Cognitive Vitality Reports® are reports written by neuroscientists at the Alzheimer's Drug Discovery Foundation (ADDF). These scientific reports include analysis of drugs, drugs-in-development, drug targets, supplements, nutraceuticals, food/drink, non-pharmacologic interventions, and risk factors. Neuroscientists evaluate the potential benefit (or harm) for brain health, as well as for age-related health concerns that can affect brain health (e.g., cardiovascular diseases, cancers, diabetes/metabolic syndrome). In addition, these reports include evaluation of safety data, from clinical trials if available, and from preclinical models.

T regulatory cell Therapies

Evidence Summary

While generally well-tolerated, limited clinical benefits have been seen with attempts to boost the immunosuppressive capacity of Tregs thus far, but next generation approaches may be better.

Neuroprotective Benefit: Treg dysfunction has been observed in neurodegeneration and disease progression. Minor benefits observed with current approaches have not been durable, requiring sustained dosing, which may not be practical long-term.

Aging and related health concerns: Benefits observed in organ transplant and autoimmune conditions have been variable and modest thus far, but next-generation approaches may allow for enhanced efficacy.

Safety: Low dose IL-2 and Treg cell transfer approaches are generally well-tolerated. Chronic immunosuppression from long-term use may increase the risk for infections. Infusion-related reactions, flu-like symptoms, and eosinophilia are reported adverse events.

Availability: In clinical trials	**Dose**: Low-dose IL-2 is most commonly administered in clinical trials at a dose of 1 million international units s.c. in cycles of five-day courses. Adoptive transfer of *ex vivo* expanded Tregs typically administered at 1×10^6 cells/kg	**Chemical formula:** N/A **MW**: N/A
Half-life: IL-2 <15 minutes, but novel formulations can be much longer, such as rezpegaldesleukin with a half life of approximately 10 days.	**BBB**: IL-2 is generally penetrant, though some novel formulations may not be. Adoptively transferred cells are administered peripherally, and the number of cells that reach the brain is unclear and likely highly variable.	
Clinical trials: Treg adoptive cell transfer and low-dose IL-2 have been tested in numerous trials, that were small Phase 1 proof of concept studies, primarily in autoimmune diseases, organ transplant, graft vs host disease, and neurodegenerative disease.	**Observational studies**: Treg numbers and/or suppressive capacity has been found to be altered in the context of autoimmune and some neurodegenerative diseases.	

What is it?

T regulatory cells (Tregs) are a subset of immune cells important for maintaining immune tolerance [2]. They suppress the responses of cytotoxic effector cells, which helps protect against autoimmunity and against excessive inflammatory damage during immune responses toward external threats. Tregs can take on a variety of functions and activities depending on the cell environment, and thus are best understood in a context dependent manner. There are several subtypes of Tregs that differ depending on origin and cytokine production. Briefly, natural Tregs originate in the thymus, while induced Tregs are generated from conventional CD4+ T cells in the periphery into different subsets, including IL-10 producing Tr1 cells, TGF-β producing Th3 cells, and IL-35 producing iTr35 cells [2]. These different subsets vary in terms of their T cell receptor (TCR) repertoire, immunosuppressive capacity, and

functionality, such that different subsets of Tregs may preferentially contribute to Treg dysfunction associated with different disease states. The classic phenotype of Tregs is defined by the expression of CD4, high expression of the IL-2 receptor alpha (CD25) and expression of the transcription factor FoxP3 ($CD4+CD25^{hi}FoxP3+$) [2]. However, due to the heterogeneity amongst different Treg populations in terms of subset and activation status, different studies use different sets of cell markers to define Tregs. As a result, many discrepancies across studies analyzing Tregs in different populations may simply stem from studies capturing information about different subsets of Tregs, which may be differentially impacted in different disease states and stages.

Treg based therapeutic approaches are in development for several conditions characterized by altered immune tolerance [3]. The main indications to date have been for enhancing tolerance toward self-antigens in autoimmune conditions, and to enhance tolerance to foreign antigens in the context of organ transplant. Some studies have also tested these approaches in the context of neurodegenerative disease, particularly amyotrophic lateral sclerosis (ALS).

The two primary approaches are to augment the number and function of endogenous Treg populations, through the use of low-dose IL-2 therapy, and the administration of *ex vivo* expanded autologous Tregs through adoptive cell transfer, often in combination with low-dose IL-2 to enhance the survival of the transferred Tregs [2; 3]. To date, the clinical benefits of these approaches have been limited, but novel formulations, genetic modifications, and methodological advances are in development, which may enhance the efficacy of these approaches.

Neuroprotective Benefit: Treg dysfunction has been observed in neurodegeneration and disease progression. Minor benefits observed with current approaches have not been durable, requiring sustained dosing, which may not be practical long-term.

Types of evidence:

- 2 Phase 1 clinical trials in AD
- 5 Phase 1 or Phase 2 trials in ALS
- 9 observational studies of Treg profiling in AD
- 4 observational studies of Treg profiling in PD
- 3 observational studies of Treg profiling in ALS
- Numerous laboratory studies

Human research to suggest prevention of dementia, prevention of decline, or improved cognitive function:

It has not yet been established whether Treg-related therapies can impact cognitive trajectories, however, there is evidence from blood-based biomarker studies that Treg profiles are impacted over the course of several neurodegenerative diseases, such that Treg capacity tends to be higher earlier in the disease course, and high capacity is associated with slower disease progression.
Although there are discrepancies across studies in terms of the relationship between Treg levels and disease status, these are likely attributable to differences in immunotyping methodology. Treg phenotypes can be influenced by the isolation protocols and assays used [4]. Additionally, Tregs can be defined in different ways based on cell surface markers, such that different studies may be capturing changes in distinct subpopulations of Tregs. The most commonly accepted marker profiles for Tregs are $CD4+CD25^{hi}CD127^{lo}$ and $CD4+CD25^{hi}FOXP3+$, which indicates that Tregs are CD4+ T cells with high expression of the IL-2 receptor alpha (noted by the surface marker CD25), low to no expression of the IL-7 receptor (noted by the surface marker CD127), and expression of FoxP3, the master transcription factor regulator of Tregs [3]. CD25 is also an activation marker, thus measures of CD4+CD25+ cells reflect activated CD4+ cells in general, rather than just Tregs [5]. Additionally, there are many subsets of Tregs which vary based on their antigen exposure, suppressive capacity, and cytokine secretion [2].
A major distinction is between naïve Tregs, and antigen exposed or activated Tregs. Naïve T cells are generally defined by the marker CD45RA, while activated or memory T cells are characterized by the lack of this marker, and/or expression of CD45RO [3]. There is a high proportion of naïve cells in early life, which shifts in adulthood toward more activated/memory cells, due to the increasing burden of antigen exposure throughout life. Activated Tregs have higher immunosuppressive capacity relative to naïve Tregs, thus measures of Treg immunosuppression can be influenced by the relative percentage of activated to naïve cells [6]. In the context of disease, chronic antigen stimulation can lead to immune cell exhaustion and senescence, in which case high levels of activated cells could be an indication of Treg dysfunction, and lower suppressive capacity.

Alzheimer's Disease (AD): Several studies have found that the level and function of Tregs varies over the course of the disease, such that there is an elevation early in disease, which is thought to be a compensatory response to heightened neuroinflammation [7]. Chronic antigen (likely Aβ) stimulation exhausts the Tregs, leading to increasingly dysfunctional Tregs, which can no longer appropriately respond to the ongoing inflammatory processes, and may actually exacerbate pathology. Additionally, changes in the microenvironment of the brain, such as the profile of inflammatory cytokines, may lead

to the skewing of Treg cells toward other cell types, such as proinflammatory Th17 cells [8]. This hybrid population loses its immunosuppressive capacity, and may instead contribute to immune-mediated damage. The changes in the immune environment over the course of disease may explain why Treg depletion at early stages was found to accelerate disease progression, while depletion showed benefits at later stages in AD animal models [9; 10].

One study (n=37) found that blood levels of both Tregs (CD4+FoxP3+) and the pro-inflammatory cytokine IL-1β were elevated in patients with amnestic mild cognitive impairment (MCI), relative to controls, while Treg levels were no longer significantly elevated in patients who had progressed to AD [11]. A separate study indicated that levels of proinflammatory Th17 cells (CD3+CD8−IL-17A+IFNγ−) were increased in patients with MCI due to AD [12]. The increase in both proinflammatory and immunosuppressive (Tregs) cells in the early stages of AD suggests that the elevation of Tregs early in disease is reflective of a compensatory response to pathological inflammatory processes. The percentage of Tregs (9.24%), along with the Treg-associated cytokine (47.02 ng/mL), TGF-β, were also found to be higher than controls (8.19%) or patients with moderate-to-severe AD (7.42% and 34.83 ng/mL, respectively) in a different cohort (n=114) [13]. There was a positive correlation between the percentage of Tregs and cognition, based on Mini-Mental State Examination (MMSE) score (partial correlation coefficient 0.445). An increase in Tregs in MCI relative to controls or severe AD patients has been observed in another study (n=105) which also found that Tregs from MCI patients had enhanced suppressive capacity against Aβ-stimulated T cells [14]. This was driven by an increased proportion of PD-1 negative Tregs (CD4+/CD25^{hi}/Foxp3+/PD1^{neg}) in MCI, as this population has enhanced suppressive capacity [14]. PD-1 can be a marker of T cell exhaustion and dysfunction in some contexts. A shift toward an increasing percentage of PD-1 positive Tregs with disease progression could be an indication of Treg exhaustion, likely as a consequence of chronic antigen stimulation.

With age, there is a shift in the T cell compartment from less naïve cells to more activated/antigen-experienced subtypes such as memory and effector T cells [15]. Several studies have found this shift to be exacerbated in the context of AD, which is thought to stem from chronic stimulation with Aβ and other disease protein-related antigens [5; 16]. One study found a positive association between CD4+ T effector cells, which are antigen-experienced cells, with disease progression in AD [12]. A similar shift is seen in the Treg population, such that Tregs are more likely to be CD4+FoxP3+ antigen-experienced cells with a memory phenotype, such that increases likely stem from clonal expansion [15]. Levels of resting/naïve Tregs (CD25^{dim}CD45RA+) were found to be reduced in patients with mid-stage AD, with a shift toward more activated (CD25^{bright}CD45RA^{neg}) cells [15; 17]. With repeated antigen-stimulation,

these activated Tregs start to undergo immunological senescence [15]. Activated Tregs in patients with mild-to-moderate AD were found to express the senescence marker KLRG1 [5]. Tregs from AD patients were also more likely to express CD95, the Fas receptor, which promotes cell death [18]. CD95 is expressed on effector and memory cells following antigen stimulation. CD95 has context dependent effects, but high levels of CD95 can trigger cell death, and in conjunction with CD28, has also been associated with senescence. This suggests that chronic stimulation results in a population of exhausted, dysfunctional Tregs which can no longer effectively combat inflammatory processes, and may contribute to disease progression.

Parkinson's Disease (PD): Alterations in the profile of circulating Tregs have been observed in PD patients. The immune profile was found to differ between AD and PD patients, such that dysfunction in regulatory subsets may play a larger role in the context of AD [18]. Additionally, several studies have observed more prominent Treg dysfunction in PD patients with cognitive impairment. PD patients with MCI (n=60) were found to have lower levels of Tregs relative to PD patients with normal cognition (n=63), and the levels declined with more advanced stages of disease [19]. A study in 43 PD patients found that those with cognitive impairment had a lower ratio of naïve Tregs relative to activated Tregs [20], which is a similar trend to what is observed in the context of cognitive impairment in AD.

Amyotrophic lateral sclerosis: Treg numbers and function have been associated with disease progression in ALS patients. The levels of circulating Tregs and FoxP3 expression were found to be associated with rates of disease progression, such that those with high levels had slow progression, while those with low levels showed more rapid progression [21]. However, these measures were indicative of the rate of disease progression at the time of blood collection, but were not necessarily prognostic about future rates of progression because the duration of slow progression is highly variable across individuals. In a study including 89 newly diagnosed ALS patients, high levels of CD4+FOXP3^{neg} effector T cells in the blood and CSF were associated with lower rates of survival, while higher levels of activated Tregs (CD25^{hi}CD45RA−) were associated with better survival and slower progression [22]. In addition to having lower levels, the Tregs from ALS patients have reduced suppressive capacity against T effector cells in *in vitro* assays [23].

Human research to suggest benefits to patients with dementia:

A couple of Phase 1 trials have been conducted testing the effects of Treg-based therapies in AD patients.

Low-dose IL-2 (1 million units [MIU]; COYA 301) was administered s.c. in five-day courses monthly for four months in eight patients with mild to moderate AD, based on MMSE score (12-25), in an open label Phase 1 feasibility study (NCT05821153) [24]. An approximately two-fold increase in CD4+CD25^{hi}FoxP3+ Tregs was observed following each cycle, which returned to baseline prior to the initiation of the next cycle. Increased Treg suppressive function was also observed, coupled with a decrease in proinflammatory cytokines and chemokines, including IL-1β, IL-6, TNFα, IL-15, CCL2, CCL4, CCL11 and FLT3LG. Statistically significant improvement was observed on the MMSE with treatment, which along with Treg levels, returned to baseline following the discontinuation of treatment. A trend toward improvement was also observed on the Clinical Dementia Rating Sum of Boxes (CDR-SB). A Phase 2 trial is underway (NCT06096090).

A clinical trial has been registered by VT Bio testing adoptive transfer of their Treg cell preparation (VT301) in patients with mild to moderate AD (NCT05016427), however, further details regarding the trial are not currently available.

Mechanisms of action for neuroprotection identified from laboratory and clinical research:

Restoration of Treg suppressive capacity through ex vivo expansion:
Several studies have found that the ability of Tregs to suppress autologous T effector cells in *in vitro* assays was reduced in patients with AD, PD, or ALS. Tregs derived from PD patients (n=39) were found to have lower expression of CD25 and FoxP3, as well as a reduction in suppressive capacity of 22.5%, relative to controls (n=31) [25]. Suppression of T effector cells went from 42% at baseline to 84% following *ex vivo* expansion. The expanded PD Tregs were comparable with control Tregs in suppressing the inflammatory cytokines IL-6 and IL-1β *in vitro*. Similarly, the suppressive capacity of Tregs against T effector cells increased by 76.6 ± 4.61% in Tregs from MCI patients (n=46), and by 87.98 ± 4.97% in Tregs from AD patients (n=42), and showed enhanced capacity to suppress M1 macrophage-derived IL-6 and IL-1β following *ex vivo* expansion [4]. Expanded Tregs from ALS patients (n=3) increased their suppressive capacity against T effector cells from 0%-1.7% to 27.8%-44.25% [23]. The *ex vivo* expansion protocols involved culturing patient-derived Tregs with IL-2 and rapamycin for 10-14 days.
These studies suggest that Tregs from patients with neurodegenerative diseases, even at more advanced stages, maintain the capacity to be expanded into functionally immunosuppressive Tregs, which is critical for the therapeutic potential of adoptive cell transfer with Tregs. The functional deficits observed in patients may then stem from the *in vivo* cell environment, and/or the protocol may selectively expand the pool of functional Tregs.

Antigen specific Tregs:

Clinically tested Treg cell transfer therapies have primarily involved the infusion of *ex vivo* expanded polyclonal Tregs. The limited efficacy observed to date, particularly in conditions with an autoimmune component, may be related to the lack of specificity toward the disease-associated antigens in the pool of infused cells. While the polyclonal Tregs may induce a more immunosuppressive environment generally, they may be less able to suppress the specific immune cells driving tissue damage. Preclinical studies have found superior modulation of disease-related immune responses through the use of antigen-specific Tregs. Benefits have been observed in rodent models of AD and PD, using Aβ-specific and alpha-synuclein-specific Tregs, respectively [26; 27; 28; 29; 30]. Various approaches have been tested, including the generation of Tregs with a transgenic Aβ-specific T cell receptor (TCR), the generation of Tregs with a chimeric antigen receptor (CAR) targeting Aβ, and via the culturing of isolated T cells with Aβ and bee venom phospholipase A2 prior to expansion and adoptive transfer. While both polyclonal and Aβ-specific Tregs exhibited anti-inflammatory properties, only the Aβ-specific cells appreciably impacted Aβ levels [27]. Similarly, alpha-synuclein Tregs, generated through the co-culturing of CD4+ cells with alpha-synuclein presenting dendritic cells prior to expansion, provided a better preservation of motor function in the MPTP rodent model of PD, relative to polyclonal Tregs [30].

Clinical trials in ALS

Treg therapies have been tested in ALS patients based on the finding that higher Treg function was associated with slower rates of disease progression. Tested strategies include low-dose IL-2, the combination of low-dose IL-2 with adoptive cell transfer, and the combination of low-dose IL-2 with abatacept, which is a human recombinant fusion protein containing the extracellular domain of the cytotoxic T lymphocyte antigen 4 (CTLA-4) fused to the Fc portion of immunoglobulin G1(CTLA-4 Ig). Low-dose IL-2 is a common feature of these strategies because while IL-2 is an important cytokine for the proliferation and survival of T cells generally, IL-2 receptor alpha (CD25) is most highly expressed on Tregs, thus low doses will preferentially bind to Tregs [3]. Unlike other activated T cell populations, Tregs cannot produce their own IL-2, thus the binding of IL-2 to its receptor on Tregs acts as a sink that prevents it from acting on other immune cell populations. Determining the optimal concentration of IL-2 for selective targeting of Tregs is difficult, as it may vary from person to person, which likely explains why, across indications, trials testing low-dose IL-2 monotherapy have generally been associated with the lowest degree of clinical benefit [31].

Low dose IL-2: MODEST POTENTIAL BENEFIT

No changes in Treg levels or clinical course were observed in a pilot study testing subcutaneous low-dose IL-2 in five ALS patients for one year, which may be related to a failure of the endogenous Tregs in the patients to productively respond to IL-2 [32].

The Phase 2a placebo-controlled IMODALS RCT tested low-dose IL-2 (aldesleukin; 2 million international units [MIU] or 1 MIU) once daily for five days every four weeks for three cycles and assessed the change from baseline in the percentage of CD4+ Tregs in patients with ALS (<75 years old, <5 years disease duration, riluzole treatment > 3 months, and a slow vital capacity ≥ 70% of normal) (n=36) [33]. The Treg percentage increased +6.2% ±2.2 in the 2 MIU group, +3.9% ±1.2 in the 1 MIU group, and declined by -0.49% ±1.3 in the placebo group after the first dosing round, and there may be cumulative effects with successive dosing rounds. The suppressive activity of the patient-derived Tregs also increased in *in vitro* assays following low-dose IL-2 treatment. No significant changes were observed on measures of disease progression, including the ALS Functional Rating Scale (ALSFRS-R) score, slow vital capacity, or plasma NFL levels in any of the groups.

Low-dose IL-2 was tested in the Phase 2b MIROCALS RCT (NCT03039673) at a dose of 2 MIU or placebo in five-day cycles every 28 days over 18 months in 220 ALS patients also taking riluzole. The primary outcome was time to death at 21 months, with a secondary outcome of disease progression based on the ALSFRS-R slope of change. Topline results indicated there was a 19% reduction in the risk of death at 21 months with low dose IL-2 which did not reach statistical significance [34]. However, a prespecified analysis adjusted on CSF phosphorylated neurofilament (pNFH) levels found that low-dose IL-2 was associated with a 73% reduction in the risk of death, with benefits driven by individuals with less extensive motor neuron damage. A 24% decrease in the ALSFRS-R slope of change was also observed with IL-2 adjusted on CSF pNFH. In response to the results of this study, a licensing agreement was reached between the MIROCALS European consortium and the French Biotech, ILTOO Pharma for the development of low-dose IL-2 for ALS (Press release).

Adoptive cell transfer + low-dose IL-2: POTENTIAL BENEFIT

A proof-of-principle study tested the safety and feasibility of administering autologous *ex vivo* expanded Tregs isolated following leukapheresis, a process that separates and collects white blood cells, in three ALS patients with different rates of disease progression [32]. The intravenous infusion of Tregs (1×10^6 cells/kg) was administered in four doses over two months and then in four doses over four months, in conjunction with subcutaneous low-dose IL-2 (2×10^5 IU/m^2) three times per week throughout the

course of the study. All three participants experienced increases in immunosuppressive Treg function and a slowing of disease progression. The rate of decline, based on the ALSFRS-R and Appel ALS (AALS) scores, slowed during the periods of infusion, and then accelerated in the interim periods. Larger gains in Treg immunosuppressive activity were associated with less decline based on the AALS. Two of the participants also experienced stabilization of maximal inspiratory pressure during the treatment. The decline during the interim periods, despite the maintenance of IL-2 therapy suggests that while IL-2 may support the survival of the Tregs, the benefits were driven by the transferred cells.

A follow-up RCT included seven ALS patients, six of whom continued into an open-label extension study along with two additional participants [35]. Two of nine participants were excluded because their Tregs could not be expanded. Participants in the active group were infused with leukapheresis derived, *ex vivo* expanded Tregs (1×10^6 cells/kg) every four weeks in combination with low-dose IL-2 (2×10^5 IU/m^2) three times per week for 24 weeks. There was a 26.4% difference in Treg suppressive function at the end of 24 weeks, with a 6.4% decline in the placebo group, and a 20% increase in the active group. Tregs were administered at a dose of 2×10^6 cells/kg and 3×10^6 cells/kg at four-week intervals in the open-label study. Treg suppressive activity also increased by approximately 20% relative to baseline in the 24-week open-label extension. No dose response was observed. Six of the participants experienced slow to no progression, while two, with elevated levels of inflammatory and oxidative stress markers, experienced rapid progression.

Low-dose IL-2 + CTLA-4 Ig: POTENTIAL BENEFIT

A proof-of-concept open label trial tested low-dose IL-2 with CTLA-4 Ig (COYA 302) for 48 weeks in four ALS patients. CTLA-4 Ig modulates the T cell co-stimulatory signal mediated through the CD28–CD80/86 pathway, which regulates the production of IL-2 and cell death [36]. CTLA-4 Ig, which is used clinically for autoimmune diseases, inhibits T cell activation, leading to the loss of proinflammatory effector cells. Low-dose IL-2 is designed to preferentially expand immunosuppressive Tregs, which express the highest levels of the IL-2 receptor alpha (CD25), while the CTLA-4 Ig is designed to dampen the proinflammatory response. Since the proinflammatory environment is thought to contribute to Treg dysfunction, shifting the milieu to a less inflammatory state is expected to increase the ability of IL-2 to increase levels of functional Tregs. Disease progression was assessed based on the rate of change on the ALSFRS-R, in which higher scores indicate less disability. Topline results indicate that treatment was associated with a slowing of disease progression (Press release). At baseline, the average ALSFRS-R score was 33.5 ±5.9 points, with a decline of 1.1 points per month. During treatment, participants were stable for the first 24 weeks, and experienced a marginal decline of less than two points (32 ±7.8) by the end of 48 weeks. Participants continued to decline at the baseline rate following the cessation of treatment, suggesting

that the treatment meaningfully slowed disease progression, but continuous treatment is necessary for the maintenance of benefits. The clinical benefits were seen in conjunction with a significant increase in Treg suppressive activity. Similar to what was observed with disease progression, the level of Treg suppressive activity declined following the cessation of treatment.

Biomarkers: Analysis of fluid analytes from participants in several of these clinical trials has identified biomarkers associated with disease progression, which may also have utility for determining which patients would most likely benefit from Treg-related therapies, as the trials to date suggest that slower progressing patients may achieve greater clinical benefit. Oxidative stress-related markers have been found to be indicators of disease progression. A longitudinal serum biomarker analysis from a phase 1 trial (NCT03241784) testing Treg therapy in ALS patients found that oxidized low-density lipoprotein (ox-LDL) levels were elevated in ALS patients relative to healthy controls, particularly in rapidly progressing patients [37]. A similar association was observed for LOX-1, a soluble receptor for ox-LDL. Levels of ox-LDL were reduced in response to Treg therapy and rebounded during the interim periods, despite being on maintenance low dose IL-2. In the trial testing low dose IL-2 plus CTLA-4 Ig, serum levels of the oxidative stress biomarker 4-HNE were elevated in ALS patients and associated with the rate of disease progression (Press release). Reductions in 4-HNE were observed with treatment.

Together these studies suggest that Treg dysfunction is a feature of several neurodegenerative diseases, including ALS, and that combination Treg therapies may help restore Treg immunosuppressive function, and potentially impact disease. However, the treatments do not induce long lasting changes, and must be continuously repeated/maintained in order to observe benefit. The use of long-term low dose IL-2 could pose risks of infection due to chronic immunosuppression, while long-term repeated dosing with autologous Tregs may not be clinically feasible, depending on the duration of the disease.

APOE4 interactions: Not established.

Aging and related health concerns: Benefits observed in organ transplant and autoimmune conditions have been variable and modest thus far, but next-generation approaches may allow for enhanced efficacy.

Types of evidence:

- 1 systematic review of Treg trials in organ transplant

- 1 Phase 2a basket trial in autoimmune diseases
- 2 clinical trials testing rezpegaldesleukin (long-lasting IL-2) in autoimmune diseases
- 3 clinical trials testing Treg approaches in T1D
- Numerous laboratory studies

The two major types of Treg therapies which have undergone clinical testing are low-dose IL-2 therapy, which is designed to boost endogenous Tregs, and adoptive cell transfer, which is designed to supply exogenous Tregs, which have typically been modified in a manner to try to enhance their suppressive capacity. There are challenges and limitations to both approaches, which will be discussed in more detail in the Safety section. These approaches have primarily been tested in the context of autoimmune disease, which is characterized by a loss of immune tolerance to self-antigens, as well as in organ transplantation, in order to promote tolerance to the transplanted organ.

Autoimmune disease: POTENTIAL BENEFIT VARIES
Tregs are key players in the regulatory immune system that allows the body to maintain tolerance to self-antigens. Alterations in regulatory subsets are commonly observed in the context of autoimmune disease, where the loss of tolerance to particular self-antigens results in immune-mediated tissue damage. As a result, autoimmune diseases have been some of the primary indications for the clinical testing of Treg-based therapies.
Low-dose IL-2 was tested in an open-label Phase 2a disease-finding basket trial (NCT01988506) including 81 participants with one of 13 different autoimmune conditions. Low-dose IL-2 (either diluted Proleukin® or ready-to-use ILT-101®) was administered at a dose of I million IU/day for the first five days and then once every two weeks [38]. The trial achieved its primary endpoint of change in Tregs on day eight compared to baseline, with a mean fold increase in Tregs percentage of 1.9 ± 0.5. The overall response rate at six months based on the Clinical Global Impression (CGI) scale was 51%, with responders observed in every indication tested except sclerosing cholangitis. Based on the results, Sjögren's syndrome, systemic sclerosis, and Behçet's diseases were identified as the most promising candidates to conduct future studies on this type of therapy.
One of the challenges of low-dose IL-2 therapy is that it has a very short half-life (<15 min). Novel formulations have been developed that are designed to stabilize IL-2 and extend its *in vivo* half-life. One formation developed by Nektar Therapeutics is rezpegaldesleukin (NKTR-358; LY3471851), a PEGylated form of recombinant human IL-2, which has a half-life of 7.4–12.9 days. It has been clinically tested in collaboration with Eli Lilly, in atopic dermatitis and systemic lupus erythematosus (SLE). In a Phase 1b trial in patients with moderate to severe atopic dermatitis (n=43), rezpegaldesleukin was tested at doses

of 12 µg/kg or 24 µg/kg s.c. twice weekly for 12 weeks, and patients were followed out to 48 weeks (Corporate Presentation). Significant improvement was observed on the percent change from baseline on the EASI score with the higher dose, which was generally maintained through the follow-up period. A placebo-controlled Phase 2 RCT testing three s.c. doses of rezpegaldesleukin (300 mcg Q2W; 900 mcg Q2W; 1800 mcg Q2W) in 291 patients with moderately-to-severely active SLE did not meet its primary endpoint of a 4-point reduction in the SLEDAI-2K score (Press release).

Type 1 diabetes (T1D): While Treg-based therapies have been tested in various autoimmune conditions, some of most extensive testing has occurred in the context of T1D. A Phase 1 trial (NCT01210664) testing autologous *ex vivo* expanded polyclonal Tregs isolated from the peripheral blood of adult patients with T1D (n=16) found that while transferred cells remained phenotypically stable, the circulating level declined over 75% within 90 days, suggesting low viability of the cells in this population [39]. Another Phase 1 trial in this population (n=9) sought to enhance the survival of the transferred cells through the administration of low-dose IL-2 (5-day courses at a dose of 0.33 or 1 MIU) [39]. The combination had limited effects on boosting Treg levels, no effect on insulin secretion, and in some patients resulted in the induction of cytotoxic cell subsets (CD8+ and NK cells). A placebo-controlled Phase 2 RCT (NCT02691247) testing of autologous *ex vivo* expanded autologous Tregs at a high (20×10^6 cells/kg) or low (1×10^6 cells/kg) single doses in 110 children and adolescents with newly onset T1D recently reported that the regimen failed to improve β-cell function in this cohort [40]. These studies suggest that polyclonal Tregs may have limited clinical utility for T1D. There are several companies/groups developing antigen specific Tregs, which have the potential to be more effective, based on preclinical studies [41].

Organ transplantation: LIMITED BENEFIT WITH CURRENT APROACHES, POTENTIAL BENEFIT FOR NEXT-GEN TREG THERAPIES

The major challenge of organ transplantation is getting the body to accept non-self-tissue and not mount an immune response against it. Since Tregs play an important role in immune suppression and mediating tolerance to self-antigens, boosting Treg activity is a strategy to enhance tolerance to the transplanted organ while potentially allowing for a reduction in the use of other immunosuppressive drugs. Treg-related therapies have primarily been clinically tested in early phase trials in kidney transplant, liver transplant, and in the context of graft-versus-host disease. The studies have shown that these types of approaches are generally feasible, but efficacy has been quite limited to date.

The use of low-dose IL-2 therapy alone has been the least effective method, with limited induction of Tregs in the transplanted tissue, and potentiation of rejection risk through the concomitant induction of pro-inflammatory immune subsets [42].

Other approaches include trials testing the infusion of autologous *ex vivo* expanded polyclonal Tregs [42]. The most comprehensive of these was the multicenter ONE study, which tested the safety and feasibility of using six different regulatory cell subsets, including four Treg cell products in *de novo* kidney transplant patients [42]. The tested products included, expanded polyclonal Tregs derived from peripheral blood at four concentrations (1×10^6; 3×10^6; 6×10^6; 10×10^6 cells/kg) five days post-transplant, as well as another polyclonal Treg product administered at seven days post-transplant.
Another strategy is the use of Tregs enriched for donor-specific antigens. One approach is to perform the *ex vivo* expansion of autologous Tregs in the presence of donor peripheral blood mononuclear cells (PBMCs), or PBMC-derived antigen presenting cells, such as B cells or dendritic cells. Two different methods of developing Tregs reactive to donor antigens were tested as part of the ONE study. One method involved co-culturing patient Tregs with irradiated donor PBMCs in the context of co-stimulation blockade with belatacept (CTLA-4 Ig) (NCT02091232). Three recipients of live donor kidneys also received donor antigen reactive Tregs ($CD4+CD25+CD127^{lo}$) 7-11 days post-transplant along with a modified immunosuppression regimen without induction [43]. None of the Treg recipients experienced acute donor rejection. An accumulation of Tregs into the transplanted kidney was observed during biopsy eight months later. The participants have all been on stable tacrolimus monotherapy for over six years and continue to show good kidney function with no evidence of rejection.
While this type of Treg therapy has allowed some patients to reduce their load of immunosuppressive medication, the vast majority of patients need to remain on some type of immunosuppressive regimen [42]. Furthermore, some patients experienced increases in opportunistic infections, which calls into question the safety of immunosuppressive Treg approaches compared to more traditional immunosuppressive medications. Complications have been associated with this approach, particularly in liver transplant patients. The process of withdrawing immunosuppressive medication, precipitated rejection episodes in several patients that received donor antigen reactive Tregs in one study (NCT02474199), while the deLTa trial (NCT02188719) had to be terminated due to issues with the manufacturing of donor antigen reactive Tregs [42].
The tolerance toward transplanted organs could potentially be enhanced through the modification of Tregs. These approaches include transducing Tregs with donor-specific transgenic TCRs, or engineering Tregs to express a CAR, which is a hybrid TCR in which a single-chain variable region fragment (scFv) of a BCR with known antigen specificity replaces the extracellular portion of the TCR [42]. The latter approach is currently being tested in a proof-of-concept study using HLA-A2-specific CAR Treg in kidney transplant patients in which there is an HLA-A2 antigen mismatch (NCT04817774). This approach is intended to minimize rejection because mismatches in major histocompatibility complex (MHC) proteins

are the major triggers for immune-mediated organ rejection [44]. The study is sponsored by Sangamo Therapeutics.

Safety: Low dose IL-2 and Treg cell transfer approaches are generally well-tolerated. Chronic immunosuppression from long-term use may increase the risk for infections. Infusion-related reactions, flu-like symptoms, and eosinophilia are reported adverse events.

Types of evidence:

- 1 systematic review of safety in trials testing low-dose IL-21
- 1 review of Treg trials in organ transplantation
- 5 Phase 1 or Phase 2 trials in ALS
- 1 Phase 2a basket trial in autoimmune diseases
- 1 clinical trial testing rezpegaldesleukin (long-lasting IL-2) in atopic dermatitis
- 3 clinical trials testing Treg approaches in T1D
- 1 clinical trial testing low-dose IL-2 in AD
- Numerous laboratory studies

Treg based therapies have generally been found to be safe and well-tolerated in early phase clinical trials [3; 31]. The impacts of boosting endogenous Tregs or administering expanding Tregs may vary depending on the background disease state, such as the inflammatory state. In some cases, pro-inflammatory immune subsets may also be stimulated, such that both the safety and efficacy of these approaches may be quite variable across patients. Most studies used dosing regimens for a period of several months or a year, but the evidence suggests that efficacy wanes upon cessation, and thus must be maintained in order to achieve sustained benefits. The feasibility and safety of continuous long-term treatment with these approaches has not been established.

Low-dose IL-2

Safety: A systematic review of trials testing subcutaneous low-dose recombinant IL-2 found that it was not associated with an increased risk for thromboembolic events [45]. Furthermore, the risk was lowest in trials using doses ≤ 1 MIU, which is the level generally used in Treg therapy trials. The most common adverse events in these very low dose trials were injection site reactions, flu-like symptoms, headache, and dyspnea. In contrast to high-dose IL-2, which is meant to stimulate immune responses, low-dose IL-2 is meant to semi-selectively target Tregs, leading to a dampening of immune responses. As a result,

the systemic toxicities related to high-dose IL-2, such as vascular leak syndrome, hypotension, and organ dysfunction, have generally not been observed with the administration of low dose IL-2 [46]. However, low-dose IL-2 has been found to stimulate non-Treg immune cell populations in some cases, including proinflammatory subsets, which could compromise the safety and efficacy of the treatment. In adults with T1D, the addition of low-dose IL-2 to promote the survival of exogenously administered autologous Tregs led to the expansion of activated Natural Killer (NK) cells, mucosal associated invariant T cells, and cytotoxic clonal CD8+ T cells [39]. In the context of autoimmune conditions, like T1D, the expansion of these proinflammatory, cytotoxic subsets could potentially exacerbate tissue damage. Elevations of eosinophils have also been observed following low-dose IL-2 therapy. In ALS patients, the adverse events most frequently associated with low-dose IL-2 were injection site reactions and flu-like symptoms [33]. The latter is a known side effect of IL-2, and were only observed at the highest tested dose (2 MIU). Eosinophils counts were also significantly elevated in the higher dose group. In the basket trial testing low-dose IL-2 in 13 different autoimmune conditions, mild to moderate increases in eosinophils were also observed [38]. Adverse events were generally mild to moderate in this study, with injection site reactions, fatigue, flu-like symptoms, gastrointestinal events, and headache, as the most commonly reported. There was one severe adverse event, a case of hives. In AD patients treated with low-dose IL-2, the most common adverse events were injection site reactions, mild leukopenia, flu-like symptoms, dizziness, and nausea [24].
No serious adverse events were observed with the combination of low-dose IL-2 with CTLA-4 Ig (COYA2) in ALS patients, and the most common adverse event was mild injection site reactions (Press release). Similarly, there were no serious adverse events with long-acting IL-2 (rezpegaldesleukin) in patients with atopic dermatitis (Corporate Presentation). The most common adverse events were injection site reactions and infections. Adverse events resulting in study discontinuation were mild headache and nausea, a mild abscess, moderate hives, and moderate asymptomatic eosinophilia.

Challenges: The primary challenge of low-dose IL-2 therapy is the selection of the therapeutic dose [31]. If the dose is too low then it will be ineffective at stimulating Tregs, but if it is too high then it can also expand effector cell populations. As a result, IL-2 has a relatively narrow therapeutic window for Treg expansion, and this window is likely to vary from condition to condition, and even from patient to patient. Another challenge is the very short half-life of IL-2 (< 15 minutes), requiring frequent dosing for a sustained response.

Treg cell transfer

Safety: Treg cell transfer, typically in conjunction with low-dose IL-2, has generally been well tolerated [3]. Fasciculations, or muscle twitches, were observed following Treg infusions in ALS patients, but otherwise there were no serious adverse events, infusion-related adverse events, or clinically significant changes in safety laboratories or electrocardiogram findings in the trials in this population [32; 35]. Inconsistency in the degree of immunosuppression is another concern, as the use of polyclonal Tregs may result in systemic immunosuppression, which could increase the risk for infections. Alternately, in the context of organ transplant, an insufficient degree of immunosuppression may result in acute rejection [42].

Challenges: The most prominent challenge for adoptive cell transfer with autologous Tregs is the time intensive nature of the process, and difficulty in bringing it to scale [3]. The Tregs are typically isolated from each individual patient, expanded *ex vivo*, and cultured for several weeks, before the purified cell product can be administered back to the patient. Although standard methods can be applied, not all patients may be suitable candidates for a given approach. There are multiple ways of isolating the Tregs from the patient, which vary in terms of invasiveness and reliability. Tregs can be isolated from peripheral blood samples, umbilical cord, the thymus, or leukapheresis products [47]. Tregs make up a small percentage of the total CD4+ cell population (1-5%), and levels may be even lower in certain disease settings, such that the volume of blood needed becomes unfeasible, which may necessitate more invasive methods [47]. To date, trials have generally used leukapheresis, which is the process of removing white blood cells from circulation. Depending on their health status, this procedure may not be tolerable for all patients. Due to the low numbers, the Tregs need to be expanded *ex vivo* several thousand-fold, typically in the presence of IL-2 and rapamycin [47]. Having a cell product of sufficient cell number and purity is technically challenging, but critical. Stemming from technical and/or biological reasons, Treg samples from some patients cannot be adequately expanded to the level needed for clinical use. The expansion process itself could impact the survival and functionality of the Tregs *in vivo* [3]. The survival and migration of the Tregs to the desired part of the body can also be highly variable. Most studies have used polyclonal Tregs, which may have limited therapeutic capacity [3]. Antigen specific Tregs are expected to be more effective, however, they may be less stable *in vivo*. There are reports that chronic antigen exposure may make the Tregs more prone to skewing toward an effector phenotype, particularly if they are transferred into a proinflammatory environment [2; 3].

Drug interactions: Treg-based therapies are likely to interact with other immunomodulatory drugs. Since they are designed to be immunosuppressive, the combination with other immunosuppressive agents may increase the risk for infection.

Sources and dosing:

Treg therapies are currently in clinical development. Clinical trials testing low-dose IL-2 tend to use a dilution of Proleukin® (aldesleukin), an approved recombinant IL-2 used for metastatic renal cell carcinoma and metastatic melanoma. Other low-dose IL-2 formulations in clinical development include a formulation from Coya Therapeutics (COYA 301) which is also being tested as a combination product (COYA 302) with CTLA-4 Ig, a formulation from ILTOO Pharma (ILT-101), and a long-acting formulation from Nektar Therapeutics (rezpegaldesleukin). Clinical trials typically used doses of I MIU every other day, or in five day cycles every few weeks.

There are dozens of companies working on developing Treg-based therapies. The vast majority of these cell products are being developed for autoimmune disease, organ transplant, graft vs. host disease, and cancer. There are a few companies developing Treg therapies for neurodegenerative diseases, aside from multiple sclerosis (MS), the main indication is ALS. Some of these companies are listed below.

Abata Therapeutics is developing TCR targeted Treg therapy for progressive MS.
Treg therapeutics is developing a 'tolerogenic induction therapy' designed to induce tolerogenic dendritic cells, which can then generate tolerogenic Tregs for several indications, including MS and stroke.
Cellenkos is developing off the shelf cryopreserved allogenic umbilical cord blood derived Tregs designed to expand *in vivo* for ALS and PD.
PolTreg is developing CAR Tregs for progressive MS and ALS.
Sonoma Biotherapeutics is developing Treg cell therapy for neuroinflammation.
VT BIO is developing Treg cell therapy for AD.
Rapa Therapeutics, a spinout from the National Cancer Institute, is developing autologous hybrid TREG/Th2 cells for ALS.

Research underway:

There are numerous groups developing Treg-based therapies for a variety of indications in different stages of preclinical and clinical development. Some notable trials for neurodegenerative indications are listed.

Coya Therapeutics is planning a Phase 2 trial for COYA 302 (low dose IL-2 + CTLA-4 Ig) for patients with ALS and a Phase 1 trial for patients with FTD (NCT06395038), as well as an ongoing Phase 2 trial for low-dose IL-2 in patients with mild to moderate AD, with an estimated completion date in 2025 (NCT06096090). These trials are being conducted through the Methodist Hospital Research Institute in Houston, Texas.

There is an ongoing Phase 2 trial testing low-dose IL-2 in patients with early AD (NCT05468073) sponsored by the Centre Hospitalier St Anne, with an expected completion date in 2025.

Cellenkos, Inc. is testing their neurotropic, allogeneic, umbilical cord blood derived T regulatory cells in a Phase 1 trial in ALS patients (NCT05695521), with an expected completion date in 2026.

Rapa Therapeutics is testing autologous hybrid TREG/Th2 cells in an open-label Phase 2/3 expansion study in ALS patients (NCT04220190), with an expected completion date in 2025.

VT BIO is testing a T regulatory cell preparation in patients with mild to moderate AD in a Phase 1 trial (NCT05016427). The status of the trial is currently listed as Unknown.

Search terms:

Pubmed, Google: Treg (T regulatory cell) therapy, Treg adoptive cell transfer, Low-dose IL-2

- Alzheimer's disease, ALS, neurodegenerative disease, autoimmune disease, organ transplant, clinical trials, safety

Websites visited for Treg therapies:

- Clinicaltrials.gov
- PubChem (IL-2)
- DrugBank.ca (IL-2)

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