Tregs prevented colitis development	216
Necrotizing enterocolitis model		
Tregs i.v.	Transfer of wild-type Tregs decreased enterocolitis severity in HO-1 heterozygous pups	217
TNBS colitis		
Chimeric receptor Tregs i.v.	Significant amelioration of hapten-specific colitis and improved survival	98

(Continues)

TABLE 2 (Continued)

Part 2. In vitro expanded Tregs		
Disease models and Treatment	**Biological effect**	**References**
Multiple sclerosis		
MOG-induced experimental autoimmune encephalomyelitis, EAE		
Minocycline-generated DC i.v.	Increased CD4+CD25+FoxP3+cells, suppressed EAE development	218
MOG-specific CAR-Tregs i.n. or i.p.	Intranasal injection of CAR-Tregs suppressed ongoing encephalomyelitis	101
siRNA-silenced lymph node cells	Increased IL-10+ CD4cells and CD4+CD25+FoxP3+cells suppressed EAE development	219
MBP-induced experimental autoimmune encephalomyelitis, EAE		
FoxA1+ Tregs i.v.	Induced CD4+FoxA1+CD47+CD69+PD-L1(hi)FoxP3$^-$(neg) Treg; FoxA1 Tregs kill activated T cells via PDL1. Adoptive transfer inhibited EAE	220
MBP-specific Tr17 i.v.	RORγt+ Treg (Tr17) cells induction, inhibition of CNS inflammation	221
Psoriasis		
TNFα-induced psoriasis in TNFα transgenic mice		
FoxP3+cells i.v.	Inhibition of pro-inflammatory phenotype macrophages and reduced psoriasis symptoms	222
Rheumatoid arthritis (RA)		
Collagen-induced arthritis		
B cell–induced iTregs i.v.	Induction of LAG3+ Tregs (IL-4+, IL-10+, TGF-β+), reduced osteolysis in hind footpads	223
Collagen-specific Tregs i.v.	Tr1 Tregs (IL-10+, IL-4$^-$, GITR+, CD39+, granzyme B+) induction, reduced clinical symptoms in preventive and curative setting	224
Collagen-specific Tregs i.v.	Reversed collagen-induced arthritis progression and TNFα production	225
iPSC-Tregs transduced with TCR and FoxP3 genes i.v.	Suppression of joint inflammation, osteoclast activity, and Th17 production	226
Mesenchymal stem cells plus Tr1 cells i.v.	Collagen-specific CD4+CD25+FoxP3+ Tregs induction, superior prevention of clinical disease development compared to MSC or Tr1 only	227
Other diseases		
Experimental autoimmune cholangitis		
Tregs i.v.	Transfer of control Tregs, but not of TGF-β RII-negative mice reduced inflammatory responses by IL-10 production	228
Autoimmune thyroiditis		
Ag-specific Tregs i.v.	Tregs from B cell–deficient animals have a stronger suppressive function	229
Autoimmune hepatitis		
Tregs cocultured with hepatic stellate cells i.v.	Reduced liver injury and inflammation	230
Autoimmune neuritis		
Induced Tregs i.v.	Reduction of infiltration in sciatic nerve	231

Note: Abbreviations: CAR: chimeric Ag receptor; CEA: carcinoembryonic Ag; CTLA4: T lymphocyte–associated protein 4; DAC: 5-aza-2'deoxycytidine; DC: dendritic cell; DSS: dextran sulfate sodium; EAE: experimental autoimmune encephalomyelitis; EPIT: epicutaneous immunotherapy; GAD65: glutamate decarboxylase 65; GITR: glucocorticoid-induced TNFR family related gene; HDM: house dust mite; HO-1: heme oxygenase-1; HSP65: heat shock protein 65; i.p.: intraperitoneal; i.t.: intratracheal; i.v.: intravenous; IBD: inflammatory bowel disease; IGFR2: insulin-like growth factor 2; ihTNF: inducible human tumor necrosis factor; iPSC-Treg: induced pluripotent stem cell regulatory T cell; iTreg: inducible regulatory T cell; IVIG: intravenous immunoglobulin; LAG3: lymphocyte-activation gene 3; LPS: lipopolysaccharide; MBP: myelin basic protein; MOG: myelin oligodendrocyte glycoprotein; MSC: mesenchymal stem cells; NDO: nondigestible oligosaccharides; NK T cell: natural killer T cell; NO: nitric oxide; NOD: nonobese diabetic; o.g.: oral gavage; OIT: oral immunotherapy; OX40: tumor necrosis factor receptor superfamily member 4, CD134; PD1: programmed cell death protein 1; PDL1: program death ligand 1; pTreg: peripherally induced regulatory T cell; RA: rheumatoid arthritis; s.c.: subcutaneous; siRNA: small interfering RNA; T1D: type 1 diabetes; TGF-β: transforming growth factor β; TGF-βRII: TGF-β receptor II: Thy-1: CD90; TLR4: toll-like receptor 4; TNBS: trinitrobenzene sulfonic acid; TNCB: trinitrochlorobenzene; TNF: tumor necrosis factor; TNFR2: tumor necrosis factor receptor 2; Tr1: regulatory T-cell type 1; USP7: ubiquitinase 7.

TABLE 3 Clinical trials of Treg cell–based therapies in immune and inflammatory diseases

Part 1. In vivo-expanded Tregs		
Disease models and Treatment	Biological effect	References/ID
Graft-versus-host disease (GVHD): prophylaxis		
Polyclonal tTregs, CD4+CD25+sorted	Combining donor Tregs and Tcons in this first clinical trial prevented GVHD and enhanced immune recovery. At a median follow-up of 12 mo (range, 9-21), 12 of 26 (46.1%) patients were alive and disease free	[46]
Polyclonal tTregs, CD4+CD25+sorted	95% of patients achieved full-donor type engraftment and 15% developed ≥ grade 2 acute GVHD.	[47]
Acute GVHD		
Polyclonal tTregs, CD4+CD25+FoxP3+	Suspended (logistics)	NCT02526329
Polyclonal tTregs	Withdrawn	NCT02118311
Chronic GVHD		
Polyclonal tTregs, CD4+CD25+FoxP3+	Recruiting; estimated study completion date: February 2022	NCT01903473
Polyclonal tTregs, CD4+CD25^{high} sorted	Recruiting; estimated study completion date: August 2019	NCT03683498
Polyclonal tTregs, CD4+CD25+ CD127$^{low/-}$	Recruiting; estimated study completion date: March 2022	NCT02749084
Steroid-refractory chronic GVHD		
Polyclonal tTregs, CD4+CD25+sorted	Unknown; estimated study completion date: July 2016	NCT01911039
Polyclonal tTregs, CD4+CD25+sorted	Recruiting; estimated study completion date: December 2019	NCT02385019
Polyclonal tTregs, CD4+CD25+sorted	Active, not recruiting; estimated study completion date: November 2020	NCT01937468
Polyclonal tTregs	Ongoing	EudraCT2012-000301-71
Transplantation		
Liver		
Polyclonal tTregs	Completed, expanded with anti-CD3/anti-CD28 mAbs + IL-2; 3 of 17 patients consented pre–liver transplantation; 6 of 6 patients consented post-transplantation for ACT. Treg transfer was safe, transiently increased circulating Tregs and reduced anti-donor T-cell responses	NCT02166177 [95,232]
Type 1 diabetes		
Polyclonal tTregs	Direct infusion, unknown; estimated study completion date: December 2020, enrolling by invitation	NCT03162237
Part 2. In vitro expanded Tregs		
Disease models and Treatment	**Biological effect**	**References/ID**
GVHD prophylaxis		
Polyclonal tTregs, CD4+CD25+	Expanded with anti-CD3/anti-CD28 mAbs + IL-2; the incidence of grade II to IV acute GVHD was reduced (43% vs 61%). No deleterious effect on the risks of infection, relapse, or early mortality was observed.	[56] INCT00602693
iTregs (IL10-DLI)	PBMC, expanded with host-derived cells + IL-10; fast immune reconstitution in 5 of 12 patients. In 4 of these 5 patients, complete remission was observed; they remained free of immunosuppression for 7.2 y after haplo hematopoietic stem cell transplantation. Transient GVHD was observed.	[93] ALT-TEN
Allo-Ag-reactive Tregs	Expansion with recipient DCs, estimated study completion date: December 2020, recruiting	NCT01795573
Polyclonal tTregs, CD4+CD25+	Ongoing	EudraCT 2012-002685-12

(Continues)

TABLE 3 (Continued)

Part 2. In vitro expanded Tregs		
Disease models and Treatment	**Biological effect**	**References/ID**
Chronic GVHD		
Polyclonal tTregs, CD4+CD25+ CD127^{-}	Expanded with anti-CD3/anti-CD28 mAbs + IL-2; In the 2 treated patients, alleviation of symptoms and reduction of pharmacologic immunosuppression were observed; completed	[57] NKEBN/458-310/2008
Polyclonal tTregs, CD4+CD25^{high}	Expanded with anti-CD3/anti-CD28 mAbs + IL-2; 2/5 patients showed improvement of chronic GVHD, the other showed stable chronic GVHD symptoms for up to 21 mo. Immunosuppressive treatment could be reduced. One patient developed a malignant melanoma and another had Bowen skin cancer 4 mo and 11 mo after Treg transfusion, respectively; completed	[94] EK206082008
Steroid-refractory chronic GVHD		
Polyclonal t Tregs	Ongoing	EudraCT 2016-003947-12
Transplantation		
Liver		
darTregs, CD4+CD25+ CD127$^{low/-}$	Expansion via anti-CD3/anti-CD28 mAbs + IL-2 + TGF-β Estimated study completion date: December 2015	NCT01624077
darTregs, CD4+CD25+ CD127$^{low/-}$	Estimated study completion date: April 2020, recruiting	[57] NCT02474199
darTregs, CD4+CD25+ CD127$^{low/-}$	Estimated study completion date: January 2022, recruiting	[57] NCT02188719
iTregs	Expansion of recipient lymphocytes with irradiated donor cells in the presence of anti-CD80/86; 7 of 10 patients were successfully weaned off treatment and immunosuppressive therapy was discontinued. The other 3 patients developed mild rejection during weaning, after which conventional low-dose immunotherapy was resumed.	[50]
darTregs, CD4+CD25+ CD127$^{low/-}$	Estimated study completion date: February 2025, not-yet recruiting	NCT03654040
darTregs, CD4+CD25+ CD127$^{low/-}$	Estimated study completion date: February 2025, recruiting	NCT03577431
Kidney		
Polyclonal expanded Tregs, CD4+CD25+	Completed; awaiting results	[233] NCT0212988
Polyclonal tTregs, CD4+CD25+ CD127$^{low/-}$	Recruiting; estimated study completion date: November 2014	NCT01446484
Polyclonal tTregs, CD4+CD25+ FoxP3+	Recruiting; estimated study completion date: April 2017, no results posted	[233] NCT02371434; EudraCT 2013-001294-24
Drug-conditioned iTregs	Expansion with kidney donor PBMC + belatacept, active-not recruiting; estimated study completion date: May 2018	[233] NCT02091232
Polyclonal tTregs	Recruiting; estimated study completion date: December 2019	NCT03284242
Polyclonal expanded Tregs, CD4+CD25+	Expansion with MACS GMP Exp-ACT beads + IL-2 + TGF-β + Sirolimus; safe at all tested Treg doses, with no adverse infusion-related side effects, infections or rejection events up to 2 y post-transplant; estimated completion date: December 2021, active-not recruiting	[49] NCT02145325
Polyclonal tTregs, CD4+CD25+FoxP3+	Active-not recruiting	EudraCT 2017-001421-41
Subclinical rejection in kidney transplantation		
Polyclonal tTregs, CD4+CD25+CD127^{-} sorted	Estimated study completion date: December 2016	[57] NCT02088931
Polyclonal tTregs (phase I) and Donor-allo-Ag-reactive Tregs (darTregs), (phase II)	Estimated study completion date: October 2021, recruiting	NCT02711826

(Continues)

TABLE 3 (Continued)

Part 2. In vitro expanded Tregs		
Disease models and Treatment	**Biological effect**	**References/ID**
Type 1 diabetes		
Polyclonal tTregs; CD3+ CD4+CD25+ CD127^{-}	Expansion via anti-CD3/anti-CD28 mAbs + IL-2, completed; half a year after diabetes onset (4-5 mo after Treg infusion), 8 out of 10 patients still required 0.5 UI/kg/day of insulin (2 patients with out insulin)	[97] ISRCTN06128462
Polyclonal tTregs; CD4+CD25+ CD127$^{low/-}$	Expansion via anti-CD3/anti-CD28 mAbs + IL-2, completed; no infusion reactions or cell therapy-related high-grade adverse events were observed. C-peptide levels persisted up to 2 + y after transfer in more than 50% of the patients; completed	[96] NCT01210664
Polyclonal tTregs	Estimated study completion date: November 2020, recruiting	NCT02932826
Polyclonal tTregs; CD4+CD25+ CD127$^{low/-}$	Estimated study completion date: March 2021, recruiting	NCT03444064
Polyclonal tTregs; CD4+CD25+ CD127$^{low/-}$	Expansion via anti-CD3/anti-CD28 mAbs + IL-2, estimated study completion date: December 2021, suspended	NCT02772679
Inflammatory bowel disease		
Crohn's disease		
Polyclonal tTregs	Estimated study completion date: September 2021, not-yet recruiting	NCT03185000
Autologous Ag-specific Tr1 (Ova-Treg)	Terminated; TxCell ended trial on October 11, 2016, due to challenges in Ovasave manufacturing (with suspension of the manufacturing site and transfer to a new manufacturing site)	NCT02327221/ EudraCT 2014-001295-65
Refractory Crohn's disease		
Autologous Ag-specific Tr1 (Ova-Treg)	PBMC expansion via anti-CD3/anti-CD28 mAbs + IL-2 + IL-4 + ovalbumin, completed; 5 wk posttreatment, clinically improvement in 6 of 8 patients who received 10 Mio cells. The clinical effect was maximal 5 wk after treatment and subsequently reduced progressively.	[234] EudraCT2006-004712-44
Systemic lupus erythematosus, SLE		
Polyclonal tTregs; CD4+CD25+ CD127$^{low/-}$	Estimated study completion date: August 2021, active-not recruiting	NCT02428309
Anti-CD19 CAR-T cells	Unknown status, no results posted	NCT03030976
Other diseases		
Multiple sclerosis		
Polyclonal tTregs; CD4+CD25+CD127^{-}	Data not available	VAC2.0; EudraCT 2014-004320-22
Uveitis		
Polyclonal tTregs; CD4+CD25+FoxP3+	Estimated study completion date: December 2017, suspended	NCT02494492
Amyotrophic lateral sclerosis		
Polyclonal tTregs; CD4+CD25+	Estimated study completion date: March 2018, active, not recruiting	NCT03241784
Autoimmune hepatitis		
Polyclonal tTregs; CD4+CD25+ CD127^{-}	Unknown status, Expansion via anti-CD3/anti-CD28 mAbs + IL-2 + retinoic acid; no results posted	NCT02704338
Pemphigus		
Polyclonal tTregs; CD4+CD25+CD127$^{low/-}$	Estimated study completion date: September 2020, recruiting	NCT03239470
Neuromyelitis optica		
CAR-T cells (tanCART19/20)	Estimated study completion date: August 2020, not-yet recruiting	NCT03605238

Treg-specific IL-2 complexes target cells expressing very high levels of CD25, such as thymus-derived Tregs. In humans, low-dose IL-2 immunotherapy has been effectively used in SLE, hepatitis C virus-induced cryoglobulinemic vasculitis, and chronic GVHD [77,78] as well as tested in RA, ankylosing spondylitis, psoriasis, Behcet's disease, granulomatosis with polyangiitis, Takayasu arteritis, Crohn's disease, ulcerative colitis, autoimmune hepatitis, and sclerosing cholangitis.[79] Alternative approaches to modulate IL-2–IL-2R engagement to modulate Treg functions and favor tolerogenic responses consist in mutated IL-2 molecules, also called "muteins",[75,80] or modified IL-2Rs.[81]

Also administrations of natural dietary compounds such as retinoic acid,[82,83] hydrolyzed whey proteins,[84] nondigestible oligosaccharides,[85] D-mannose,[86] the flavonoid luteolin,[87] ω-3 fatty acids maresin-1,[88] and microbiota-based approaches using bacterial pigments such as violacein,[89] cell wall components of *S pneumoniae*,[90] or whole probiotic strains [91,92] have been successfully employed to expand the Treg population in vivo.

3.2.2 | Ex vivo-expanded/selected Tregs

Treg infusion has been particularly effective in transplantation trials. In the initial therapeutic applications of ACT, blood-derived Tregs were followed by infusion of conventional T cells, prevented GVHD, and favored immunologic reconstitution in 26 out 28 patients with high-risk hematologic malignancies who underwent HLA-haploidentical hematopoietic stem cell transplantation.[46] Also, ACT treatment using ex vivo-expanded Tregs alone reduced and prevented acute GVHD [46,47,56,93] and chronic GVHD,[57 94] with several trials still being ongoing (Table 3.2) Similarly, Treg ACT were successfully employed in several phase I studies to prevent solid organ (liver, kidney) rejection, with several phase 2 trials ongoing or in planning.[60,78,79,95]

Adoptive Treg therapy was also pioneered in autoimmune diseases for patients affected by T1D. Ex vivo-expanded Tregs can both prevent and reverse T1D in mice,[36] and phase 1 clinical trials in T1D determined that a subset of the transferred Tregs were long-lived and survived for at least 1 year.[96] Two published clinical trials of ACT with autologous polyclonal Tregs in T1D have been reported: one in children within two months of diabetes onset [97] and one in adults.[96] ACT was well tolerated with evidence of efficacy despite limited power to infer definitive conclusions. Similarly, evidence in animal models [12] has supported clinical trials of Treg ACT in SLE (NCT02428309) [20] and in a growing list of other autoimmune and autoinflammatory diseases. As for Ag-specific Tregs, donor-allo-Ag-reactive Tregs (darTregs) are currently in clinical trials for liver and kidney transplantation and autologous Ag-specific induced type 1 Tregs (iTr1) for refractory Crohn's disease (part 2 in Tables 2 and 3).

3.2.3 | Ag-specific CAR-Tregs

Although there are no phase 2 clinical trials yet ongoing, several preclinical studies (Table 2, part 2) describe successful application of Ag-specific Treg technology in murine models [8] of experimental colitis,[98-100] experimental autoimmune encephalomyelitis (EAE),[101] T1D,[67] and allergic airway inflammation.[102] In preclinical models, the use of human Tregs engineered against known pathogenic antigens promoted suppression in vitro and in vivo, as with BAR-T cells targeting factor VIII A2 or C2 domains [8,65] and CAR-Tregs recognizing the HLA molecule A*02 [103-106] indicate the feasibility of developing human Ag-specific CAR-Tregs. The lack of identified targeted protein(s) in many immune-driven diseases may be overcome by the bystander effect of Tregs through local suppression of T cells with different Ag specificity.[65,66,98,100,103,107] Specificity could also be achieved by engineering CAR for a tissue-specific Ag in order to direct recruitment of CAR-Tregs to the affected tissue.[108,109]

3.2.4 | CAR-T cells to kill B cells producing autoantibodies: pemphigus as an example

Pemphigus vulgaris (PV) is a life-threatening blistering skin disease caused by IgG autoantibodies directed to the keratinocyte adhesion protein desmoglein 3 (Dsg3).[110] Temporary clinical improvement is seen with the anti-CD20 monoclonal Ab rituximab [111,112] associated with depletion of $CD20^+$ memory B cells.[113] Thus, specific elimination of anti-Dsg3 memory B cells has the potential to cure the disease without the risk of general immunosuppression.[111,112] Recently, human cytotoxic CAAR-T cells have been engineered to express Dsg3 fused to components of the intracellular domains of the TCR activation complex (CD137-CD3).[17] This auto-Ag–based CAR directed cytotoxic T cells to kill autoreactive B cells (expressing Dsg3-specific BCR) without affecting B cells with other specificities. The circulating anti-Dsg3 IgG in PV patients [110,113] promoted CAAR-T cell survival through CD137 signaling.[17] In human skin-xenografted mice, CAAR-T cells did not exhibit cytotoxicity against tissues expressing biological ligands of Dsg3, such as desmosome components,[17,114] indicating a good safety profile. Moreover, CAAR-T cells did not react in vivo with cells expressing FcγR (including monocytes and neutrophils) which could potentially carry anti-Dsg3 IgG, or with immature bone marrow B cells displaying a polyreactive BCR repertoire.[17] Development of CAAR-T cells thus expands the range of targeted approaches to treat autoantibody-mediated diseases with well-identified autoantigens and points at more applications of this approach for autoimmune diseases with similar biology.

4 | TARGETING SEVERE PHENOTYPES OF ALLERGY, ASTHMA, AND COPD: A STEP AHEAD FOR ACT IN IMMUNE-DRIVEN DISEASES

4.1 | Targeting IgE-mediated responses and complex atopic disorders

Treg dysfunction is implicated in the failed tolerance toward foreign proteins in most allergic diseases of the skin, airways, and gut.[3,115-119]

The potential use of Tregs in the prevention of allergic diseases would be particularly relevant in high-risk atopic children. As suggested by preclinical studies (Table 2b), an antigen-specific Treg population against the most common antigens could be developed ex vivo that could protect against the clinical manifestations associated with allergen contact. Severe forms of food allergy could be potentially treated with Ag-specific ACT since the allergens are largely identifiable.[15,117] Clinical responses to oral immunotherapy (OIT) for severe food allergy are paralleled by restoration of Treg numbers and function, though long-term tolerance has not been consistently achieved so far. Notwithstanding, in recent studies pretreatment with omalizumab facilitated rapid oral desensitization to peanut and restored Treg function.[120] Murine models have suggested that combining OIT with low-dose IL-2 may aid tolerance.[121] While no clinical study with Treg ACT have been reported for food allergy to date, preclinical studies clearly show that increased tolerogenic responses are involved in preventing allergen sensitization and suppressing Ag-driven inflammatory responses in the gut (Table 2, part 2).

A cure for severe allergies could be envisioned through ACT by targeting long-lived IgE-producing memory B and plasma cells with CAR-T cells. This approach was tested using CAR-T cells carrying the α chain of the high-affinity IgE receptor, FCεRI, recognizing the transmembrane form of IgE (mIgE) present only on the B-cell lineage and using FCεRI α mutants with low affinity to IgE. FCεRIα mutant-CAR-Tregs did not activate other cells carrying surface-bound IgE or trigger degranulation of LAD2 mast cells, while they exerted potent and specific T-cell responses on $mIgE^+$ murine B cells.[122]

The CAAR-T and BAR-Treg approach is conceptually applicable to IgE-mediated food allergy where a small number of allergen-specific memory B cells maintain the clinical reactivity to food allergens throughout life, exposing patients potentially to life-threatening anaphylaxis even after minimal Ag exposure. Memory B cells, rather than long-lived IgE^+ plasma cells (PC), appear to be responsible for this lifelong reactivity.[123] Tregs could be engineered with BARs with the extracellular domain displaying the allergenic molecular determinant, to directly bind to Ag-specific memory B cells causing their permanent anergy or elimination and permanent loss of specific IgE-mediated responses. However, off-target effects on basophils and mast cells must be avoided, possibly by concomitant use of biologicals targeting IgE.

Along the same lines, also severe forms of atopic dermatitis (AD) may be amenable to ACT, as AD arises from the combination of an altered skin barrier and dysregulated immune reactions mainly driven by T-cell dysfunction.[124] AD patients display drastically increased numbers of circulating IgE of varied specificity and affinity [125] and a significant proportion of AD subjects (23%-91%) display IgE antibodies against skin proteins (autoallergens).[126] Some studies found also a correlation between disease severity and the occurrence of autoallergy.[127,128] Chronic tissue damage would expose otherwise hidden intracellular antigens, thus facilitating IgE-sensitization.[129] Low-avidity but potentially autoreactive T-cell clones might escape negative selection in the thymus [130,131] and could be activated by the strong inflammatory milieu in AD lesions, ultimately accounting for the generation of autoreactive IgE.[129] In this regard, autoallergy might also arise from IgE-sensitization against fungal antigens, as atopic skin is often colonized by a variety of fungi and bacteria [124] and cross-reactivity between skin and fungal proteins has been demonstrated in vitro.[132,133] Although the clinical relevance of autoallergy has been questioned,[129] skin autoallergens were able to induce T-cell proliferation in AD patients.[134] Taken together, these data raise the potential of ACT with Tregs or CAR–T and B cells for restoring self-tolerance in recalcitrant AD.[13,135] Moreover, CAR-directed Tregs might contribute to repair of the skin barrier, with subsequent decrease in the local availability of autoallergens.[26] Also, the use of other regulatory cells, such as Bregs and myeloid-derived suppressor cells, may be investigated for future cell-based approaches against autoimmunity, allergic, and chronic inflammatory diseases, either alone or in combination with Tregs.

4.2 | Targeting lung inflammation, tissue damage, and autoantibodies in severe asthma and COPD

Transfer of polyclonal, ex vivo-expanded $CD4^+CD25^+$ Tregs can suppress Ag-driven responses and prevent tissue remodeling in lung disease models,[136,137] with Ag-specific Tregs being 10 times more potent than polyclonal Tregs.[138] Skuljec and colleagues [102] have provided a proof-of-concept study for CAR-T cell therapy in experimental asthma.[139] Adoptive transfer of CAR-Treg cells directed against the carcinoembryonic Ag (CEA) transgenically expressed in airway epithelial cells led to preferential CAR-Treg localization in the airway mucosa and draining lymph nodes. This was associated with suppression of eosinophilic inflammation, mucus production, airway hyperresponsiveness, T-cell proliferation, Th2 cytokine secretion, and reduced specific IgE levels.

Therapeutic targeting of airway remodeling is a major unmet need both in asthma and COPD.[135] Epithelium-driven fibrosis is pathogenically relevant, and ACT could address airway remodeling. Adoptive transfer of CAR-T cells against fibroblast activation protein, a fibroblast- and disease-specific gene, in a mouse model of cardiac fibrosis resulted in significantly reduced fibrosis and restoration of function after injury.[140] Similarly, CAR-Tregs may repair bronchial epithelia damaged by amphiregulin release.[27]

Local tertiary lymphoid tissue formation, developing in the airway mucosa during chronic inflammatory responses, may enable the generation of autoantibodies toward immunogenic components released upon tissue damage by degranulating eosinophils. Autoantibodies against nuclear antigens and tissue components/cell types (airway epithelium, endothelium, extracellular matrix, cell junction proteins) have been reported in asthma [141] and in a subset of patients with severe eosinophilic asthma.[142] This raises the potential of using directed CAR-Treg ACT in severe and therapy-refractory asthmatics. Cellular and Ab-mediated autoimmunity is also found in stable COPD,[143] with oligoclonal B cells found in bronchus-associated lymphoid follicles suggesting a role in local Ag-specific autoimmunity.[143] Severe emphysema has been associated

with lung oligoclonal $CD4^+$ and $CD8^+$ T cells in man [144] and in murine disease models [145,146] with cytotoxicity against the bronchial epithelium.[144] Serum and/or lung autoantibodies are often found in both "healthy" smokers and COPD patients [143,147] and the serum IgG_1 Ab titer against carbonyl-modified self-proteins correlates with disease severity. High levels of autoimmune IgA are evident in severe COPD [148] although the driver of lymphoid follicle development and IgA production requires elucidation.[149] This will enable the production of distinct types of regulatory and other CAR–T and B cells that could be disease-modifying.[14]

4.3 | Integrating ACT with ongoing therapies in chronic lung inflammatory diseases: opportunities for patients with severe disease phenotypes

Patients with chronic inflammatory diseases are often on complex drug treatments, which may impact the clinical efficacy of CAR-T cells. Such action may be positive, but also negative, which altogether should be considered when initiating personalized therapy for severe asthmatics and COPD patients.[150,151] As an example of potential positive synergy, CAR-T cells directed against IgE-producing B cells [122] may be used in patients with severe asthma where repeated dosing of expensive biologicals, as the anti-IgE-specific monoclonal Ab omalizumab, is currently required. The development of BAR-Tregs, originally designed to suppress B cells producing anti-factor VIII antibodies, indicate that CAR technology is suitable to target soluble antigens and both T cell- and Ab-mediated responses. Thus, this approach could suppress the production of anti-IgE auto-Abs in severe asthmatics [141] that decrease the effectiveness of omalizumab in severe prednisone-dependent asthmatics.[141,152] Raising the issue of a possible negative influence, metformin, a treatment for diabetes mellitus that acts through AMP-activated protein kinase (AMPK), can suppress the ability of CD19-CAR–T cells to induce cytotoxicity of tumor cells.[150] Future studies will determine whether the efficacy of CAR-T cells in cancers and in severe chronic inflammatory diseases, which often co-exists with metabolic syndrome, will be enhanced by metformin in combination therapies.[45]

5 | TREG CELL THERAPIES IN SEVERE ALLERGIC AND CHRONIC INFLAMMATORY LUNG DISEASES: LIMITATIONS AND CHALLENGES

Clinical trials for Treg ACTs in immune-related diseases (Table 3)[45,153] increasingly indicate the technical feasibility and good efficacy of these approaches. Along with evidence on CAR-Tregs in preclinical stage (Table 2), these studies are also providing important insights on current limitations and challenges facing their application. The main issues currently confronted refer to managing the stability and plasticity of Tregs, to directing their homing to the desired sites, and to safety concerns.

Concerning the latter, the adverse events observed following Treg-based ACT are overall milder and are qualitatively different from those occurring when conventional CAR-T cells are used, reflecting their different immunomodulatory strategies. Adverse effects using $CD8^+$ cytotoxic CAR-T cells may be severe and consist in over-activation of the immune system [cytokine release syndrome (CRS) and CAR–T cell–related encephalopathy syndrome (CRES), hemophagocytic lymphohistiocytosis], sudden tumor lysis (tumor lysis syndrome), and on-target/off-tumor recognition (B-cell aplasia and acute respiratory distress syndrome).[154-156] Major trials indicated an incidence of CRS of 77% in patients treated for acute lymphoblastic leukemia,[157] while for non-Hodgkin lymphoma patients the reported incidence with two different CAR-T cell products was 57% [158] and 93%.[159]

The occurrence of CRS for future treatments using CAR-Tregs is yet to be assessed but is deemed unlikely. However, in case of a phenotype shift of infused Tregs to conventional T cells possibly caused by a strong pro-inflammatory microenvironment in the recruitment sites, safety strategies for their rapid elimination exploiting "suicide cassettes," initially developed from conventional CAR-T cell, are in place [reviewed in 69]. A suicide system tested in Treg ACT for GVHD entails the retroviral transduction in donor T cells of an inducible "suicide" proapoptotic gene construct. When exposed to a drug, an inducible caspase 9 (iCasp9) gene is activated, triggering rapid and specific death of the transduced CAR-T cells.[160] CAR-Treg share with conventional CAR-T cell-based ACT the potential risk of viral vector-specific toxicity for transgene insertion (related to the potential of viral vectors to replicate) [161]; the standardization of CRISPR-Cas9 genome editing methods represent a major step ahead in development of new synthetic CAR/TCR-T cell therapy not only for improving safety, but also to increase the stability of Treg phenotype.

The most expected adverse events for Treg-based ACT are rather linked to generalized immunosuppression, possibly favoring infectious diseases and tumors.[60,96] Patient data analysis following ACT with nTregs for GVHD treatment in hematopoietic transplantation indicated an increased risk of viral reactivations (mainly human herpesvirus 6 or cytomegalovirus) only at short term postinfusion (30 days), while Tregs were detectable.[162] No opportunistic infections were observed after Treg ACT in pediatric patients newly diagnosed for T1D followed for 4 months [97] nor malignancies or infections in adults patients with T1D followed up to five years.[96] The use of engineered Tregs and expanded Ag-specific Tregs might reduce these risks,[8,29] but severe immunosuppression toward pathogens carrying specific antigens may be observed in the case of unpredicted antigenic specificity (eg, cross-reactivity). Large studies with long-term follow-up constitute an important unmet need to measure the actual risk of generalized or pathogen-specific immunosuppression—as well as malignancies—associated with Treg-based ACT.

Another potential risk specific for this approach is the worsening, rather than improvement, of the targeted disease. This negative outcome was initially attributed mainly to the use of Tregs contaminated by other T cells due to errors in the purification procedure

TABLE 4 Molecule- vs cell-based therapies: synopsis

Feature	Small-molecule drugs and biologicals	Adoptive Treg cell therapy (engineered-Treg cells and Ag-specific Treg cells)
Dose	Controlled at time of administration	Controlled by cell decision-making based on proliferation, activation, and death
Distribution	Diffusion and transport Controlled PK/PD	- Ag-dependent migration tissue-targeting approach - Bystander effect in the tissue expressing the Ag
Main activity	Singular, determined by the targeted molecule/pathway	Multiple, determined by cell activity: - immunosuppression/immunomodulation - cell killing of autoreactive cells - tissue repair properties (independent from immunomodulation activity)
Therapeutic effect on disease	Control of the disease/ disease-modifying effect	Disease-modifying effect/potential cure of the disease
Duration of therapeutic effect	Usually determined by pharmacokinetics (few hours-some wk)	Dependent on cell biology features (mo/y): - survival - proliferation - phenotype plasticity - phenotype stability
Numbers of treatment	Several	One/few
Adverse effects	From nonserious to fatal	Potentially few and non-serious
Titration/change of strategies	Easily implementable	Not immediate (setup of salvage strategies are under development)
Type of treatment	Universal or phenotype/ endotype-driven	Prepared for each patient (until validation of universal CAR technique)
Cost	Low/high dependent on type and approval time	Very high dependent on the procedure needed to prepare Treg cells (until setup of universal Treg cells)
Main strength of Treg ACT	-	- Cure of the disease (change of the immune balance toward homeostasis) - Multiple effects, determined by cell activity
Main weaknesses of Treg ACT	-	- Risks dependent on cell purification/ preparation, including contamination and vector-related effects - Organ-centered immunosuppression - Cell phenotype instability
Main unmet needs for implementation of Treg cell therapy in chronic inflammatory and allergic diseases	-	- Definition of most suited Treg subset to be used - Ways to implement Treg phenotype stability - Ways to favor survival/expansion of infused Treg cells - Longitudinal studies of safety and tolerability - Definition of disease-specific molecular targets when feasible - Definition of severe chronic inflammatory disease patient subsets likely to have favorable cost/benefit profile for ACT adoption - Synergy with SMD/biologicals - Effect on patients with major comorbidities

or the incorrect choice of cell selection marker, for example, CD25, which is also transiently upregulated on activated T cells in some autoimmune diseases.[13,74,163] Isolation and manufacturing cells for ACT is a major procedural endeavor that has evolved in the last decade into well-standardized, GMP-compliant protocols.[58] Untoward effects and risks of Treg-based ACT are now mostly related to issues regarding phenotype stability and persistence of the infused Tregs.

Despite the stability of epigenetic processes in several Treg subsets [157] and some positive evidence for maintenance of Treg phenotypes,[95] ensuring the persistence and stability of the suppressive phenotype of infused Tregs is a critical issue for the success of current and future ACT applications.[1,13,42,60,96,153,158-161] Loss of FOXP3 expression is associated with Treg conversion to conventional phenotypes in human disease and in animal models, driven by high levels of pro-inflammatory cytokines and low IL-2 [164,165]; therefore, sustaining FOXP3 levels represent a key strategy to maintain Treg phenotype and related suppressive functions. Stabilization of Tregs can be obtained In vitro by many drugs, from immunosuppressive small molecules (eg, glucocorticoids, rapamycin, fingolimod) to epigenetic drugs (eg, HDAC inhibitors and

hypomethylating agents), TGF-β, trans-retinoic acid and vitamin D or even biologicals that favor Treg expansion, for example, the anti-CD3 antibody.[11,166-168] Therefore, the association of Treg therapy with such treatments could be an effective strategy to be investigated. Several strategies to take advantage of IL-2 function to sustain Treg persistence and stability are also currently considered (Section 3.2.1, Table 2.1).

In addition, if CAR-Tregs recruited in inflammatory microenvironments convert into other T cell phenotypes they may not only lose efficacy, but they may even become pathogenic. Relevant to allergic diseases, in a food allergy mouse model with enhanced T cell IL4R signaling, allergen-specific pTregs were found in lower number and with inferior suppression function in OVA-sensitized mice compared to cells in WT controls. These cells exhibited a marked STAT6-dependent, Th2-like phenotype with high expression of GATA-3 and IL-4 that was responsible of clinical anaphylaxis upon Ag rechallenge, as deletion of *IL-4* and *IL-13* in Treg cells fully abrogated this clinical response. Importantly, this Th2-like reprogramming was also found in Ag-specific Treg cells of food allergic pediatric patients.[169] These data suggest that tTregs may be a more stable population to employ in ACT, as they there are selected on the basis of their strong Ag affinity.[162] The use of CRISPR technology could enable the engineering of Treg populations with a blunted reprogramming ability, by removing for example IL-4, IFN-γ, and IL-17 genes, or by making them resistant to inflammatory signals known to destabilize Tregs, such as IL-6, by deleting the IL-6 receptor gene. The effect of this latter modification needs to be tested in specific disease models, given the pleiotropic effects of IL-6.[170] Conversely, CAR-Tregs could be engineered to stably express IL-10 and/or TGF-β.[24] Furthermore, to improve T-cell homing and limit off-target activity, studies in tumor models indicate that CAR-T cell homing can be better directed to the targeted site by ectopic co-expression of chemokine receptors whose ligands are locally overexpressed [reviewed in 69,153].

Several technical aspects also limit ACT with Tregs, such as the scarcity of reagents and instruments specifically designed for Treg manufacturing and the difficulty in obtaining a sufficient number of Tregs.[15,29] The development of Ag-specific CAR or TCR Tregs is expected to provide higher cell yields. Further research is needed to better characterize important therapeutic determinants, such as the most appropriate Treg subset to be engineered, the optimal doses and, ultimately, the immune-driven diseases and phenotypes in which the ACT could be most impactful, given its current very high cost.[15,29,58]

6 | CONCLUSIONS

Therapeutic approaches for allergic and chronic inflammatory lung diseases have evolved from general immunosuppression, delivered through glucocorticoids and some SMDs, to more targeted therapies with newer SMDs, including LTRA and PDE4 inhibitors, and the introduction of biologicals. Improved integration and addressing common unmet needs for SMD and biologicals—such as definition of adequate biomarkers predicting therapeutic response [5,6]—will hopefully render these strategies even more effective in facing the global increase in the social and economic burden of these diseases.[171,172] In this setting, T cell–based therapeutics take a further step and pursue the ambitious and so far elusive goal of disease-modifying strategy, by addressing the breaks in immune tolerance originally preventing overexpressed inflammatory responses and tissue damage. Basic immunological questions, potential clinical scenarios, manufacturing issues, and cost effectiveness are all critical aspects of ACT approaches currently under intense scrutiny in therapeutic research and development.[7,15,16] The painstaking evidence generated in the last two decades clearly shows that engineering Tregs with specific TCR or CAR could yield therapeutic tools able to expand beyond disease control the treatment goal for autoimmune, allergic, and chronic lung inflammatory diseases [24,173] while the next generation of immune cell–based therapies is already advancing, exploiting gene-editing techniques.[174] To this end, a necessary research endeavor will be to identify disease-specific antigens that can be effectively targeted without incurring in critical off-target effects.

Disease control by SMD and biologicals and disease modification by cell-based therapies are strategies with specific pharmacological qualities, scopes, and challenges [7] (Table 4). A common key need is the identification of disease biomarkers to assist in the critical aspect of patient selection.[5] The overarching goal of all three approaches is providing precision therapy when is most needed, balancing efficacy and economical sustainability. Rational integration of the three pillars—SMD, biologicals, and cell-based therapies—may be an effective way to approach this goal.

CONFLICT OF INTEREST

IMA, CBV, RB, LB, OB, LC, AK, GN, FAR, FRW, CS, and FLS have no conflicts of interest regarding the subject of this manuscript. GC has unrestricted educational grants and/or lecture fees and/or grants for travel and accommodation to participate to scientific meetings from AstraZeneca, Biofutura, Boehringer Ingelheim, GSK, and Menarini Group. ZD is employed by a CRO (QPS-NL) who performs phase I/II studies for various biotech and pharmaceutical companies; ZD also received honoraria for consultancy activities (within the past 3 years) from the following pharmaceutical companies: ALK, Aquilon, AstraZeneca, Boehringer Ingelheim, Gilead, HAL-Allergy, and Sanofi-Genzyme-Regeneron. IEG received advisory fees from ALK and Novartis, and lecture fees from AstraZeneca, Novartis, Chiesi, and Diater; AGAK has served as an investigator, speaker, and/or advisor for AbbVie, Abbott, Janssen, Eli Lilly, MSD, Pfizer, Celgene, Novartis, Actelion, Leo, Amgen, and Alk-Abello, and does not hold any shares or other financial interest in any related pharmaceutical company; OP received lecture fees from Allergy Therapeutics, Amgen, AstraZeneca, Inmunotek, Novartis, Sanofi Genzyme, and Stallergenes and participated in advisory boards from Novartis and Sanofi Genzyme. CBV received the FPU fellowship from MINECO, and KFC has received honoraria for participating in Advisory Board meetings of the pharmaceutical industry regarding treatments for asthma and chronic obstructive pulmonary disease

and has also been renumerated for speaking engagements. BvE is partly employed by Nutricia Research.

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