

REVIEW ARTICLE

TNF, TNF inducers, and TNFR2 agonists: A new path to type 1 diabetes treatment

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Summary

In the past decade, interest in the century-old tuberculosis vaccine, bacillus Calmette-Guerin (BCG), has been revived for potential new therapeutic uses in type 1 diabetes and other forms of autoimmunity. Diverse clinical trials are now proving the value of BCG in prevention and treatment of type 1 diabetes, in the treatment of new onset multiple sclerosis and other immune conditions. BCG contains the avirulent tuberculosis strain *Mycobacterium bovis*, a vaccine originally developed for tuberculosis prevention. BCG induces a host response that is driven in part by tumour necrosis factor (TNF). Induction of TNF through BCG vaccination or through selective agonism of TNF receptor 2 (TNFR2) has 2 desired cellular immune effects: (1) selective death of autoreactive T cells and (2) expansion of beneficial regulatory T cells (Tregs). In human clinical trials in both type 1 diabetes and multiple sclerosis, administration of the BCG vaccine to diseased adults has shown great promise. In a Phase I trial in advanced type 1 diabetes (mean duration of diabetes 15 years), 2 BCG vaccinations spaced 4 weeks apart selectively eliminated autoreactive T cells, induced beneficial Tregs, and allowed for a transient and small restoration of insulin production. The advancing global clinical trials using BCG combined with mechanistic data on BCGs induction of Tregs suggests value in this generic drug and possible immune reversal of the type 1 diabetic autoimmune process.

KEYWORDS

autoimmunity, bacillus Calmette-Guerin (BCG), regulatory T cells (Tregs), tumour necrosis factor (TNF), tumour necrosis factor receptor 2 (TNFR2), type 1 diabetes

1 | INTRODUCTION

Autoreactive T cells directed to diverse insulin-secreting islet cell proteins are the predominant cause of type 1 diabetes, yet there are currently no approved therapies that selectively target these self-directed cytotoxic T cells. Rather, immune-directed treatments like immunosuppressants and lymphocyte-directed or cytokine-directed antibodies that kill or harm autoreactive T cells also can produce undesirable immune side effects in the host.

There is evidence that type 1 diabetes and other, diverse forms of autoimmunity are diseases driven by immune imbalances due to too

few or poorly functioning regulatory T cells (Tregs), a subtype of T lymphocytes that help maintain tolerance to self-antigens and suppress or quiet the immune response, and too many cytotoxic T cells.¹ This is illustrated as an alerted autoimmune microenvironment at the site of autoreactivity (Figure 1).

Preclinical and clinical evidence suggests that the regulatory cytokine tumour necrosis factor (TNF), inducers such as bacillus Calmette-Guerin (BCG), or TNF receptor 2 (TNFR2) agonistic antibodies may provide a pathway to targeted and selective destruction of the autoreactive T cells in type 1 diabetes.²⁻⁴ In addition, this treatment approach may also provide clinical benefits by inducing Tregs that suppress pathologic T cells. TNF can directly and selectively kill the autoreactive T cells both in diabetes-prone non-obese diabetic (NOD) mice as well as in humans with type 1 diabetes.⁵⁻⁷ Clinical trials in type 1 diabetes and another autoimmune condition, multiple sclerosis, are currently investigating this strategy, especially as it relates to medications that induce elevation of TNF levels. Therapies that could

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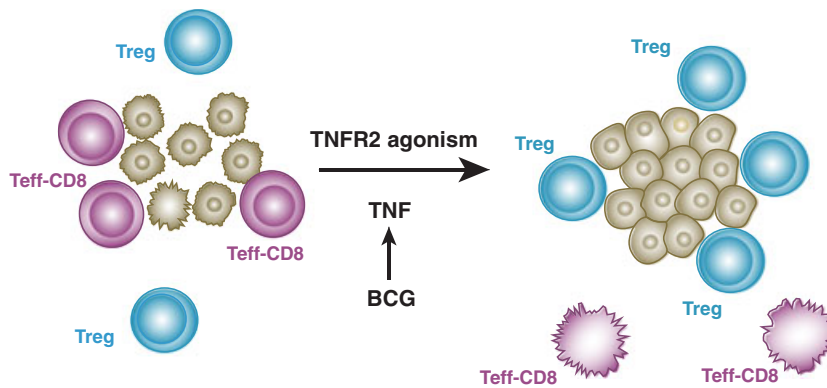


FIGURE 1 The autoimmune microenvironment. At the tissue site in autoimmune diseases, there are too few Treg cells and too many T effector cells or cytotoxic T cells. The hypothesis of this review is that TNF or TNFR2 agonism can alter this abnormal microenvironment and convert it to a non-disease state

both expand Tregs and kill autoreactive T cells such as has been suggested by TNFR2 agonism or TNF induction might be able to change the autoreactive microenvironment of the disease site (Figure 1).

In this review, the role of the TNF pathway and the use of the BCG vaccine and other TNF inducers or agonists as treatments for established type 1 diabetes are discussed, focusing on recent human data and supporting data from other autoimmune diseases.

1.1 | Signalling defects in the TNF pathway in type 1 diabetes make insulin-autoreactive T cells vulnerable to apoptosis in the presence of elevated TNF or TNFR2 agonism

In type 1 diabetes, insulin-autoreactive T cells mediate destruction of the pancreatic beta cells that produce insulin, leading to insulin deficiency, hyperglycemia, and the need for lifelong insulin injections. Despite insulin treatment, many patients with type 1 diabetes will develop disease-related microvascular and macrovascular complications, underscoring the need for new, effective interventions. Directly addressing autoimmunity through interventions focused on the TNF pathway is a promising new approach that is currently being investigated in human clinical trials.

The rationale behind this approach is based on over 2 decades of work that identified signalling defects in the NF- κ B pathway of autoimmune mice^{8,9} and then shown in mice and humans that these intracellular signalling errors in insulin-autoreactive T cells make them selectively vulnerable to death upon exposure to or induction of TNF.^{5,7,10,11} In addition, TNFR2 agonism has also been shown to effectively and selectively kill insulin-autoreactive CD8+ T cells in blood samples taken from patients with type 1 diabetes.⁵

Autoreactive T cells, but not normal T cells, are selectively vulnerable to TNF-induced apoptosis. This is a result of genetic or functional defects that prevent the transcription factor signalling in the NF- κ B pathway, thereby preventing this pro-life survival factor from entering the nucleus to express pro-survival genes. In normal T cells, TNF activates the proteasomal cleavage of NF- κ B from IKBa. After cleavage, NF- κ B enters the nucleus and initiates transcription of an array of anti-apoptotic proteins that counteract the pro-apoptotic effects of TNF.¹² Thus, NF- κ B activation in normal cells is protective, preventing cells from death upon TNF exposure. Conversely, in type 1 diabetes and some other forms of autoimmunity,

this process is disrupted. NF- κ B cannot be activated to counteract TNF-induced apoptosis, leaving the cell vulnerable to death. In experiments performed in culture, in vitro cell death of a subpopulation of autoreactive CD8 T cells, but not CD4 cells, has been demonstrated by incubating TNF with blood samples from patients with type 1 diabetes and other autoimmune diseases known to have defects in the NF- κ B pathway.⁵

Soluble TNF can bind to both TNF receptor 1 (TNFR1) and TNFR2, although preferential binding is towards TNFR1, which is ubiquitously expressed, whereas TNFR2 is infrequently expressed in the normal immune system. The natural ligand for TNFR2 is trimeric membrane TNF, most often expressed on monocytes. The engagement of TNF with its receptors results in contrasting responses, depending on the receptor. In non-diseased cells, TNFR1 is linked to cell death pathways, and, therefore, sole engagement of TNFR1 results in cell death. By contrast, sole engagement of TNFR2 causes normal CD8 T cells to proliferate. This paradoxical response between engagement with TNFR1 and TNFR2 is due to both the activation state of the T cells and that the TNFR2 receptors have very different TNF signalling pathways. The TNFR1 intracellular domain is a cell death domain, whereas the TNFR2 intracellular domain is linked to NF- κ B expansion. In diseased CD8 T cells from autoimmune patients (which all express TNFR2), TNF or TNFR2 agonism causes selective cell death through an altered signalling pathway. This ability of TNF or TNFR2 engagement to be selective for CD8-driven cell death was functionally tested in the human disease setting of type 1 diabetes and other autoimmune diseases. Fresh autoimmune CD8 T cells from autoimmune patients were incubated with either TNF, TNFR1 agonistic antibodies, or TNFR2 agonistic antibodies. The human autoimmune data were clear: only TNFR2 agonists, but not TNFR1 agonists, had the ability to target and kill the autoreactive T cells from diverse human autoimmune diseases, including cytotoxic T cells in type 1 diabetes, as well as autoimmune cells in multiple sclerosis, Graves' disease, Crohn's disease, and other forms of autoimmunity.⁵ Additionally, murine autoimmune data show that TNF, TNF induction, and TNFR2 agonism all relieve the burden of autoreactive cells by selectively inducing cell death and also prevent disease transfer.^{12,13} Of the 2 receptors that TNF acts upon, TNFR2 has more limited cellular expression and is primarily expressed on T cells, subsets of neurons, and a few other cell types such as Tregs and diseased cytotoxic T cells. TNFR1's more ubiquitous expression, which correlates with toxicity data, even in baboons, suggests that TNFR2 agonism would be expected to have a

better toxicology profile. In patients with type 1 diabetes, the subpopulation of T cells vulnerable to TNF or TNFR2 agonist-induced death was traceable to insulin-autoreactive CD8 T cells.⁵

Overall, signalling defects through the TNF pathway, whether through NF- κ B or activators of NF- κ B, such as the proteasome complex and the ubiquitination process, may provide an important opportunity for intervention in type 1 diabetes.

1.2 | Induction of TNF or TNFR2 agonism triggers expansion of Tregs

In addition to eliminating the autoreactive cells, TNF induction or TNFR2 agonism may work through a second mechanism, by triggering the induction or expansion of Tregs. Tregs are a subset of CD4 T cells that help to prevent or treat autoimmunity by maintaining self-tolerance, immune homeostasis, and suppression of cytotoxic T cells.¹ However, clinical application of Tregs in autoimmunity has been hampered by the difficulty in obtaining and safely applying sufficient quantities of Tregs, whether generated in vitro or stimulated in vivo, to be effective.

In humans with longstanding type 1 diabetes, repeat BCG administration (2 doses) induced proliferation of Tregs in a Phase I clinical trial,¹⁴ which appeared to contribute to early clinical benefits including suppression of disease and transient restoration of low-level insulin secretion.

Similar to BCG vaccinations, TNFR2 agonism induces proliferation of Tregs in the mouse¹⁵ and human.⁴ TNFR2-expressing Tregs are highly suppressive and appear to be the most suppressive Tregs identified to date in the mouse and human.¹⁶⁻¹⁹ Agonism of TNFR2 through use of agonistic antibodies or transmembrane TNF induces IL2R (CD25), TNF, and TRAF2 expression, all of which are elements of the TNFR2 signalling pathway for Treg expansion.⁴ Interestingly, the Tregs observed after expansion through TNFR2 agonism in a study by Okubo et al are a homogenous population, contrasting to the heterogeneous population observed when a TNFR1 agonist is used to trigger expansion in human cell cultures.⁴ This homogeneity is important, because heterogeneous progeny are capable of releasing pro-inflammatory cytokines and also can include cell populations with antagonistic properties. Further, the Tregs in the same study exerted an immunosuppressive function that may be beneficial in type 1 diabetes.

On an historical note, it is important to point out that the triggering of TNF release by the host immune system after BCG vaccination or tuberculosis exposure defines the innate immune response. The ability of BCG or other select microbial pathogens to trigger systemic "cachectin" release is well documented.²⁰ After the cloning of cachectin, it was renamed TNF. Although many immune cells can produce and release TNF after BCG exposures, macrophages, in general, release the largest amount of this cytokine after exposure to this infection.

1.3 | TNF induction and TNFR2 agonism as a treatment for type 1 diabetes, lessons from NOD mice

Historical evidence supports the premise that autoimmune-prone NOD mice with both type 1 diabetes and Sjögren's syndrome are amenable to treatments with adjuvants that stimulate TNF, such as

BCG or its heat-inactivated equivalent, complete Freund's adjuvant (CFA). Like many other interventions, BCG and CFA can prevent type 1 diabetes onset if administered to young NOD mice.²¹⁻²⁵ What distinguishes BCG and CFA from many other forms of immunotherapy in mice, however, is that BCG and CFA not only can stop new-onset diabetes, they can also reverse end-stage diabetes.^{11,26} Data in animal models have shown that double dosing of BCG was more effective than single dosing in preventing diabetes onset in NOD mice.²⁷ In addition, for BCG or CFA to be effective in the NOD mouse model, the immune adjuvant needed to be administered after the autoimmune disease had started the attack in the pancreas. If BCG, CFA, or TNF was administered at birth into NOD mice, disease prevention did not occur, and some data have shown that BCG administered at birth in the mouse could accelerate disease expression.²⁸ The NOD mouse data also show in NOD mice-administered CFA, BCG, or TNF-upregulated Tregs appeared in treated mice a potential benefit due to the possible disease-reversing effects due to the induction of these suppressive Treg expansion.^{29,30}

Unlike many other "cures" of type 1 diabetes in mice, BCG or CFA prevented or reversed type 1 diabetes while imposing little or no toxicity. Although the mechanism for efficacy is mediated through TNF, systemic toxicity prevents the use of direct TNF administration at high doses in the human. However, TNF induction with a safe vaccine such as the BCG vaccine or via TNFR2 agonism may be a way to correct signalling defects and address autoimmunity through selective destruction of autoreactive T cells and induction of Tregs that suppress cytotoxic T cells.

1.4 | TNF induction with the BCG vaccine

In the past decade, interest in the nearly century-old tuberculosis vaccine, BCG, has been revived for potential therapeutic uses in new indications, including type 1 diabetes and other forms of autoimmunity. BCG has an excellent longstanding record of safety and tolerability. The most common adverse event, which is still rare, is suppurative lymphadenitis, which is self-limiting and does not require treatment. The BCG vaccine's main appeal for investigations in the treatment of autoimmunity is its ability to induce TNF. BCG contains the avirulent tuberculosis strain *Mycobacterium bovis* and was first introduced into humans in 1921 as a vaccine to prevent tuberculosis. Since BCG's introduction, an estimated 3 billion people worldwide have received the BCG vaccine. However, because of declining incidence of tuberculosis in Europe and the US, routine BCG vaccination has been discontinued at these sites. By contrast, the BCG vaccine is broadly and uniformly administered in developing countries.

In type 1 diabetes, the potential benefit of BCG vaccinations in established type 1 diabetes was first demonstrated in the NOD rodent model. As previously discussed, TNF induction with CFA or BCG in the NOD mouse reversed advanced disease by killing disease-causing autoimmune cells and restoring insulin secretion.^{11,26} CFA and BCG are similar products in that they contain *Mycobacteria*: CFA contains inactivated *Mycobacterium* and BCG contains avirulent *Mycobacterium* that has historically undergone many rounds of in vitro selection to decrease virulence in humans. CFA is a research tool, whereas BCG is a clinical product manufactured according to Current Good

Manufacturing Practices (CGMPs) monitored by the US Food and Drug Administration. It is therefore suitable for use in the human.

Recent human clinical trials in type 1 diabetes and multiple sclerosis in adults who were administered the BCG vaccine show therapeutic promise even after disease onset or in long established autoimmunity,^{14,31-34} although early trials, conducted without an awareness of strains nor necessary dosing, showed variable effects. In an early, uncontrolled trial of 17 patients who were newly diagnosed with type 1 diabetes, a single-dose of BCG led to a temporary remission in 11 patients (65%).³⁵ However, subsequent controlled clinical trials using a single low-dose of BCG as the TICE strain and epidemiologic studies in children with new onset type 1 diabetes³⁶⁻³⁸ did not show a benefit to BCG vaccination. The reason for the negative findings may be due to insufficient (ie, single) dosing, as well as the use of different BCG strains such as TICE, which even has low efficacy even in mouse studies. Interestingly, the first study by Shehadeh et al dosing BCG in new onset type 1 diabetic subjects used an older BCG strain that is known to induce strong host TNF responses.³⁵ By contrast, the later type 1 diabetes studies frequently used the TICE strain of BCG which has lower efficacy in terms of NF- κ B induction and TNF production, as well as lower protective responses against tuberculosis.^{39,40} With a more advanced understanding of the mechanism of action of BCG, it is now possible to pair biomarkers for early indications of efficacy in the clinical trials. BCG's mechanism of action was not known until approximately 10 years ago, and it was not appreciated that strain differences and longer follow-up of subjects were essential.

About a decade after the first study of BCG in humans with type 1 diabetes, a double-blind, placebo-controlled, Phase I trial of repeat BCG vaccination was conducted in adults with longstanding type 1 diabetes (mean duration of diabetes: 15.3 years) at a single center in North America.¹⁴ Six type 1 diabetic subjects were randomly assigned to BCG or placebo (2 injections spaced 4 weeks apart) and were compared with self, a healthy paired control ($n = 6$) or reference subjects with ($n = 57$) or without ($n = 16$) type 1 diabetes, depending on the outcome measure, over a 20-week period. Within several weeks, repeat BCG vaccinations led to a large increase in dead insulin-autoreactive T cells entering into the circulation (ie, selectively eliminated insulin-autoreactive T cells) and transiently induced beneficial Tregs for 4 to 6 weeks after vaccination. In these human studies, the Connaught strain of BCG (manufactured by Sanofi) was utilized. These findings were not only seen in the BCG-treated patients, but also in 1 placebo-treated patient who developed an acute infection with the Epstein-Barr virus (EBV) after enrollment. EBV infection is known to trigger robust release of TNF. Therefore, the response of the participant who unexpectedly developed EBV underscores the benefits of triggering innate immunity²⁰ in longstanding type 1 diabetes.⁴¹

In 2 BCG-treated subjects and the 1 EBV-infected subject, treatment transiently restored a small amount of insulin production as measured by C-peptide levels, as detected by an ultrasensitive assay. As expected from the long safety history of the BCG vaccine, no major adverse events were reported. This trial was the first to demonstrate that, by transiently arresting the autoimmune response that underlies type 1 diabetes, BCG treatment or EBV infection can pave the way for some degree of C-peptide restoration, even after in patients with a long duration since type 1 diabetes diagnosis.

Long-term follow-up of Phase I participants continues, including analysis of patients in the placebo arm who have now received BCG in an open-label crossover portion of the study. A randomized, placebo-controlled Phase II trial is also currently underway, in which subjects will be followed for 5 years. The Phase II trial has enrolled type 1 diabetic subjects with long standing diabetes who demonstrate some small amounts of remaining C peptide as measured with an ultrasensitive assay. The endpoint of this trial is a >10% lowering of HbA1c values. Data from multiple sclerosis trials show that clinically significant immune alterations after BCG administration may take time to occur.³¹ Therefore, a trial duration of 5 years has been selected for the Phase II type 1 diabetes study, as a clinical outcome, not just a biomarker outcome, is desired.

The positive findings from the Phase I trial are consistent with trials of BCG vaccination in multiple sclerosis, an autoimmune disease in which autoreactive T cells are also susceptible to TNF-triggered cell death. In trials conducted in Italy, BCG vaccination was found to decrease multiple sclerosis disease activity and prevent progression of brain lesions in patients with relapsing-remitting multiple sclerosis.^{32,33} These studies were followed by a double-blind, placebo-controlled Phase II trial testing BCG vaccination in subjects with early symptoms of multiple sclerosis (ie, clinically isolated syndromes [CIS]), but who had not yet been definitively diagnosed with advanced multiple sclerosis.^{31,34} A single dose of BCG was administered to 33 subjects, while an additional 40 subjects received the placebo. Vaccinated subjects were significantly less likely to develop lesions within 6 months of vaccination, and the number of T1-hypointense lesions was lower in the BCG group at 6, 12, and 18 months. Further, at the end of 5 years, 58% of subjects who received BCG did not progress to multiple sclerosis, compared with 30% of those who received the placebo. Overall, clinical benefits after BCG administration in new onset multiple sclerosis were durable and even enhanced at 5 years³¹ There were no major adverse events, and the frequency of all adverse events did not differ between treated and placebo groups. A Phase III clinical trial is now underway (Personal correspondence with G. Ristori, Rome, Italy).

1.5 | TNFR2 agonism in type 1 diabetes

Unlike TNF induction through the BCG vaccine, which is currently advancing in human clinical trials, TNFR2 agonism as a treatment for type 1 diabetes is still in the preclinical stage, although diverse studies in various autoimmune settings are showing benefit. As mentioned earlier, a TNFR2 agonist has been demonstrated to selectively kill autoreactive T cells from patients with type 1 diabetes in culture.⁵

Further in vitro studies show that a Treg activation defect in type 1 diabetes can be corrected with TNFR2 agonism.⁴² Activated Tregs (aTregs) prevent or halt various forms of autoimmunity. Okubo et al have shown that type 1 diabetics have elevated numbers of resting Tregs (rTregs, CD4(+)/CD25(+)/Foxp3(+)/CD45RA) and a decrease in aTregs (CD4(+)/CD25(+)/Foxp3(+)/CD45RO) compared with controls ($n = 55$ type 1 diabetics, $n = 45$ controls, $P = 0.01$), associated with a trend for less residual C-peptide secretion from the pancreas ($P = 0.08$) and poorer HbA1C control ($P = 0.03$). TNFR2 agonism was used as a method for stimulating conversion of rTregs to aTregs, which

corrected the activation defect. Further, TNFR2 agonism was superior to standard protocols and to TNF in proliferating Tregs. The TNFR2 agonist-expanded Tregs were homogeneous and functionally potent by virtue of suppressing autologous cytotoxic T cells in a dose-dependent manner comparable to controls. Thus, targeting the TNFR2 receptor for Treg expansion *in vitro* demonstrates a means to correct the activation defect in type 1 diabetes.⁴³

Often when the benefit of TNF induction through BCG is described, it has been hard to reconcile with the vast autoimmune literature that shows some subsets of autoimmune subjects benefit from anti-TNF therapy, such as subjects with rheumatoid arthritis who benefit from anti-TNF antibody therapies. Previously, it was presumed that anti-TNF therapies “took away” TNF as their mechanism for clinical benefit. It is worth mentioning that new data perhaps explains this paradox. It is known from the rheumatoid arthritis literature that anti-TNF therapies in the form of Remicade and Adalimumab induce Tregs in autoimmune patients. These drugs are antibodies that bind TNF. Another approved drug for rheumatoid arthritis is Etanercept, a soluble TNFR2 antibody-receptor linked antibody; Etanercept does not induce Tregs *in vivo*.⁴⁴ In culture, human Tregs expand with TNF are potent Tregs.^{18,45-48} It is now appreciated that the therapeutic anti-TNF antibody Adalimumab induces *in vivo* Tregs but by binding preferentially to membrane surface TNF with trimerization that induces TNFR2 on Treg cells, both *in vivo* and *in vitro*.⁴⁹ Because TNFR2 Tregs are the most suppressive Tregs known to exist naturally as well as the abundant Treg subtype in cancer conferring suppression, this means anti-TNF therapy is not working through TNF reduction, but instead through TNF trimerization and TNFR2 induction of potent Tregs. Thus, adalimumab is expanding functional and potent TNFR2 Tregs well with potent suppressive activity.⁴⁹

Recent data show that TNFR2 restricts the pathogenicity of CD8 T cells in murine colitis.⁵⁰ Additionally, a TNFR2 agonist expands *in vitro* potent host Treg cells and *in vivo* also protects from acute graft versus host disease in a mouse.⁴³ In the human, exogenous TNFR2 activation protects from acute graft-versus-host disease (GVHD) via host Treg cell expansion.⁵¹

2 | CONCLUSIONS

Administration of TNF inducers or TNFR2 agonists may represent a new treatment strategy for type 1 diabetes. TNF induction and TNFR2 agonism have been associated with selective death of autoreactive T cells in type 1 diabetes, induction of Tregs, and even early clinical signs of restoration of pancreatic islet function *in vivo*. In current type 1 diabetes clinical trials, the selection of BCG as an immuno-intervention is based on the elimination of autoreactive T cells, the protective host TNF response (including induction of Tregs), and potential long-term modulation of the immuno-inflammatory profile of vaccinated subjects.

CONFLICTS OF INTEREST

DLF is an employee of Harvard Medical School and Massachusetts General Hospital and receives no research support from for-profit companies for the advancement of the BCG clinical trials which are wholly supported by philanthropy.

ETHICS STATEMENT

This is a review article that cites the peer-reviewed papers of our group and other groups. Therefore, the study designs are detailed in the primary papers, and this review does not include a study design with the direct ethical statements of the human study or animal study designs. For our own published studies cited in this paper, all animal studies go through the office of human studies or the animal studies offices to confirm the ethical treatment of humans and animals in research.

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