

Vascular and cerebral benefits of Tofacitinib in rheumatoid arthritis: evidence in rat adjuvant-induced arthritis

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Abstract

Background: To determine vascular and cerebral effects of Tofacitinib in the adjuvant-induced arthritis (AIA) model in rat.

Methods: At the first signs of arthritis (day 10-12 post-immunization), Tofacitinib (10 mg/kg s.c., twice daily) or vehicle were administered for 3 weeks in AIA rats. A group of non AIA served as controls. Body weights and arthritis scores were daily monitored. Anhedonia was explored with the saccharin preference test at day 4 (preclinical), day 11 (early) and day 28 (acute arthritis) post-immunization. At the end of the treatment, aorta, brain and blood were collected for analysis of endothelial function using acetylcholine (Ach)-induced relaxation, brain-derived neurotrophic factor (BDNF) protein levels in the hippocampus (Hip) and prefrontal cortex (PFC), and IL-1 β , TNF α , IL-17A plasma levels. Radiographic score of paws was also assessed.

Results: As compared to vehicle, Tofacitinib reduced body weight loss, arthritis and radiographic scores ($p < 0.001$) but did not change plasma levels of pro-inflammatory cytokines. Tofacitinib fully prevented AIA-associated reduction in Ach-induced relaxation. As compared to controls, vehicle-treated AIA exhibited low BDNF levels in PFC ($p < 0.001$) and anhedonia at all times examined ($p < 0.05$). Tofacitinib did not improve anhedonia, but resulted in elevation of BDNF levels both in PFC ($p < 0.05$) and Hip ($p < 0.001$). A positive correlation was observed between brain BDNF levels and endothelial function ($p < 0.05$).

Conclusion: Restoration of normal endothelial function and elevation of brain BDNF levels by Tofacitinib in AIA rats support a positive effect of this new antirheumatic drug on cardiovascular and neuropsychiatric comorbidities of rheumatoid arthritis.

Introduction

Rheumatoid arthritis (RA) is one of the most common chronic autoimmune diseases characterized by joint destruction and bone lesion as a result of production of multiple inflammatory cytokines. TNF α , IL-1 β and IL-17A produced by synoviocytes and leucocytes play a pivotal role in the articular manifestations of RA (1). Thus, their inactivation by biologic disease-modifying antirheumatic drugs (bDMARDs) has revolutionized treatment of RA. However, the failure of these drugs to induce remission in all patients has led to the development of Jakinibs. Unlike bDMARDs, Jakinibs inhibit the janus kinase (JAK)/STAT pathway, a signal transduction pathway downstream of cytokines other than TNF α , IL-1 β and IL-17A including IL-2, IL-6, IL-12/23, IFN γ (2). Among the Jakinibs, Tofacitinib is a first-generation Jakinib that inhibits JAK 1, JAK 3 and to a lesser extent, JAK 2 (2). It has been approved for the treatment of moderate to severe active RA in adult patients who have responded inadequately to, or who were intolerant to one or more DMARDs including synthetic DMARDs and bDMARDs. Tofacitinib was even superior to methotrexate, the first-line synthetic DMARDs in reducing signs and symptoms of RA and in inhibiting progression of structural damage (3).

RA is not only associated with articular manifestations but also with frequent cardiovascular and neuropsychiatric comorbidities (4, 5). While DMARDs are efficient to control disease activity, there is no consensus on their potential to reduce these comorbidities. In fact, the exact reasons for the excess CV risk are not well known even though a role of TNF α and IL-1 β is largely suspected. Indeed, exposure of cultured endothelial cells to these cytokines induced endothelial activation (6) and endothelial dysfunction (ED) (7), two events recognized as initial step for atherosclerosis development (8). Likewise, the exact reasons for the high prevalence of neuropsychiatric manifestations in RA including impaired cognition (executive function, memory), anxiety and depression are also unknown. However, neuroinflammation, sedentary behaviour and ED that have been associated with impaired cognition and depression in the general population (9–11) are of particular relevance in RA. Our recent demonstration that cerebral levels of brain-derived neurotrophic factor (BDNF) were decreased in adjuvant-induced arthritis (AIA) rats provided a potential molecular mechanism underlying neuropsychiatric comorbidities in RA (12, 13). Indeed, cognitive ability including mood is largely dependent on BDNF, a neurotrophin playing a crucial role in neuroplasticity and neurogenesis (14). In agreement with this, BDNF^{+/-} rats exhibited anhedonia and anxiety-like behaviour (15) and the Val66Met polymorphism that results in abnormal BDNF signalling (16) increased the risk of mental diseases (17, 18). It is noteworthy that brain BDNF levels are decreased in diseases associated with ED (19, 20) or after immune challenge (21).

The major concern for addressing whether Jakinibs may improve or not vascular or brain function in clinical studies is that on the one hand, Jakinibs are usually used in combination therapy, and in the other hand, RA patients take multiple medications including drugs which can impact upon vascular or brain function. Thus animal models of RA provide the unique opportunity to determine the impact of one given drug without the influence of other medications, in rat cohorts with reproducible polyarthritis severity. In this context, the aim of the present study was to explore the vascular and cerebral effects of an anti-inflammatory dosage of Tofacitinib in a rat model of RA. For this purpose, we used the rat adjuvant-induced arthritis (AIA) model that is a benchmark model for pharmacological studies on antirheumatic drugs in RA, with high predictive and face validity including ED and depression-like symptoms (12, 22). AIA rats were treated from the first signs of inflammation for 3 weeks with Tofacitinib or vehicle. ED, brain BDNF levels, serum levels of cytokines and anhedonia as a sign of depression were investigated.

Methods

Animals

Six-week-old male Lewis rats (n = 65) were purchased from Janvier (Le Genest Saint Isle, France). Animals were kept under a 12 h-12 h light: dark cycle and allowed free access to food and water. The experimental procedures were approved by the local committee for ethics in animal experimentation n°2015-001-CD-5PR of Franche-Comté University (Besançon, France), and complied with the “Animal Research: Reporting In Vivo Experiments” ARRIVE guidelines. Anaesthesia was induced by volatile (isoflurane 4%, Virbac, Carros, France) and non-volatile (pentobarbital, 60 mg/kg, i.p.) anaesthetic agents for arthritis induction and organ removal, respectively.

Induction And Clinical Evaluation Of The Arthritis Model

Adjuvant arthritis was induced by a single intradermal injection at the base of the tail of 120 μ L of 1 mg of heat-killed *Mycobacterium butyricum* (Difco, Detroit, MI) suspended in 0.1 mL of Freund's incomplete adjuvant (Difco, Detroit, MI). The AIA model is characterized by rapid onset and progression of a robust and easily measurable polyarthritis, clinically characterized by severe erythema and diffuse soft swelling with complete ankyloses, paw malformations, reduced locomotor activity, frequently associated to ears and tail inflammation, weight loss, anorexia and diarrhea (23). Rats were weighed and monitored 7 days per week for clinical signs of arthritis. The scoring system was employed as follows (24): arthritis of one finger scores 0.1, weak and moderate arthritis of one big joint (ankle or wrist) scores 0.5 and, intense arthritis of one big joint scores 1. Tarsus and ankle were considered as the same joint. Sum of joints scores of 4 limbs leads to an arthritis score of maximum 6 to each rat. A group of non-arthritic age-matched rats was used as controls (n = 15) and received saline at the base of the tail.

Drug Treatment

Tofacitinib citrate was provided by Pfizer. It was dissolved in 33% DMSO in PEG300 and administered daily by subcutaneous (s.c) route (1 mL/kg) from the first signs of arthritis (day 10–12 post-immunization) for 21 days, according to different protocols described in the “dose finding” results.

Saccharin Preference Test

Depression-like state was assessed by testing of anhedonia, a core feature of major depressive disorder and a key diagnostic criterion according to DSM-5 (American Psychiatric Association – APA 2013), using the saccharin preference test (SPT). For 2 consecutive nights (from 6:00 PM to 9:00 AM), rats (n = 2 to 3/cage) were given free access to drinking spouts containing tap water or a saccharin solution at 7.5 mM while the two spouts contained water during the diurnal period. The position of the spouts was switched between the two nights. Spouts were weighted at the beginning and the end of the two nights. Index of saccharin preference was calculated for each cage from the ratio of saccharin solution intake over total liquid intake during second night. A reduced saccharin preference indicates anhedonia. Using such protocol, we previously reported anhedonia in AIA rats (12).

Tissue Collection

Twenty-one days after treatment initiation, blood was withdrawn from the abdominal artery and centrifuged to obtain plasma, divided into aliquots and stored at -80 °C until analysis. Thoracic aortas were removed and immediately used for vascular reactivity studies. Brain regions that are connected to the neuroanatomical circuit involved in depression namely the prefrontal cortex (PFC) and hippocampus (Hip) (25) were collected and stored at -80 °C until homogenization in 7 volumes of ice-cold buffer, centrifugation and determination of protein concentration (Lowry method) in supernatant. Hind paws were removed and placed in 4% formalin until assessment of radiographs.

Radiographic Ex-vivo Analysis Of Joints

Radiographs of hind paws were performed with a BMA High Resolution Digital X Ray (40 mV, 10 mA) – D3A Medical Systems (Orleans, France). A score of 0 to 20 was determined for each paw using a grading scale modified from Ackerman et al. (1979) (26). This score used the scale: 0 (normal), 1 (slight), 2 (mild), 3 (moderate), and 4 (severe) abnormalities in the tissue for each of 5 characteristic features of AIA. Radiographs take into account: (a) the soft tissue swelling, (b) the osteoporosis as measured by bone density, (c) the loss of cartilage shown by narrowing of the joint spaces; (d) the bone erosions and (e) the heterotopic ossification defined as proliferation of new bone tissue. The maximum score for each rat is 40.

Vascular Reactivity

At the end of the treatment period, thoracic aorta was excised, cleaned of connective tissue, and cut into rings of approximately 2 mm in length. Rings were suspended in Krebs solution (moles/liter: NaCl 118, KCl 4.65, CaCl₂ 2.5, KH₂PO₄ 1.18, NaHCO₃ 24.9, MgSO₄ 1.18, glucose 12, pH 7.4), maintained at 37 °C and continuously aerated with 95% O₂, 5% CO₂, for isometric tension recording in organs chambers. In some rings, endothelium was mechanically removed. The completeness of endothelial denudation was confirmed by the absence of relaxation to the endothelium-dependent agonist acetylcholine (Ach, 10⁻⁶ moles/liter). After a 90-min-equilibration period under a resting tension of 2 g, to evaluate endothelial function, rings with intact endothelium were constricted with phenylephrine (PE, 10⁻⁶ moles/liter) and endothelium-dependent relaxation to Ach (10⁻¹¹-10⁻⁴ moles/liter) was studied. Endothelium-denuded rings were used to determine the vasoconstrictive response to norepinephrine (NE, 10⁻¹¹-10⁻⁴ moles/liter) and the vasorelaxant response to the nitric oxide (NO)-donor sodium nitroprussiate (SNP, 10⁻¹¹-10⁻⁴ moles/liter) after precontraction with PE 10⁻⁶ moles/liter.

Brain BDNF Levels

Western blotting analyses were used to determine BDNF levels in PFC and Hip as previously described in details (12). Equal protein amounts (20 µg) were resolved by SDS-PAGE, electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes. After blockade of non-specific binding sites, membranes were incubated for 3 h with primary antibody directed against BDNF (1/3000, rabbit monoclonal antibody, ab108319, Abcam, Cambridge, UK). Membranes were then incubated for 2 h with horseradish peroxidase-conjugated anti-IgGs as secondary antibodies (111-035-144, 1/30000, Jackson ImmunoResearch, Newmarket, UK). Protein-antibodies complexes were visualized using enhanced chemiluminescence (ECL+, GE Healthcare, Little Chalfont, UK) and the band densities were determined using ChemiDoc Imaging Systems (Biorad Laboratories).

Plasma Cytokines Levels

Plasma levels of IL-1β, TNFα and IL-17A were measured, as these cytokines were identified in preclinical and clinical studies as potential circulating biomarkers of ED (27). IL-1β and TNFα were measured by using Milliplex magnetic bead panel kits (eBioscience, Thermofisher, Courtaboeuf, France) that were

analyzed with a Luminex MAGPIX system (Luminex Corporation; Houston, TX) and Milliplex Analyst software (Millipore; St. Charles, MO) while IL-17A was measured by using an ELISA kit (eBioscience, ThermoFisher, Courtaboeuf, France). The limits of detection provided by the manufacturer for IL-1 β , TNF α and IL-17A were 13, 2.88 and 1 pg/mL, respectively.

Data And Statistical Analysis

Values are presented as means \pm SEM. Data were analyzed using GraphPad Prism or Sigma plot softwares. Contractile responses to PE were expressed as the percentage of the maximum response to KCl 100 mmoles/liter. Relaxant responses to SNP and Ach were expressed as the percentage of relaxation of the contractile response to PE 10⁻⁶ moles/liter. Emax values (values of maximal relaxation) were determined by fitting the original dose-response curves. Concentration-responses curves to Ach, SNP and PE were compared by 2-way analysis of variance (ANOVA) for repeated measures. Comparison between two values was assessed by unpaired Student *t* test or Mann-Whitney test when data were not normally distributed. The analysis of the relationship between two parameters was determined by linear regression analysis and Spearman's correlation coefficient was calculated between these variables. *P* < 0.05 was considered statistically significant.

Results

Dose-finding study in male Lewis rats with AIA

As only 4 studies in the literature administered Tofacitinib in the AIA model (28–31), and only one in male Lewis rats (29), a first set of experiments was dedicated to the determination of an efficient dosage and regimen on arthritis and radiographic scores. At the day of first inflammatory symptoms (i.e. day 10–12 post-immunization), AIA rats (*n* = 5/group) were treated by s.c. route (1 mL/kg) for 21 days with:

- Tofacitinib at 10 mg/kg/day once daily (10 qd)
- Tofacitinib at 10 mg/kg/day twice daily (10 bid)
- Tofacitinib at 20 mg/kg/day twice daily (20 bid)
- Vehicle (33% DMSO in PEG300) twice daily

As shown in Fig. 1A, Tofacitinib significantly reduced arthritis score in AIA rats regardless of the dose. However, 10 bid and 20 bid administrations had a better efficacy on arthritis score than 10 qd. Thus, the delay for the anti-inflammatory effect was 2 days for 10 bid and 20 bid instead of 5 days for 10 qd. In addition, at the end of the treatment, Tofacitinib reduced arthritis score by -63% and -68% for 10 bid and 20 bid, respectively, *vs* -39% for 10 qd. As regards the structural damage (Fig. 1B), as compared to vehicle, Tofacitinib 10 bid and 20 bid significantly reduced radiographic score with no difference between dosages whereas 10 qd was ineffective. On the basis of this preliminary study, the dosage 10 bid was chosen for a second set of experiments that randomized male Lewis rats in 3 groups: AIA rats treated either with Tofacitinib (10 bid, Tofacitinib-AIA, *n* = 15) or vehicle (33% DMSO in PEG300, 1 mL/kg, s.c.,

twice daily, Vehicle-AIA, n = 15) for 3 weeks, and control age-matched rats (Controls, 3% DMSO in PEG 300, 1 mL/kg, s.c., twice daily for 3 weeks, n = 15).

Tofacitinib decreased arthritis severity in AIA

As shown in Table 1, AIA induced a severe arthritis associated with body weight loss, joint damage and increased plasma levels of pro-inflammatory cytokines as compared to controls. Tofacitinib dramatically reduced arthritis severity, radiographic score and reduced the body weight loss as compared to Vehicle-AIA rats (Table 1). By contrast, Tofacitinib did not significantly change plasma levels of IL-1 β , TNF α and IL-17A (Table 1).

Table 1
Effect of Tofacitinib on physiological and biological parameters

	Control	Vehicle-AIA	Tofacitinib-AIA
Body weight (g)	312 \pm 20	216 \pm 3 ^{###}	261 \pm 5 ^{***}
Arthritis score	n.d.	3.8 \pm 0.2	0.9 \pm 0.2 ^{***}
Radiographic score	1.8 \pm 0.5	30 \pm 1.5 ^{###}	8 \pm 1.1 ^{***}
IL-1 β (pg/mL)	55.6 \pm 8.9	158.5 \pm 14.5 ^{###}	131.6 \pm 11.3
TNF α (pg/mL)	15.9 \pm 1.7	36.2 \pm 4.0 ^{###}	29.7 \pm 2.0
IL-17A (pg/mL)	3.7 \pm 0.9	11.5 \pm 2.3 ^{###}	13.7 \pm 2.0
All parameters were measured at day 33 post-immunization after 21 days of treatment (s.c.) with vehicle or Tofacitinib (10 bid) or in age-matched control rats. Values are expressed as means \pm SEM (n = 9–15 rats per group). ^{###} p < 0.001 vs control, ^{***} p < 0.001 vs Vehicle-AIA.			

Tofacitinib fully prevented endothelial function in AIA

The effect of Tofacitinib on endothelial function was assessed from the concentration-response curves to Ach of aorta collected from controls (n = 11), vehicle-AIA (n = 12), Tofacitinib-AIA (n = 12). As shown in Fig. 2A confirming ED in AIA rats (27), the relaxant effect of Ach was significantly lower in vehicle-AIA than controls. Tofacitinib fully prevented ED. Thus, there was no difference in Ach-induced relaxation between Tofacitinib-AIA and controls. To ascertain that this effect was not due to impaired response of vascular smooth muscle cells to vasoconstrictive stimulus or to the relaxant effect of NO, the effect of Tofacitinib on NE-induced vasoconstriction and SNP-induced vasodilation was determined on endothelium-denuded rings. Our results showed that Tofacitinib-AIA rats displayed similar constriction to NE (Fig. 2B) and similar relaxation to the NO-donor SNP than Vehicle-AIA rats (Fig. 2C)

Tofacitinib enhanced brain BDNF levels

PFC and Hip BDNF levels were measured in controls (n = 12), Vehicle-AIA (n = 12) and Tofacitinib-AIA (n = 11). Supplemental Fig. 1 shows whole uncropped images from PFC and Hip samples in each of the group of rats studied. We first examined inter-individual variabilities in PFC and Hip BDNF levels among controls, Vehicle-AIA and Tofacitinib-AIA. Variation coefficient (standard deviation/mean ratio) was closed to 25% except for PFC BDNF levels in Tofacitinib-AIA rats where it reached 42% (data not shown). Then, to assess the effect of arthritis on BDNF levels, we compared on a same gel half of control and Vehicle-AIA rats (series 1), these rats having been immunized on the same day. The remaining rats that were immunized on another day (series 2) were compared on another gel. The same procedure was used to compare Vehicle-AIA and Tofacitinib-AIA rats. The results on BDNF for series 1 and 2 are shown in supplemental Fig. 2. Then, we pooled rats of series 1 and 2. BDNF levels in Vehicle-AIA group were expressed as percentage of BDNF levels obtained in controls while BDNF levels in Tofacitinib-AIA group were expressed as percentage of BDNF levels obtained in Vehicle-AIA group. The results are shown in Fig. 3. As compared to control rats, Vehicle-AIA rats exhibited reduced BDNF levels in PFC (-46.3%, $p < 0.001$, Fig. 3A) but not in Hip (-4.9%, n.s., Fig. 3B). Tofacitinib resulted in a significant elevation of BDNF levels both in PFC (+ 20%, $p < 0.05$, Fig. 3C) and Hip (+ 132%, $p < 0.001$, Fig. 3D).

Tofacitinib Did Not Change Preference For The Saccharin Solution

The results of saccharin preference test are summarized in Fig. 4. As compared to controls, saccharin preference was significantly lower in both Vehicle-AIA and Tofacitinib-AIA rats at the preclinical phase (day 6 post-immunization) and throughout the inflammatory phase (day 14, 21 and 28 post-immunization) with no difference between the two groups.

Correlations between endothelial function, arthritis score, cytokines and BDNF levels in AIA rats

Correlations were tested in AIA rats treated with vehicle or Tofacitinib (Table 2). Endothelial function (represented by the Emax of Ach) correlated negatively with arthritis score ($r = -0.569$, $p = 0.00588$) and positively with BDNF in either PFC ($r = 0.504$, $p = 0.0234$) or Hip ($r = 0.54$, $p = 0.014$). BDNF levels in Hip but not in PFC negatively correlated with arthritis score ($r = -0.717$, $p < 10^{-4}$). Cytokines levels were associated neither with arthritis score nor endothelial function or BDNF levels.

Table 2

Correlations between endothelial function, arthritis score, cytokines and BDNF levels in AIA rats

	Arthritis score	Emax (Ach)	BDNF PFC	BDNF Hip
Emax (Ach)	r=-0.569, p = 0.00588 (n = 22)	n.d.	r = 0.504, p = 0.0234 (n = 20)	r = 0.54, p = 0.014 (n = 20)
BDNF PFC	r=-0.389, p = 0.0655 (n = 23)	r = 0.504, p = 0.0234 (n = 20)	n.d.	n.d.
BDNF Hip	r=-0.717, p < 10⁻⁴ (n = 23)	r = 0.540, p = 0.014 (n = 20)	n.d.	n.d.
IL-1 β	r = 0.151, p = 0.449 (n = 27)	r=-0.0773, p = 0.733 (n = 21)	r=-0.223, p = 0.314 (n = 22)	r = 0.207, 0.35 (n = 22)
TNF α	r = 0.261, p = 0.187 (n = 27)	r=-0.101, p = 0.657 (n = 21)	r=-0.286, p = 0.193 (n = 22)	r = 0.106, p = 0.632 (n = 22)
IL-17A	r=-0.098, p = 0.630 (n = 26)	r = 0.0548, p = 0.805 (n = 22)	r=-0.131, p = 0.545 (n = 25)	r = 0.140, p = 0.518 (n = 23)
Correlations were determined in AIA rats treated with vehicle and Tofacitinib. Endothelial function was expressed as the Emax of acetylcholine (Ach). PFC: prefrontal cortex, Hip: hippocampus. Data in bold are significant (p < 0.05). n = number of rats. n.d.: not determined.				
Brain BDNF levels were measured in the prefrontal cortex (PFC) and hippocampus (Hip) in non AIA control rats (Control), AIA rats treated with either vehicle (Vehicle-AIA) or Tofacitinib (Tofacitinib-AIA) using western blotting analysis. Bar graphs show effect of AIA on PFC (A) and Hip (B) and effect of Tofacitinib on PFC (C) and Hip (D) levels. Values are expressed as means \pm SEM (n = 11–12 rats per group). *p < 0.05, ***p < 0.001. See Suppl. Figure 2 for details.				

Discussion

While the antirheumatic effect of Tofacitinib is largely documented in RA patients and to a lesser extent in animal models of arthritis (2, 3, 28–31), the effect of this Jakinib on two major comorbidities of RA namely vascular and brain dysfunctions has never been investigated. The present study investigated the effect of Tofacitinib on arthritis and radiographic scores, endothelial dysfunction, anhedonia, brain BDNF levels and circulating proinflammatory cytokines levels in AIA rats. Strength of study is that these parameters were all measured in a same rat. The major findings are that Tofacitinib given at a dosage

efficient on arthritis and radiographic scores in AIA rats fully prevented endothelial function and substantially increased brain BDNF levels.

ED is well documented in RA patients and in animal models of RA (22, 27). ED that affects not only peripheral conductance and resistance vessels but also the cerebral vasculature (27, 32) is largely suspected in high incidence of coronary artery diseases and stroke in RA. The exact mechanisms underlying ED in RA are poorly understood even though a contribution of TNF α and IL-1 β is suspected from evidence that the exposure of cultured endothelial cells to these cytokines induced endothelial activation and dysfunction, attested by overexpression of adhesion molecules and decreased nitric oxide availability, respectively (6–8). In addition, IL-17A that also plays a pivotal cytokine in RA pathophysiology, was found to correlate with ED in RA patients (33) even though a causal link between IL-17A and ED remains speculative. The present results showed that Tofacitinib totally prevented AIA-associated aortic ED. This new data is in agreement with studies in which JAK inhibition was reported to reduce the expression of adhesion molecules in cultured endothelial cells exposed to TNF α (34) or oxidized-LDL (35), and to alleviate plaque burden in a model of atherosclerosis (36). Furthermore, in line with the current guidelines recommending an aggressive control of disease activity for CV risk reduction in RA, we reported a negative correlation between arthritis score and endothelial function. By contrast, no correlation was observed between ED and circulating pro-inflammatory cytokines levels, as previously observed in the AIA model after treatment with etanercept, methotrexate, non-steroidal anti-inflammatory drugs or glucocorticoids (37–40). This suggests that improvement of ED consecutive to Tofacitinib was independent on changes in circulating levels of these cytokines. From a clinical perspective, this indicates that the measurement of circulating levels of TNF α , IL-1 β or IL-17A levels cannot be used as a surrogate marker of ED in RA. How Tofacitinib prevented ED in AIA was not investigated here. Notably, it was recently showed that endothelial-progenitor cells-mediated endothelial repair capacity (41) that conditions endothelial function (42) involved phosphorylation of JAK-2. Evidence that AIA-associated ED was improved by Tofacitinib consequently suggests that JAK 2 inhibitory effect of Tofacitinib was not high enough to counteract endothelial repair, or alternatively that AIA-associated ED is independent on defect in endothelial repair. Further studies are now needed to understand the mechanisms by which Tofacitinib improved ED in AIA and to investigate whether the benefits of Tofacitinib on ED translate into a reduction of CV events in RA patients. Supporting this hypothesis, Tofacitinib tended to reduce carotid intima-media thickness in a small cohort of RA patients (43).

The growing interest in neuropsychiatric comorbidities of RA contrasts with the paucity of studies on BDNF in this disease. Available clinical studies consistently reported high levels of BDNF in the blood of RA patients (44, 45). This finding was somewhat surprising with respect to the numerous studies that reported low circulating BDNF levels in patients with depression (46), mild cognitive impairment or Alzheimer disease (47) and suggests that systemic inflammation is a confounding factor when peripheral BDNF is used as a marker of brain health. Notably, blood BDNF levels were lower in RA patients treated with etanercept as compared to patients not treated with this drug but did not correlate with inflammatory parameters (48). Using the AIA model, we previously reported a disconnection between peripheral and cerebral BDNF, high peripheral BDNF levels (+ 30% in serum of AIA vs controls) coexisting

with low (-50%) BDNF levels in both PFC and Hip (12). In the present study, the decrease in brain BDNF levels was restricted to PFC. The reason for this is not known. However, in the present study, control and rats were treated with the vehicle of Tofacitinib (33% DMSO in PEG 300) while control rats did not receive this vehicle in the previous study. Thus, an interaction between BDNF and DMSO cannot be excluded. With regards to the pivotal role of cerebral BDNF in neuroplasticity, neurogenesis and cognition, the most important result of the present study is that Tofacitinib robustly increased BDNF levels both in PFC and in Hip. In addition, evidence that brain BDNF levels irrespective of the examined region positively correlated with endothelial function in AIA rats (Table 2) supports the hypothesis that improved BDNF levels are consecutive to improved ED. These data fit well with the previous evidence that restoration of ED in AIA rats by administration of an arginase inhibitor was efficient to elevate brain BDNF levels (12). Notably, in this latter study, arginase inhibitor - unlike Tofacitinib - did not reduced arthritis score, suggesting that changes in BDNF levels in AIA might be disconnected from immune process involved in joint inflammation. In accordance with this, the present data revealed inconsistent associations between brain BDNF levels and arthritis score in AIA. Indeed, no association was observed between PFC BDNF levels and arthritis score even though a negative correlation was identified between Hip BDNF levels and arthritis score. Our data also reported no association between brain BDNF and plasma cytokines levels in AIA rats. From a clinical perspective, these data suggest that BDNF-dependent cognitive function in RA can be predicted neither from disease activity nor from blood pro-inflammatory cytokines levels.

It is rather difficult to explore neuropsychiatric morbidity of RA in AIA rats. Indeed, AIA model resulted in bilateral inflammation of hindpaws and consequently locomotor deficit which is a confounding factor in interpreting behavioral tests routinely used to explore memory such as Morris water maze, novel object recognition test or depression using forced swim test. Nevertheless, hippocampal long-term potentiation (49) as an *in vitro* model of memory and anhedonia as a core symptoms of depression (12) were previously reported in AIA rats. The new data provided by the present study is that anhedonia preceded the appearance of inflammatory symptoms and was maintained throughout the symptomatic phase, suggesting that immunization rather than inflammation contributed to anhedonia. Supporting this, Tofacitinib that increased BDNF levels did not reverse anhedonia. These apparent conflicting data between mood and brain BDNF levels highlights the need for a better understanding of the pathophysiology of depression in RA, that would not be only secondary to disease-related processes (50), and to identify which of the BDNF-dependent cognitive functions might be improved by Tofacitinib.

In conclusion, this study provided the first evidence that Tofacitinib fully prevented endothelial function and increased BDNF levels in the prefrontal cortex and hippocampus, two cognition-related brain regions in AIA. These data suggest that Tofacitinib might be an attractive option to reduce CV and neuropsychiatric risks in RA.

Abbreviations

AIA: adjuvant-induced arthritis; Ach: acetylcholine; BDNF: brain-derived neurotrophic factor; CV: cardiovascular; ED: endothelial dysfunction; Hip: hippocampus; JAK: Janus kinase; NE: norepinephrine;

PE: phenylephrine; PFC: prefrontal cortex (PFC); RA: rheumatoid arthritis; SNP: sodium nitroprussiate

Declarations

Acknowledgements

Not applicable

Ethics approval

All animal experiments conformed to the “Animal Research: Reporting In Vivo Experiments” ARRIVE guidelines, under approved protocols (APAFiS #8699-2017010210088460) of the local committee for ethics in animal experimentation of Franche-Comté University (2015-001-CD-5PR # Comité d’Ethique Bisontin en Expérimentation Animale, CEBEA N°58, France).

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

PT, CP, CM and CD conceived, designed and coordinated the research. PT, AQ, MP, MC, MT carried out the experiments, collected the data, performed the statistical analysis, prepared the figures, and helped to draft the manuscript. PT, AQ, APT, CP, CM and CD analyzed the data. CM, PT and CD were the major contributors in writing the manuscript. All authors read and approved the final manuscript.

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Figures

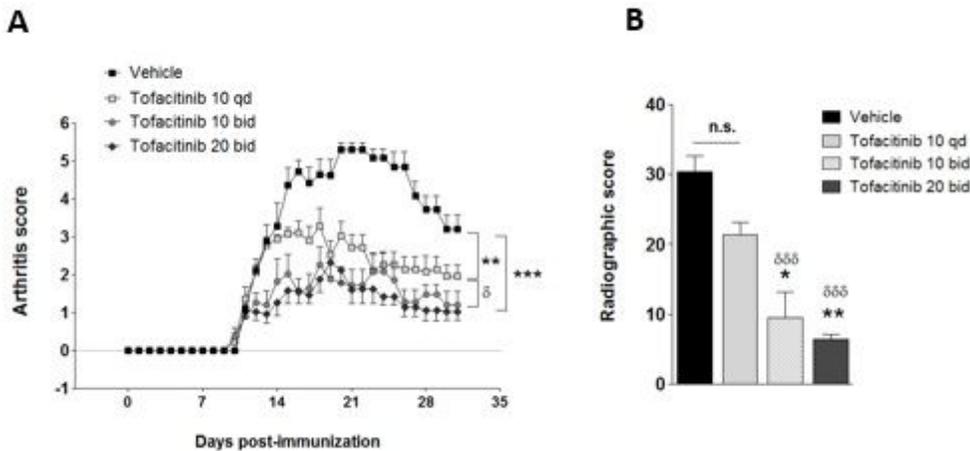


Figure 1

Dose finding study in AIA male rats AIA rats were treated subcutaneously with Vehicle or Tofacitinib at 10 mg/kg once daily (10 qd), 10 mg/kg twice daily (10 bid) or 20 mg/kg twice daily (20 bid) from day 10 to day 33 post-immunization. (A) Arthritis scores were plotted over time after AIA induction. (B) Radiographic scores were evaluated at the end of the treatment period. Values are means \pm SEM (n=5 per group). *p<0.05, **p<0.01, ***p<0.001 vs AIA treated with Vehicle, δ p<0.05, $\delta\delta\delta$ p<0.001 vs Tofacitinib 10 qd; n.s.: not significant.

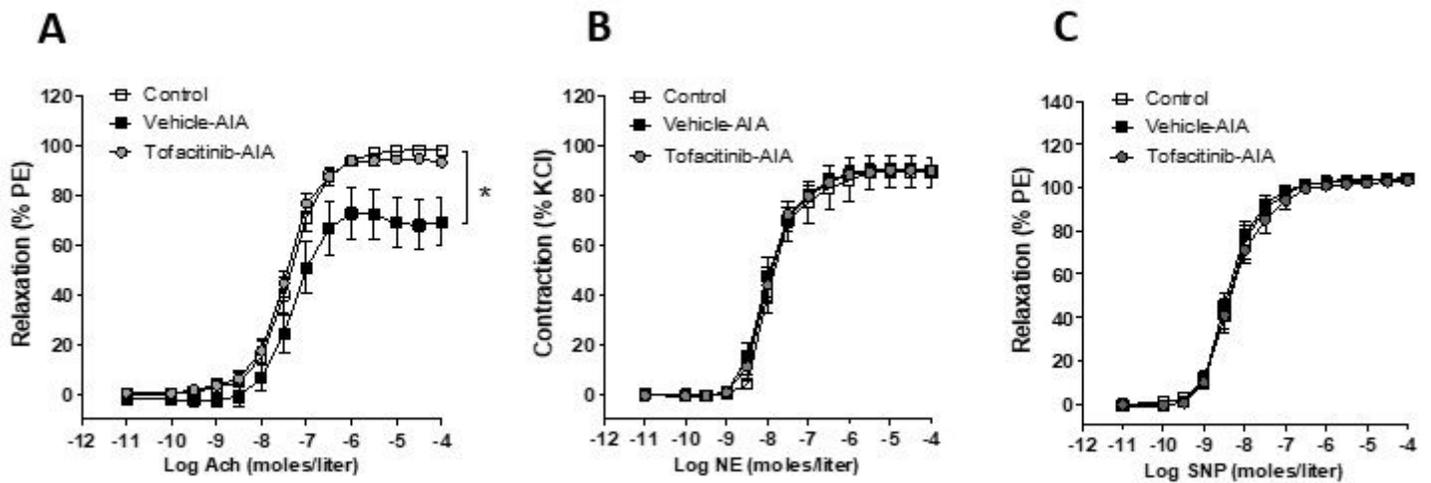


Figure 2

Effect of Tofacitinib on endothelial function Thoracic aortic rings were collected from controls and AIA rats treated with Vehicle or Tofacitinib at the end of the treatment period (3 weeks after the onset of arthritis). Concentration-response curve of (A) Ach in endothelium-intact aortic rings precontracted with PE 10^{-6} moles/liter and (B) NE on endothelium-denuded aortic rings, (C) SNP on endothelium-denuded aortic rings precontracted with PE 10^{-6} moles/liter. Values are means \pm SEM (n = 7-12 aortic rings from 11-12 rats per group). * $p < 0.01$ vs Vehicle-AIA.

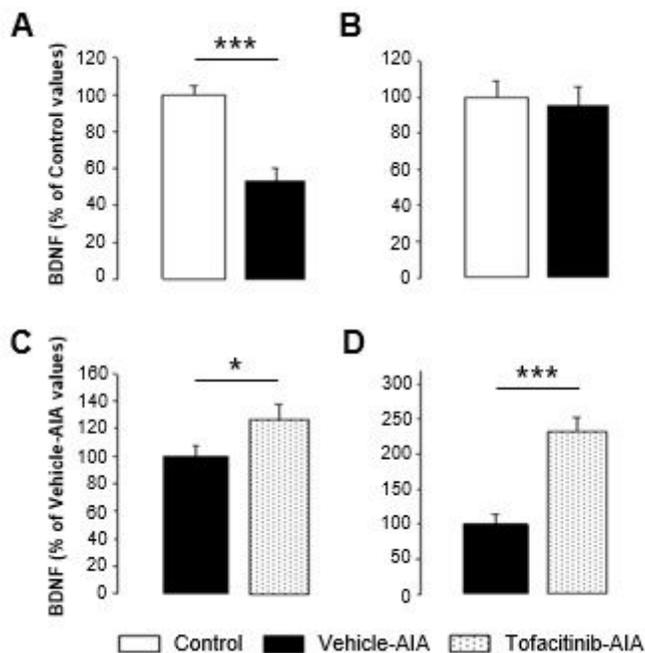


Figure 3

Effect of Tofacitinib on brain BDNF levels Brain BDNF levels were measured in the prefrontal cortex (PFC) and hippocampus (Hip) in non AIA control rats (Control), AIA rats treated with either vehicle (Vehicle-AIA) or Tofacitinib (Tofacitinib-AIA) using western blotting analysis. Bar graphs show effect of AIA on PFC (A) and Hip (B) and effect of Tofacitinib on PFC (C) and Hip (D) levels. Values are expressed as means \pm SEM (n=11-12 rats per group). *p<0.05, ***p<0.001. See Suppl. Fig. 2 for details.

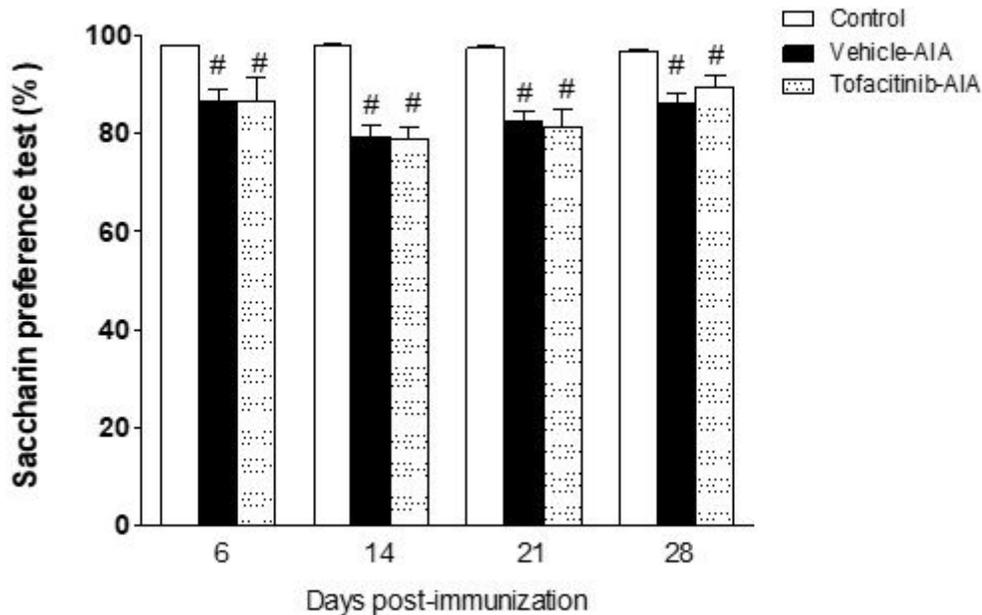


Figure 4

Effect of Tofacitinib on anhedonia in AIA Anhedonia was assessed from the saccharin preference test in control rats (Control, n=15) or AIA rats treated with vehicle (Vehicle-AIA, n=15) or Tofacitinib (Tofacitinib-AIA, n=15). Saccharine preference was calculated as a percentage of the volume of saccharin intake over the total of fluid intake. #p<0.05 vs Control.

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