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Blockade of B-cell-activating factor suppresses lupus-like syndrome in autoimmune BXSB mice

Hanlu Ding a, Li Wang a,*, Xiongfei Wu b, Jun Yan c, Yan He c, Bing Ni d, Wenda Gao e, Xuemei Zhong f

a Department of Nephrology, Sichuan Provincial People’s Hospital, Chengdu, China
b Department of Nephrology, Southwest Hospital, The Third Military Medical University, Chongqing, China
c Department of Nephrology, Daping Hospital, The Third Military Medical University, Chongqing, China
d Department of Immunology, Institute of Immunology PLA, The Third Military Medical University, Chongqing, China
e The Transplant Institute, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA
f Department of Medicine, Boston University Medical Center, Boston, MA, USA

Abstract

B-cell-activating factor (BAFF), a member of the tumour necrosis factor superfamily, plays a critical role in the maturation, homeostasis and function of B cells. In this study, we demonstrated the biological outcome of BAFF blockade in BXSB murine lupus model, using a soluble fusion protein consisting of human BAFF-R and human mutant IgG4 Fc. Mutation of Leu235 to Glu in IgG4 Fc eliminated antibody-dependent cell cytotoxicity (ADCC) and complement lysis activity, and generated a protein devoid of immune effector functions. Treatment of BXSB mice with BAFF-R-IgG4mut fusion protein for 5 weeks resulted in significant B-cell reduction in both the peripheral blood and spleen. Treated mice developed lower proteinuria, reduced glomerulonephritis and much delayed host death than untreated animals. Thus, BAFF blockade with BAFF-R-IgG4mut protein is an effective strategy to treat B-cell-mediated lupus-like pathology. Moreover, compared with other IgG isotypes with undesired effector functions, mutant IgG4 Fc should prove useful in constructing novel therapeutic reagents to block immune molecule signalling in various diseases.

Keywords: BAFF • receptor antagonist • lupus, autoimmunity • BXSB mice

Introduction

Lupus nephritis is characterized by loss of B-cell tolerance to self-antigen and the presence of abnormally activated circulating B cells [1], contributing to tissue damage by secreting autoantibodies [2]. In disease models, activated autoreactive B cells can also drive the loss of tolerance in the T-cell compartment [3]. B-cell-activating factor (BAFF, also called TALL-1, zTNF4, BlyS and TNFSF13b) is a member of the TNF superfamily that is essential for B-cell survival, proliferation and immunoglobulin secretion [4–7]. Transgenic mice overexpressing BAFF develop symptoms characteristic of systemic lupus erythematosus (SLE), with high levels of rheumatoid factors, circulating immune complexes, anti-DNA autoantibodies and immunoglobulin deposition in the kidneys. The animals eventually succumb to an immune complex-mediated, lupus-like nephritis [8–10]. Even in the absence of T-cell help, BAFF-transgenic mice that were completely deficient in T cells still develop an SLE-like disease, indicating a critical role of BAFF in B-cell autoimmunity [11]. The levels of BAFF are also elevated in the serum of patients with SLE, correlated with circulating levels of anti-dsDNA antibodies and with clinical disease activity [12–15]. Thus, inhibition of BAFF signalling is a potential therapeutic option for treating lupus nephritis.

In addition to interacting with BAFF receptor (BAFF-R, BR3, TNFRSF13C), BAFF also binds to two other receptors. One is transmembrane activator and calcium-modulator and cyclophilin ligand (CAML) interactor (TACI, TNFRSF17), and the other is B-cell maturation antigen (BCMA, TNFRSF17). Compared with BAFF-R, these two receptors have more restricted functions: TACI controls T-cell-independent B-cell antibody response, isotype switching and B-cell homeostasis, whereas BCMA supports the survival of bone marrow plasma cells (for review, see reference [16]). Of the three receptors for BAFF, only BAFF-R is specific and the primary receptor for transmitting the BAFF-dependent B-cell survival signal, whereas TACI and BCMA also bind to the related ligand APRIL (a proliferation-inducing ligand, TNFSF13) [9, 17].

*Correspondence to: Dr. Li WANG,
Department of Nephrology,
Sichuan Provincial People’s Hospital. Chengdu,
Sichuan, 610072, P. R. China.
E-mail: scwangli62@163.com

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Strategies to block BAFF signalling have been tested in animal models of diseases with promising success. For example, treatment with a soluble TACI-Ig fusion protein in NZB/W F1 mice, a mouse model of lupus nephritis, inhibited the development of proteinuria and prolonged survival of the animals [9]. Adenovirus-mediated expression of TACI-Ig also blocked the development of hypergammaglobulinaemia, decreased the numbers of splenic plasma cells in C57BL/6 (B6) Ipr/lpr mice and reduced the extent of glomerulonephritis and proteinuria, improved survival in MRL.Ipr/lpr mice [18].

Nevertheless, because of the complexities of receptor-ligand cross-interaction as well as the species difference between human beings and mice, one should take extra caution in interpreting the experimental data. For instance, Ramanujam et al. compared the effects of BAFF-R-Ig, which blocks only BAFF, with those of TACI-Ig, which blocks both BAFF and APRIL, in a murine SLE model [19]. They found that both reagents similarly inhibited disease activity, but non-selective blockade with TACI-Ig further interfered with the development of T-cell-dependent IgM response, probably because of blockade on APRIL and TACI/BCMA interaction [19]. Although BAFF-R-Ig seems more specific, unlike human BAFF-R, mouse BAFF-R can also weakly bind to mouse APRIL [20], and hence high doses of soluble BAFF-R-Ig may inhibit mouse APRIL in addition to BAFF.

Another important issue when applying receptor immunoglobulin fusion proteins as cytokine antagonists is that, in most of the applications people often use human or mouse IgG1 Fc tail as the fusion partner. The IgG1 isotype has strong antibody-dependent cell cytotoxicity (ADCC) and complement activation capacity. Hence, an IgG1 fusion protein may become lytic to a cell if it binds to its cell surface ligand [21]. As BAFF is also synthesized as a membrane-bound protein [4], BAFF-R-IgG1 could kill BAFF+ cells including monocytes, thus complicating the interpretation of BAFF blockade data and may cause unwanted side effects when used as a therapeutic agent. In this study, we characterized the biological activity, both in vitro and in vivo, of a human BAFF-R-IgG4mut fusion protein in BXSB murine lupus model. The ability of IgG1 to induce complement lysis was eliminated by replacing the Fc domain of human IgG1 with that of human IgG4, which does not bind C1q, the first protein in the complement cascade. Also, mutation of Leu235 to Glu in IgG4 Fc eliminated the remaining ADCC activity and generated a protein devoid of immune effector functions [21]. Here, we describe in detail how to achieve high-quality IgG4 fusion protein that supports the clinical potential of BAFF-R-IgG4mut as a therapeutic agent for lupus nephritis.

### Materials and methods

#### Animals

Male BXSB mice, aged 8 weeks, 21–22 g in weight, were obtained from the Experimental Animal Laboratory of Peking University Health Science Center (Beijing, China). All animals were housed at 22°C under a 12-hr light/dark cycle under pathogen-free conditions. The animals were monitored for the development of proteinuria every 2 weeks and serum was collected every 3 weeks to measure anti-dsDNA antibody titres. Morbidity was checked three times a week. Animal care and handling was performed with the approval of the Institutional Authority for Laboratory Animal Care of the Health Science Center.

#### Construction of BAFF-R-IgG4mut fusion protein

The Leu235 to Glu mutation in hIgG4 was created by overlapping PCR with primers: IgG4mu UP: CCAGCACCTGAGTTCAAGGGGGAATCGCTG and IgG4mu DN: DACTGATGCTCCCCCTGCAACTCGATGTCG. The coding sequence for the extra-membrane region of hBAFF-R (Genbank: AF373846) was chemically synthesized by annealing the following four long oligos with built-in mutations V20Q and L27P to eliminate aggregates [22]. BAFF-R PciI primer: ggtaacATGTcGGCAGGGGCAGGGCTGGG; BAFF-R NheI primer: TATGAGGAGCAGGGCCAGGGCTGGG; BAFF-R BamHI primer: ggcggccagctgagttcagggctgg; BAFF-R XhoI primer: 5'-cgaagcttctgagctgccagggctgg-3'. digestion of BamHI followed by ligation with XhoI and transformation into DH5α and high-efficiency transformation into DH5α TA cloning vector. The right recombinant was amplified by PCR and ligated into pSec/WG vector. The right recombinant was transfected into Flp-In Chinese hamster ovary (CHO) cells with lipofectamine reagent and stable constitutive expression cell line was generated according to the instructions of the manufacturer (Invitrogen).

#### Western blot of BAFF-R-IgG4mut fusion protein

BAFF-R-IgG4mut fusion protein was expressed and purified by Protein A affinity column chromatography as described previously [23]. Purity of the purified protein was assayed on a 12% SDS-PAGE gel. For Western blot analysis, the membrane with transferred protein was blocked in 1% blocking solution in TBS (150 mM NaCl in 50 mM Tris-HCl, pH 7.5) for 12 hrs at 4°C, and then incubated with goat anti-human BAFF-R polyclonal antibody (0.2 μg/ml, clone AF1162, R&D Systems, USA) overnight at 4°C. After washing three times with 0.5% Tween in TBS buffer, the blot was incubated for 1 hr with horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG (1:3000, Sino-American Biotechnology Co., Beijing, China). Proteins were detected with DAB reagent sets (Sino-American Biotechnology Co., Beijing, China).

#### Bioactivity assay of BAFF-R-IgG4mut fusion protein

B cells from BALB/c spleen were purified by the Miltenyi B-cell enrichment kit (Miltenyi Biotec, USA). B cells 2 × 10⁶ were seeded in 96-well plate and stimulated with 5 or 10 μg/ml anti-IgM (Southern Biotech, USA) in triplicates. In some wells, 2 ng/ml of mouse recombinant BAFF (R&D Systems) and serially diluted purified hBAFF-R-IgG4mut was added. Cell proliferation after 3 days was measured by [3H]-thymidine incorporation (0.5 μCi/well) added at the last 18 hrs of culture.
Measurement of serum BAFF levels

Serum BAFF was measured by ELISA in male BXSB animals before onset of disease (8 weeks of age) as a baseline, and then measured during disease progression. Briefly, plate-coated rabbit anti-mouse BAFF polyclonal antibody (20 μg/ml, eBioscience) was incubated for 1 hr at 37°C with sera samples diluted 1:100. After washing, rat anti-mouse BAFF monoclonal antibody (40 μg/ml, R&D Systems) as detecting antibody was then incubated for 1 hr at 37°C, the latter of which was further recognized by biotinylated antirat IgG (1:200, Zhongshan Biotechnology Co., Beijing, China) and avidin-conjugated HRP with tetramethylbenzidine as the substrate. Recombinant mouse BAFF (R&D Systems) was used as a standard (diluted from 1–1000 ng/l) to measure BAFF concentrations in 100 μl of serum samples in triplicate.

Assessment of nephritis

For monitoring the development of nephritis, proteinuria was measured as described previously [25], and urine protein concentrations of >100 mg/dl were considered positive. For histological evaluation of renal disease, mice were killed after treatment at 15 weeks of age. Kidneys were either fixed in 10% buffered formalin or prepared for cryostat sectioning. Formalin-fixed tissues were embedded in paraffin, sectioned and stained by the haematoxylin and eosin method. Cellularity was determined by counting the number of cells of 12 randomly selected glomeruli in each kidney cross-section. The degree of cellularity was scored from 0 to 3 by the following criteria as described [26]: grade 0, normal (35–40 cells/glomerulus); grade 1, mild (41–50 cells/glomerulus); grade 2, moderate (51–60 cells/glomerulus); grade 3, severe (>60 cells/glomerulus). The intensity of tubular interstitial inflammatory cell infiltrates was scored according to a 0–4 scale as previously described [27], where 0 represents no abnormality, and 1, 2, 3 and 4 represent mild, moderate, moderately severe and severe abnormality, respectively. For determining IgG deposition, frozen sections were fixed in acetone and stained with FITC-conjugated goat anti-mouse IgG mAb (Sino-American Biotechnology). The staining profiles were obtained with a fluorescent microscope (BX-50; Olympus, Tokyo, Japan) and the mean fluorescence intensity (MFI) of 15 glomeruli from each section was averaged blindly from 0 to 4+ by a pathologist who was unaware of the experimental design, as described previously [28].

Statistical analysis

Log-rank and Student’s t-tests were used. Methods were noted in figure legends. Values of P < 0.05 were considered significant.

Results

BAFF-R-IgG4mut fusion protein blocks BAFF activity in vitro

We successfully constructed the expression vector for BAFF-R-IgG4mut containing the extracellular domain of human BAFF-R gene (Genbank: AF373846). Based on amino acid sequence, the mature human BAFF-R-IgG4mut protein expressed in CHO cells has a calculated molecular mass of approximately 35.6 kD. As a result of glycosylation, the recombinant protein migrates with apparent molecular weight (MW) of 40–50 kD in SDS-PAGE under reducing conditions (Fig. 1A). The authenticity of the BAFF-R moiety was confirmed by Western blot (Fig. 1B). To test the bioactivity of BAFF-R-IgG4mut protein, we stimulated mouse B cells with anti-IgM, anti-IgM plus BAFF (2 ng/ml) or anti-IgM+BAFF+BAFF-R-IgG4mut. Low dose of exogenous BAFF did not induce B-cell proliferation by itself (data not shown), with H2O2 and Ophenylenedianime at 4 μg/ml in phosphate-citrate buffer (pH 5.0). The reaction was stopped with 2 N H2SO4 and absorbance at 490 nm was measured by an ELISA plate reader (Alexis, Montreal, Canada).
but increased anti-IgM-stimulated proliferation. Clearly, BAFF-R-IgG4mut protein exerted a dose-dependent suppression of anti-IgM/BAFF stimulated proliferation (Fig. 1C). Interestingly, in almost all the cultures with BAFF-R-IgG4mut protein added, B-cell proliferation is even lower than that induced by anti-IgM alone. This suggests that during activation by anti-IgM, B cells secrete endogenous BAFF as a growth or survival factor, and this effect can also be neutralized by BAFF-R-IgG4mut protein.

**BAFF-R-IgG4mut blocks the elevation of serum BAFF levels during murine lupus development**

To determine if administration of BAFF-R-IgG4mut can neutralize BAFF in vivo, we measured serum BAFF levels in BXSB mice at 9, 10, 12 and 15 weeks of age. An average of one-fold increase in serum BAFF was detected in 70% of the animals at 10 weeks of age, and the level reached threefold over baseline in 100% of the animals by 15 weeks of age in control mice. After treatment with BAFF-R-IgG4mut, the serum BAFF levels were significantly reduced (Fig. 2), indicating this recombinant fusion protein can be used as a BAFF blocker in vivo.

**BAFF-R-IgG4mut prevents the development of lupus-like nephritis**

To assess the effects of BAFF-R-IgG4mut on the development of lupus-like nephritis, we started the treatment of BXSB mice at 8 weeks of age before serum levels of BAFF were elevated. Proteinuria (>100 mg/dl) was first detected in the control mice (PBS or IgG) at 9 weeks of age and became prominent at 12 weeks of age (Fig. 3A). At the cessation of treatment (15 weeks of age), 100% of the mice treated with PBS or IgG4 developed renal disease with proteinuria >100 mg/dl. Although 50% of the mice treated with BAFF-R-IgG4mut developed proteinuria at 15 weeks of age, the disease course was significantly delayed ($P < 0.01$). As the hallmark of lupus is the development of anti-dsDNA autoantibodies, we measured by ELISA the levels of serum anti-dsDNA IgG in BXSB mice after different treatments. The results showed that BXSB mice treated with PBS or IgG4 possessed highly elevated levels of serum anti-dsDNA IgG, and there was an obvious
The trend of increase in antibody titres with age. The levels of anti-dsDNA IgG were suppressed in the mice 4 weeks after the initial treatment with BAFF-R-IgG4mut and were maintained at 15 weeks of age compared with PBS or IgG treatment alone ($P < 0.035$) (Fig. 3B). Animals treated with BAFF-R-IgG4mut had 100% survival at 30 weeks of age compared with 0% survival in the control group ($P < 0.01$) (Fig. 3C). Although all animals in BAFF-R-IgG4mut-treated group eventually died of disease, this may be as a result of the neutralization of this human fusion protein by murine anti-human antibody.

### Histological verification of the beneficial effects of BAFF-R-IgG4mut

The improvement of nephritis by BAFF-R-IgG4mut treatment was verified by histological examination of glomerular hypercellularity, tubular interstitial inflammatory cell infiltration and IgG deposition in renal tissue sections from mice that underwent different treatments. At 15 weeks of age, renal sections from PBS or IgG-treated mice showed mesangial hypercellularity, in conjunction with tubular interstitial inflammatory cell infiltration (Fig. 4A). The above pathological changes were diminished in BAFF-R-IgG4mut-treated mice (Fig. 4B). While there was strong deposition of IgG to glomeruli in PBS or IgG4-treated mice (Fig. 4C), the intensity of such IgG deposition was clearly inhibited in mice treated with BAFF-R-IgG4mut (Fig. 4D). In semi-quantitative analyses, BAFF-R-IgG4mut treatment showed the most significant inhibition on glomerular hypercellularity, tubular interstitial inflammatory cell infiltration and IgG deposition, compared with treatment with PBS or IgG (Fig. 4E–G). These results support the notion that blocking BAFF:BAFF-R interaction could effectively inhibit the development of lupus-like disease in mice.

### Effects of BAFF-R-IgG4mut on lymphocyte composition

To study the potential mechanisms contributing to the suppression of disease in BAFF-R-IgG4mut-treated animals, we determined the percentage of B cells (B220$^{+}$CD5$^+$) and T cells (CD3$^+$CD4$^{-}$CD8$^-$, CD3$^+$CD4$^+$CD8$^-$) by flow cytometry at 10, 12 and 15 weeks of age. At 12 weeks of age (4 weeks after treatment), there was already a significant decrease of the percentage of B cells in the peripheral blood and spleen in animals treated with BAFF-R-IgG4mut, compared with control animals (Fig. 5A). To show this effect is B-cell specific, we used the B220$^+$CD5$^+$ non-T, non-B cell population as an internal calibrator, and calculated the ratios of B or T cells versus this calibrating population. We found that starting from 12 weeks of age, BXSB mice had increased B-cell population in both the spleen and the peripheral blood. Treatment with BAFF-R-IgG4mut fusion protein significantly suppressed B-cell expansion, while having no effect on T cells (Fig. 5B). These results indicate a role for BAFF in maintaining peripheral B-cell populations and blocking BAFF:BAFF-R has a beneficial effect on inhibiting B-cell hyperplasia in murine lupus.

### Discussion

BAFF-R is expressed on T cells and a wide range of B-cell subsets, including immature, transitional, mature, memory and germinal centre B cells, as well as on plasma cells [29–32]. In this study, we used hBAFF-R-Ig instead of mBAFFR-Ig to specifically block BAFF signalling in mice. This is because mouse BAFF-R weakly binds to mouse APRIL, and hence high experimental doses of recombinant soluble mouse BAFF-R-Ig may inhibit mouse APRIL in addition to BAFF [20]. We used BXSB mice, a well-characterized mouse model that spontaneously develops an autoimmune syndrome similar to human SLE, and is characterized by hypergammaglobulinemia, autoantibody production and the development of fatal glomerulonephritis. Positive serology to self-Ags (dsDNA, ssDNA, erythrocytes and platelets) and immune complex-mediated glomerulonephritis is the hallmark of disease in BXSB mice. Histopathological changes are evident by 10 weeks of age in male
mice, leading to end-stage renal disease and 70% mortality by 40 weeks of age [33, 34].

The BAFF-R-IgG4mut fusion protein is functional as a blocker for BAFF, as shown by its ability to neutralize BAFF activity in vitro. In this study, we demonstrated that weekly treatment of BXSB mice with BAFF-R-IgG4mut fusion protein for 5 weeks resulted in significant B-cell reduction in both the peripheral blood and the spleen. Treatment with BAFF-R-IgG4mut also led to a partial suppression of serum autoantibody levels. Glomerulonephritis was reduced in conjunction with the improvement in proteinuria. This was associated with prolonged survival in treated animals. In BXSB mice, the infiltrating T cells include both CD4+ and CD8+ T cells that appear to be required for disease pathogenesis. However, BAFF blockade in this model has no effect on the activation and expansion of T cells, as CD4+ and CD8+ T-cell compositions are not different between the groups. The delayed mortality with BAFF blockade could result from reduced autoantibodies and/or from reduced numbers of B cells driving the infiltrative process [3, 35]. The results indicate that blocking B-cell extravasation is sufficient to quench autoimmune reactions and may be the major mechanism for the BAFF-R-IgG4mut fusion protein activity in vivo. Our data suggest that BAFF blockade may be effective as an interventional strategy, rather than being simply a preventative measure, in patients with established disease.

Recombinant fusion proteins consisting of the extracellular domain of immunoregulatory proteins and the Fc region of immunoglobulin G (IgG) are a novel class of protein therapeutics. A crucial component of these fusion proteins is the IgG domain. The Fc region of IgG1, the most commonly used isotype in fusion proteins, exerts immune effector function, such as complement lysis and ADCC. Depending on their potential application, fusion proteins targeting cancer cells or autoreactive immune cells may gain potency if they have the capability to induce cell death of their targets. This increase in potency has to be balanced with possible side effects if the ligand of the fusion protein is expressed on tissues other than the target cells. Even though the original design of certain receptor fusion proteins is to block their soluble cytokine ligands, one should bear in mind that some cytokines can be expressed in a cell-surface-bound form. For example, TNF-α is synthesized as a trans-membrane 26 kD protein that is transported to the cell surface where it is cleaved by a metalloproteinase (TACE) to release the soluble 17 kD protein [36]. TNFR-IgG1, brand name Enbrel™, can effectively block TNF-α and has been used to treat rheumatoid arthritis [37]. However, some unwanted side effects, such as increased incidence of infection and cancer, as well as ‘injection site reaction’ might be caused by Enbrel™ binding to cell-surface TNF-α and subsequent lysis of these cells via IgG1-mediated effector functions [37].
Like TNF-α, BAFF can also be expressed as a membrane-bound form on many types of immune cells [4]. Potential lysis of these cells during treatment with conventional BAFF-R-IgG1 could create a ‘hole’ in host immunity and may increase the incidence of infection and cancer. In our study, the ability of IgG1 fusion proteins to induce complement lysis was eliminated by replacing the Fc region of human IgG1 with that of human IgG4, which does not bind C1q, the first protein in the complement cascade [21]. ADCC is exerted by monocytes and natural killer cells when they recognize opsonized cells via their Fc receptors. Human IgG4 does not bind FcγRII and FcγRIII, but it does bind FcγRI. A crucial subdomain for this interaction has been mapped to amino acids 234–238 of IgG4 [38]. Mutation of Leu235 to a Glu in human IgG4 should completely abolish FcγRI binding and ADCC activity [21]. Thus, our study demonstrated that mutant IgG4 Fc should prove useful in constructing novel therapeutic reagents to block immune molecule signalling in various diseases.

Last, human lupus is a very complicated disease, affecting multiple organs. One should not expect any monotherapy to be the ultimate regimen. Potentially efficacious reagents might be possibly combined to achieve synergy in future therapy. Compared with untreated controls, treated animals in our study did show prolonged survival by week 30, although they all died by week 33. This sudden onset of host death is more likely because of the insufficient treatment and/or intrinsic disease mechanism, but not the toxicity of BAFF-R-IgG4mut, as histological analysis did not reveal any sign of liver abnormality after treatment with BAFF-R-IgG4mut (data not shown). It is possible that the full human sequence of BAFF-R-IgG4mut induced murine anti-human neutralizing antibodies that made its therapeutic benefit less...
prominent. This is not without a precedent. In a study by Charles Dinarello’s group [39], injection of human α-1-antitrypsin (hAAT) in mice induced anti-hAAT after 18 days. The authors further showed that it was the neutralizing antibody that limited the beneficial effect of hAAT, as pre-vaccination with hAAT abolished its protective effect on transplanted allogeneic islets, and rejection-induced hyperglycaemia correlated well with the existence of anti-hAAT titres. In our study, we did not inject BAFF-R-IgG4mut for >5 weeks, as once the anti-human antibodies are induced, the again introduced human reagent will be of limited effect. In addition, formation of mouse antibody:human antigen complex and its deposition may accelerate the disease. This issue can be controlled by using murine BAFF-R-Ig with escalating doses to cover longer period of time in future animal studies, but will not become an issue in human trials.

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The authors declare no competing financial interests.

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